Effect of green tea extract on expression of proteins involved in drug transport and metabolism and on the expression and secretion of the chemokine interleukin-8 in intestinal cell lines

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Abbreviations

ABC	ATP-binding cassette
ActD	actinomycin D
AhR	aryl hydrocarbon receptor
ALOX	Lipoxygenase-5
ANOVA	analysis of variance
ARNT	AhR nuclear translocator protein
ATP	adenosine-5`-triphosphate
BFA	brefeldin A
Caco-2	human colon carcinoma cell line type Caco-2
CCR	CC chemokine receptor
cDNA	complementary deoxyribonucleic acid
CMFDA	5-chloromethylfluorescein diacetate
COX	Cyclooxygenase
Ct	cycle threshold
CYP	cytochrome P450
CXCR	CXC chemokine receptor
EGCG	(-)-epigallocatechin gallate
EGC	(-)-epigallocatechin
EC	(-)-epicatechin
ECG	(-)-epicatechin-3-gallate
EIA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GS-MF	glutathione-methylfluorescein
GTE	green tea extract
GSH	glutathione
HBSS	Hank's balanced salt solution
H_2O_2	hydrogen peroxide
IBD	inflammatory bowel disease
IEC	intestinal epithelial cells
IFNγ	interferon-gamma
IL-1β	interleukin-1β
IL-8	interleukin-8
kDa	kilodalton
KTZ	ketoconazol

LS-180	human colon carcinoma cell line type LS180
MALT	mucosa-associated lymphoid tissue
MDCK	Madin-Darby Canine kidney
MDR	multi-drug resistance
MEM	minimum essential medium
mRNA	messenger ribonucleic acid
MRP	multi-drug resistance associated protein
MTX	methotrexate
NEM	N-ethylmaleimide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
P-gp	P-glycoprotein
PXR	pregnane X receptor
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SRB	sulforhodamine B
TEER	transepithelial electrical resistance
TNF-α	tumor necrosis factor-α
Vpl	verapamil

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Summary

Pharmacokinetic interactions often occur as a result of changes in the functional expression of drug-metabolising and transporting proteins. In recent years, interactions of herbal medicines with synthetic drugs have come into focus and drug-interactions for more than 150 herbal medicines have been reported including the most prominent hypericum. Importantly, also dietary drug-food interactions have been observed, grapefruit juice presenting the most famous herein.

Systemic elimination and uptake of xenobiotics is regulated, predominantly in the liver but also in other extrahepatic tissues including the gut, by their biotransformation and excretion. In the metabolic conversion of drugs the cytochrome p450 (CYP) enzyme family is the major catalyst of phase I drug biotransformation reactions. The isoenzymes most commonly involved in drug metabolism include CYP3A4, which is also the most abundantly expressed CYP, and CYP1A2. The absorption, distribution, and excretion of endogenous and ingested substances is mediated by membrane transporter proteins. In this context, the transporters P-glycoprotein (P-gp) and multidrug-resistance associated protein 2 (MRP2), both located in the apical membrane of enterocytes, exert a key role in the gastrointestinal tract. The interplay of CYP3A4 and P-gp in limiting oral drug availability is emphasised by their striking overlaps of substrates and inhibitors. Overexpression of particular drug transporters can even lead to the phenotype of drug resistance, which is often observed in cancer patients. However, therapy resistance has also been observed in other diseases such as inflammatory bowel disease (IBD).

Green tea (*Camellia sinensis* (L.) O. KUNTZE, fam. Theaceae) is one of the most popular beverages in the world and has been reported to exert beneficial effects on several life-style related diseases. These have been ascribed to several potential activities of green tea or single constituents thereof, such as antiinflammatory and anticancer activities. However, a potential modulation of drug metabolism by green tea described in the literature remains controversial.

The aim of the thesis was to investigate *in vitro* the influence of a commercially available green tea extract (GTE) on the gastrointestinal drug metabolism and transport. These results might be of therapeutical relevance and/or useful in the prediction of the outcome of future clinical trials. Therefore, the effect of GTE on the expression of different CYPs and membrane transport proteins was assessed in two established intestinal cell lines, LS-180 and Caco-2 cells. Additionally, the influence of GTE on metabolic or transport activity was also investigated *in vitro*.

Green tea has been reported to modulate the generation of reactive oxygen species (ROS) under cell culture conditions. This effect may lead to the generation of artefacts, especially in mRNA induction experiments. In view of this, preliminary experiments excluded an influence of GTE on hydrogen peroxide concentrations in the medium (Chapter 2).

The effect of GTE, or constituents thereof, on the mRNA expression of the two efflux pumps P-gp and MRP2 was investigated in LS-180 cells (Chapter 3). mRNA expression levels were determined by quantitative RT-PCR. Due to their location in the apical membrane of enterocytes, these transporters exert the first obstacle in the uptake of orally ingested xenobiotics. At low concentrations GTE was found not to modulate the mRNA expression of P-gp or MRP2. Functional assays using a cell line stably overexpressing human MRP2 (MDCK-MRP2) showed an inhibition of the extrusion of methotrexate, a MRP2 substrate, by GTE at high concentrations (Chapter 3). In concentrations corresponding to their content in GTE, none of the green tea constituents tested did exert this inhibitory activity on MRP2 function.
In two intestinal cell lines, Caco-2 and LS-180, the effect of GTE on CYP1A1 and CYP1A2 mRNA expression was assessed using quantitative RT-PCR (Chapter 4). CYP1A2 mRNA expression was inducible by GTE in a dose-dependent manner in both cell lines. GTE influenced CYP1A1 expression differentially in these cell lines. mRNA expression of CYP1A1 was only induced in the Caco-2 cell line. These data were confirmed on the protein level by western blot experiments. No effect of GTE on CYP3A4 mRNA expression levels was observed in LS-180 cells. However, GTE showed a dose-dependent inhibition of CYP1A2 and CYP3A4 metabolic activity *in vitro* using luminescent substrates.
These data demonstrate that dietary substances might have an impact on therapeutic effectiveness by a possible influence on drug uptake and metabolism. Additionally, the chemopreventive character implicated for GTE might be explained by an inhibition of the generation of carcinogenic intermediates by CYP1A2.

The gut mucosa represents a site of active immunological activity potentially involved in the initiation of an immune response to inflammatory stimuli. Intestinal epithelial cells are capable to secrete proinflammatory mediators like chemokines and cytokines in response to such stimulants. Chemokines are involved in the regulation of leukocyte migration from the blood into the inflamed tissue and play an important role in the generation of the respiratory burst in these cells. A prominent member of the chemokines is interleukin (IL)-8, which acts on neutrophils. An increased expression of this chemokine has been observed in enterocytes in inflammatory bowel diseases and in several gastrointestinal cancers.

Antiinflammatory and chemopreventive activities have been described for green tea in the literature. Therefore, in this thesis we wanted to clarify the effect of GTE on the potential of enterocytes to produce proinflammatory mediators. Due to inconclusive results concerning different cytokines or enzymes involved in arachidonic acid metabolism (Chapter 6.3), the focus was laid on IL-8 expression and secretion in Caco-2 cells (Chapter 6.1). Induction of IL-8 mRNA expression by GTE at highest dose was shown by quantitative RT-PCR. While this lead to an increase of intracellular IL-8 protein concentration, the excretion of IL-8 was specifically inhibited by GTE as demonstrated by EIA (Enzyme-linked immunosorbent assay) detection. GTE dose-dependently inhibited the induction of IL-8 secretion by the proinflammatory stimulus IL-1β. However, these results give evidence for an anti-inflammatory activity of GTE in enterocytes, which might result in a decreased infiltration of neutrophils in the inflamed tissue. Thus, GTE might be useful for acute treatment of intestinal inflammation.

Aim of the thesis

The major goal of the thesis was to reveal possible influences of a commercially available green tea extract (GTE), namely EFLA®942, on intestinal drug absorption and metabolism. The focus was laid on membrane transporters and phase I metabolising enzymes involved in intestinal first-pass clearance of the majority of xenobiotics. By providing *in vitro* data from intestinal cell lines we might be able to predict possible drug-drug interactions with GTE. Additionally, these results offer an estimation on the influence of GTE co-ingestion on oral bioavailability of drugs and on therapy resistance. However, it has to be emphasised that further *in vivo* studies are needed to clarify the effects of GTE on intestinal drug absorption and metabolism in humans.

Therefore, we intended to generate data concerning the influence of GTE on the following aspects in intestinal cell lines:

- mRNA expression levels of the ABC transporters P-gp and MRP-2
- Functional activity of the ABC transporter MRP-2
- Expression of CYP1A and CYP3A4 with regard to mRNA or protein levels
- Functional activity of CYP1A2 and CYP3A4
- Intestinal epithelial barrier function

Additionally, *in vitro* data were generated concerning the anti-inflammatory activity of GTE on proinflammatory mediators. The focus was set on chemokines and cytokines involved in chronic intestinal inflammation including inflammatory bowel disease (IBD). The effect of GTE on the following topics was investigated using an intestinal cell line model:

- IL-8, IL-1β, and TNF-α mRNA expression levels
- IL-8 mRNA decay
- IL-8 protein secretion
- IL-8 protein expression levels

1. Introduction

1.1 Drug absorption and metabolism in the gastrointestinal tract

Along the mammalian digestive tract, which represents the internal barrier between the environment and the organism, the gastrointestinal mucosa serves as a highly selective barrier designed to permit the absorption of nutrients from the gut lumen into the circulation and to restrict the passage of potentially toxic xenobiotics (Farhadi et al., 2003). Two major routes exist for the permeation of this barrier, namely transcellular and paracellular transport, which require the respective cellular systems. Paracellular transport is mainly controlled by tight junctions (Hollander, 1992). Additionally, the cytoskeleton plays an important role in paracellular transport, beside its critical function together with adherens junctions in the maintenance of the intestinal barrier function (Alvila, 1987). For transcellular transport membrane transport proteins play a pivotal role (Baumgart and Dignass, 2002).

The small intestine principally serves as the site for absorption of nutrients, water, and xenobiotics. Accordingly, it has become apparent that enterocytes lining the intestinal mucosa are equipped with a broad range of metabolic systems such as phase I and II drug metabolising enzymes associated with different efflux pumps (Kaminsky and Fasco, 1992; Suzuki and Sugiyama, 2000). Morphologic features of the small intestine increase its metabolic competence as well as its potential for first-pass metabolism. These include the remarkable length of 7 m in humans divided into the duodenum, jejunum, and ileum (proximally to distally), the appearance of the metabolic competent epithelium as an enterocyte monolayer, as well as further amplification of the epithelial surface by villi and crypts (Kaminsky and Zhang, 2003). However, the small intestine determines the bioavailability of the majority of orally ingested drugs, beside their physiochemical properties, by metabolism and active extrusion after absorption (Suzuki and Sugiyama, 2000). The importance of the isoenzyme cytochrome P450 (CYP) 3A4, the major phase I drug metabolising enzyme in humans, and P-glycoprotein (P-gp), the multidrug efflux pump, in limiting oral drug delivery has been suggested due to broad overlapping substrate specifities and poor oral bioavailability of joint substrates of both proteins (Wacher et al., 1996). Additionally, many compounds induce or inhibit both proteins simultaneously (Schuetz et al., 1996). Phase II drug-metabolising enzymes are able to conjugate xenobiotics with small organic donors, e.g. glutathione, by taking advantage of electrophilic functional groups already present on the molecule, or ones introduced by CYPs (McCarver and Hines, 2001). In most cases these conjugations result in detoxification and pharmacological inactivation and may render the xenobiotics to substrates for specific transport enzymes like the multidrug-resistance associated proteins (MRPs), thus facilitating excretion to the lumen.

The steroid and xenobiotic receptor (SXR), or pregnane X receptor (PXR), an orphan nuclear receptor with high abundance in liver and intestine, has been shown to exert a central role in the expression of P-gp and CYP3A4 and other ABC transporters and CYP isoforms (Lehmann et al., 1998; Synold et al., 2001). After activation by a diverse array of substances including rifampicin and hyperforin, PXR forms heterodimers with retinoic X receptor, another nuclear receptor, and then specifically interacts with a DNA sequence, the hormone-binding element. As many of the compounds that induce CYP3A4 and/or P-gp activate or bind directly to PXR, this receptor could be exploited for a screening of drug candidates, which fail to activate or inhibit this pathway (Lehmann et al., 1998; Geick et al., 2001). In addition, the constitutive androstane receptor (CAR) with high expression in the liver and intestine plays an important role in the expression of ABC transporters (Kullak-Ublick and Becker, 2003). Moreover, expression of P-gp may be induced by stress signals including heat shock, genotoxic stress, or cytokines, as respective transcription binding sites have been located in the MDR1 promotor region (Sukhai and Piquette-Miller, 2000). In the CYP1A enzyme family, each member is inducible via the aryl hydrocarbon receptor (AhR). Ligand binding to AhR induces conformational changes, which enables AhR to translocate to the nucleus, where it dimerizes with the AhR nuclear translocator protein (ARNT). These heterodimers function as transcriptional activators by binding to consensus sequences called dioxin-response elements (DRE) (Denison and Whitlock, 1995).

The general importance of drug metabolising enzymes and transport proteins is given by their control of metabolism, absorption, distribution, and excretion of endogenous substances and exogenous xenobiotics in the organism. The high abundance of these metabolising enzymes and transport proteins in the liver and in the small intestine has raised the question about the contribution of each of these organs to the first-pass metabolism of xenobiotics. The greater metabolic capacity due to a higher overall weight relative to the small intestine and a higher concentration of CYPs and of microsomal protein content, as well as the potential of absorbed systemic xenobiotics to undergo countercurrent exchange, would favour the liver (Lin et al., 1999). Nevertheless, this does not detract from the capability of the small intestine, as the first site of exposure, to metabolise or extrude orally ingested xenobiotics prior to systemic uptake. Thus, it is worthwhile to assess possible interactions of drugs of dietary origin, e.g. green tea, in view of a possible therapeutic potential and/or possible drug-drug interactions leading to reduced therapeutical effectiveness.

1.2 ABC transport proteins

The ATP-dependent efflux pumps belong to a family of ATP-binding cassette (ABC) transporters that share sequence and structural homology, which compromises the

characteristic ATP-binding cassette, consisting of two nucleotide-binding domains. So far, about 50 human ABC genes have been identified and divided into seven distinct subfamilies (ABCA-ABCG) on the basis of their sequence homology and domain organization (Dean et al., 2001). ABC transporters are widely distributed in different tissues, which highlights their importance in the transport of numerous endogenous substances in addition to exogenously administered drugs (Gottesman et al., 2002). ABC transporters are transmembrane proteins located in the plasma membrane where they can mediate anti-gradient transport of an array of distinct substances in an ATP-dependent fashion. In this thesis focus has been laid on ABC-transporters located in the apical membrane of polarized intestinal mucosal cells.

The first ABC-transporter identified as drug-resistance protein was P-gp (ABCB1, MDR1) (Juliano and Ling, 1976), while other ABC family members were discovered later (MRPs, ABCC). Remarkably, a second MDR gene (MDR2) is expressed in humans, that is specific for phosphatidylcholine translocation in cells. Overexpressed in tumour cells, P-gp causes the multidrug resistant (MDR) phenotype by active extrusion of a wide range of cancer chemotherapeutic drugs (Patel and Rothenberg, 1994). Due to the fact that not all tumour cells with this phenotype express P-gp, other ABC-transporters were identified to confer multidrug-resistance including many MRP family members as well as the recently discovered breast cancer resistance protein (BCRP, ABCG2). The latter transporter is thought to be a homodimer of two half-transporters, each containing an ATP-binding domain (Komatani et al., 2001; Gottesman et al., 2002).

1.2.1 MDR1 (ABCB1)

P-glycoprotein (P-gp), a glycoprotein of about 170 kDa, consists of 12 transmembrane domains and two nucleotide-binding sites and functions as an energy-dependent drug efflux pump that lowers intracellular drug concentrations. Beside its occurrence in cancer cells, P-gp is expressed at high levels on the apical surfaces of epithelial cells of tissues such as the liver (bile canaliculi), kidney (proximal tubule), pancreas (pancreatic ductile cell), small intestine and colon (Thiebaut et al., 1987). Besides, P-gp is also expressed in the capillary endothelium of the brain and testes (Schinkel et al., 1994). In these tissues P-gp mediates the efflux of xenobiotics and toxins into the intestinal lumen, urine, bile, and blood. Along the intestine, alterations of P-gp mRNA expression levels from duodenum to sigmoid colon were reported with the highest expression in the terminal ileum (Zimmermann et al., 2005). A strong interindividual expression of P-gp protein was observed in small intestinal biopsies (Lown et al., 1997b). Additionally to the extrusion of orally administered dugs, hence forming a first line of defence, P-gp secretes intravenously administered drugs into the gastrointestinal tract (Sparreboom et al., 1997).

P-gp has a broad substrate specificity covering a large variety of structurally diverse substances (Wacher et al., 1995). In general, these substrates are hydrophobic, and/or organic cations. Drug-drug interactions occur when a substance modulates cellular P-gp activity, which results in an altered efflux of other substrates. These may origin from a change of P-gp expression, direct inhibition of P-gp function, and/or agonist properties of the substance. Several substances have been reported to increase plasma levels of a P-gp substrate, digoxin, due to an inhibition of P-gp-mediated efflux, e.g. verapamil and ritonavir (Verschraagen et al., 1999; Ding et al., 2004). P-gp modulators such as cyclosporin A and verapamil were able to interrupt the transport of anti-cancer agents, which are substrates of P-gp, e.g. irinotecan (Ma and McLeod, 2003).

1.2.2 MRP2 (ABCC2)

Multidrug-resistance associated protein 2 (MRP2) is a member of the MRP family of membrane proteins, which so far comprises nine members (MRP1-9). All of the MRPs possess the characteristic ATP-binding cassette motive but they vary in the number of their transmembrane domains. The MRP family members that have been functionally characterized so far share the property of transporting anionic conjugates and amphiphatic organic anions. Many of these transport proteins enable the sequestration and terminal excretion of conjugates formed in phase II metabolism (Keppler, 1999).

MRP2 (ABCC2, cMOAT) is localized in the canalicular membrane of hepatocytes, in the apical membrane of proximal tubules in the kidney, and in the apical surface of intestinal epithelial cells (Gottesman et al., 2002; Keppler, 1999). In these tissues MRP2 mediates the export of metabolites into bile, intestinal lumen, and urine. Noteworthy, the MRP1 (ABCC1) isoform shares about 50% sequence homology with MRP2 and is expressed in all tissues. MRP1 and MRP3 (ABCC3), which has a similar tissue distribution as MRP2, are located on the basolateral membrane of intestinal mucosal cells, where they extrude substrates into the interstitium and the bloodstream. MRP1, MRP2, and MRP3 act as conjugate export pumps and exhibit overlapping substrate specificities, but with significant kinetic differences (König et al., 1999). The localization of MRP2 in the apical membrane of polarized epithelial cells favours a particular role in detoxification by the terminal excretion of conjugation products such as glutathione S-conjugates, glucuronides, and sulfoconjugates. Together with MRP1, MRP2 exports oxidized glutathione and therefore contributes to the GSSG/GSH ratio as well as to the antioxidative defence potential of the cell (Leier et al., 1996). In chemotherapy, MRP2 has the potential to confer resistance to chemotherapeutical agents including vincristine, doxorubicine, cisplatin, and methotrexate (Cui et al., 1999; Ma and McLeod, 2003; Hooijberg et al., 1999).

1.3 Cytochrome P450 (CYP) enzymes

The CYP superfamily is the major enzyme family responsible for the oxidative biotransformation of a variety of endogenous substrates such as steroids, prostaglandins, bile acids, and xenobiotics including drugs and carcinogens (Wrighton et al., 1996). CYPs are localized in the smooth endoplasmatic reticulum of numerous tissues. They are heme-containing monooxygenases, which mediate oxidation reactions, where an incorporation of an oxygen atom from O_2 into the target molecule takes place, while the remaining oxygen atom is reduced to water. First the substrate binds to the heme iron, where a transfer of an electron of NADPH happens. After the attachment of oxygen and an uptake of a second electron the ternary complex dissociates and CYP, hydroxylated substrate, and water are set free. The substrate specificity of CYP is determined by the ability of a potential substrate to bind in the active site of the enzyme, by the ability to promote electron transfer to the heme iron, and by the extent of which completion of the catalytic cycle occurs without uncoupling of NADPH and oxygen utilization from substrate oxidation (De Voss et al., 1997).

The CYP superfamily is the predominant player in xenobiotic phase I metabolism. Among the three CYP gene families involved for most drug metabolism in humans, namely CYP1, CYP2, and CYP3. Among the different CYP isoenzymes, CYP1A1, CYP1A2, and CYP3A4 are involved in the biotransformation of a wide array of xenobiotic compounds (Nelson et al., 1996). CYP3A is the principal subfamily responsible for CYP-mediated phase I metabolism of more than 50% of administered drugs (Zhang and Benet, 2001). Studies on intestinal CYP expression revealed CYP3A as the predominant subfamily in enterocytes (Watkins et al., 1987; Kolars et al., 1992) and in the liver (Shimada et al., 1994), comprising about 30% and 70%, respectively. CYP1A, which is one of the most abundant CYPs in the liver, participates in the metabolic activation of polycyclic aryl hydrocarbons (PAH) to mutagenic and carcinogenic derivatives, which are often highly reactive but unstable intermediates (Guengerich and Turvy, 1991). Beside the first-pass clearance, CYP-mediated drug metabolism can lead to altered bioavailability of therapeutic agents or tissue burdens of foreign compounds. Additionally, it can lead to altered drug efficacies through inactivation of an active drug or activation of a prodrug. As most drugs have extrahepatic target tissues, the extent and characteristics of target tissue drug metabolism may influence the effectiveness of treatment. Moreover, toxic compounds may be detoxified after CYP-mediated biotransformation, and inert xenobiotics may be activated to become toxic compounds. Therefore, genetic polymorphisms in CYP activity or effects of substrates including dietary compounds on this activity might influence therapeutic effectiveness and/or toxic burden of tissues. Thus, these substrates might alter susceptibility to disease induced by toxins or reactive intermediates, such as smoking-induced cancer (Whitlock, 1999).

1.3.1 CYP1A

The CYP1A subfamily consists of two members, CYP1A1 and CYP1A2. The CYP1A enzymes are responsible for the metabolism of many chemical carcinogens found in the environment and diet. CYP1A mediated metabolism of these carcinogens creates highly reactive intermediates that possess mutagenic and carcinogenic activity (Gonzalez and Gelboin, 1994). CYP1A1 metabolises important polycyclic aromatic hydrocarbon carcinogens such as benzo[a]pyrene and benz[a]anthracene, while CYP1A2 is a primary pathway for the metabolism of caffeine and also metabolises carcinogens as arylamines and aflatoxins. The expression of CYP1A1 in the small intestine seems rather to be inducible than constitutive, while CYP1A2 has not been detected in small intestinal biopsies (Ding and Kaminsky, 2003). Also data from biopsy tissues on CYP1A expression in the colon remains inconclusive (Mercurio et al., 1995; McKinnon et al., 1993). However, CYP1A was inducible in colon carcinoma cell lines (Ding and Kaminsky, 2003).

1.3.2 CYP3A

Studies on small intestinal and colonic CYP expression revealed CYP3A as the predominant subfamily in enterocytes (Watkins et al., 1987; Peters and Kremers, 1989; Ding and Kaminsky, 2003). CYP3A protein and catalytic activity decrease longitudinal along the small intestine (Wacher et al., 1998). The importance of the small intestine in determining oral drug bioavailability has been demonstrated with the CYP3A4 substrate midazolam (Paine et al., 1996). Modulation of intestinal midazolam metabolism has been demonstrated with different CYP3A4 inhibitors such as saquinavir or grapefruit juice (Palkama et al., 1999; Lown et al., 1997a). A high interindividual variability of CYP3A4 has been described (Wacher et al., 1998).

1.4 The gastrointestinal tract as a site of immunological activity

The mucosal surfaces of the gut, covered by a barrier of epithelial cells, are protected by epithelial secretion products such as mucins, defensins, and secretory antibodies as well as by epithelial specializations including tight junctions and apical surface coats. Despite their protective function, mucosal tissues are sites of intense immunological activity. Epithelial cells continuously provide the mucosal immune system with information about the external environment by transporting foreign antigens across the epithelial barrier. This transport occurs along the gastrointestinal tract and is concentrated at sites that contain organized mucosa-associated lymphoid tissues (MALTs) (Neutra et al., 2001). Single lymphoid follicles and follicle-associated epithelium (FAE) increase in frequency in the distal ileum, where they are organized in large patches (Peyer's patches), and colon, where the microbial flora is abundant and diverse. In humans the greatest frequency of FAE occurs in the rectum and in

dead-ended extensions of the intestinal lumen such as the cecum and appendix (O'Leary and Sweeney, 1986). In contrast to the villus epithelium, which is dominated by absorptive enterocytes, mucin-secreting goblet cells and enteroendocrine cells, the FAE predominantly consists of specialized M cells. These mediate transepithelial vesicular transport of foreign material directly to intraepithelial lymphoid cells and to MALTs and are therefore crucial for the induction of protective mucosal immune responses (Neutra et al., 1996). Consequently, due to their mucosal entry port function, M cells are involved in the pathogenesis of certain bacterial and viral diseases. In comparison to the villus epithelium, FAE exerts a lower expression of digestive membrane-associated hydrolases, as well as a reduced mucus, defensin, and lysozyme production, and IgA secretion into the intestinal lumen (Neutra et al., 2001). These features promote local contact of intact antigens and pathogens to the FAE. However, intestinal epithelial cells (IEC) are considered to generally participate in the initiation and regulation of a mucosal immune response. Accordingly, activated IEC secrete a variety of inflammatory mediators such as chemokines, e.g. interleukin (IL)-8 and Groα, and cytokines such as IL-1β and IL-6 (Yang et al., 1997; Woywodt et al., 1994). Corresponding mucosal inflammatory states have been observed in several gastrointestinal diseases including inflammatory bowel disease (IBD) and colorectal cancer (Sartor, 1994; Csiszar et al., 2004).

A continuous migration of activated lymphocytes, macrophages, and granulocytes from the circulation into the mucosa is a hallmark of chronic inflammation of the intestine. Further upregulation and exacerbation of destructive processes is the result of this constant influx of leukocytes, which is caused by a wide variety of different proinflammatory and chemoattractant molecules (MacDermott, 1999). These are released by phagocytes and a variety of cells like IEC in the inflamed tissue upon activation with inflammatory stimuli. The composition of leukocytes present in the inflamed intestine is probably determined by secreted chemokines as well as by the expression of the respective receptors on different cell types. Secreted chemokines establish a chemotactic gradient within the intestine, which directs the migration of leukocytes from the blood through the endothelium into the mucosa and submucosa. The characteristic tissue damage found in IBD, Crohn's disease and ulcerative colitis, result from matrix metalloproteinases from granules, whose exocytosis is induced by chemokines. Additionally, chemokines are able to activate the respiratory burst, which potentially contributes to these lesions. Thus, selective chemokine inhibitors, which could diminish the severity of the inflammatory response, are of great interest as potential therapeutic strategies in IBD.

1.4.1 Chemokines

Chemokines (chemotactic interleukins) constitute a superfamily of small (8-10 kDa), inducible, secreted, proinflammatory cytokines, which comprises about 40 known members. They

primarily act as chemoattractants and activators of specific types of leukocytes. Three classes of chemokines have been defined by the arrangement of the conserved cysteine (C) residues in the mature protein: the CXC chemokines that have one amino acid separating the first two conserved cysteins, e.g. IL-8, Groα, ENA-78; the CC chemokines in which the first two conserved cysteine residues are adjacent, e.g. RANTES, MIP-1α; the C chemokines which lack two of the four conserved cysteine residues, e.g. lymphotactin. A chemokine-like structure with three amino acids between the first two cysteins (CX_3C) at the N-terminal end of a mucin structure has also been described (Baggiolini, 1998). They differ in their biological activity on leukocytes populations depending on the pattern of expression of their receptors. Many chemokines bind to more than one receptor, and most receptors recognize more than one ligand of the corresponding subfamily. This demonstrates a characteristic redundancy and versatility of the chemokine system.

Five receptors for CXC chemokines (CXCR) and eight receptors for CC chemokines (CCR) have been characterized (Murphy, 1996). These receptors are coupled to GTP-binding proteins. Chemokine binding to leukocytes induces a shape change, based on actin remodelling, which leads to the formation and retraction of lamellipodia. Adherence of leukocytes to endothelial cells of the vessel wall before migration into the tissue is mediated by an upregulation and activation of integrins upon chemokine stimulation (Springer, 1994). Other rapid and transient responses are characteristic of leukocyte activation by chemokines including the rise of intracellular free calcium concentration, the production of oxygen radicals and bioactive lipids, and the release of the contents of cytoplasmic storage granules like proteases from neutrophils and monocytes, histamine from basophils and cytotoxic proteins from eosinophils (Baggiolini et al., 1997).

1.4.2 IL-8

The CXC chemokine family member IL-8, a protein of about 8 kDa, was identified 1987 as a novel type of neutrophil-activating cytokine (Baggiolini et al., 1989). Besides in IEC, its expression and secretion was observed in endothelial cells and fibroblasts of different tissues, keratinocytes, synovial cells, chondrocytes and even neutrophils. IL-8 is generated as a precursor of 99 amino acids and is secreted after cleavage of a signal sequence of 20 residues. Several biological active variants are generated by extracellular N-terminal cleavage, with a predominant variant of 72 amino acids. It is a basic protein and contains four cysteins, which form two disulfide bridges required for its biological activity (Baggiolini and Lewis, 1992).

IL-8 activates with high affinity two receptors, CXCR-1 and CXCR-2, which are constitutively expressed in neutrophils. Both receptors are subject to homologous and heterologous desensitisation leading to a progressive attenuation of cellular response after prolonged exposure of neutrophils to IL-8 (Ali et al., 1999). Homologous desensitisation by IL-8 involves

internalisation of agonist-occupied receptors, degradation of IL-8 by lysosomal enzymes, and reexpression of the receptor on the cell membrane (Samanta et al., 1990). Beside a mechanism for limiting cellular response, receptor internalisation, in combination with reexpression, appears to be a fundamental step for chemotaxis. Due to differences in the IL-8 concentration dependence of receptor internalisation, the two receptors seem to induce different chemotactic behaviour. In contrast to CXCR-1, CXCR-2 desensitisation and internalisation are faster and occur at lower concentrations of IL-8. Therefore, CXCR-2 function might be dominant at sites distant from the inflammation, while CXCR-1 plays a more important role in the centre of an inflammation (Chuntharapai and Kim, 1995). Coherently, the respiratory burst, which is solely mediated by CXCR-1, appears predominantly in the centre of inflammation, where it is most efficiently. CXCR-2 is too rapidly desensitised and internalised to mediate phospholipase D activation and superoxide anion production, which are needed to generate the respiratory burst (Zeilhofer and Schorr, 2000).

In colonic biopsies of IBD patients, IL-8 expression was shown to be upregulated and IL-1β and TNF-α have been proposed as pivotal mediators of the mucosal chemokine response (Puleston et al., 2005). Furthermore, increased IL-8 expression was observed in human colorectal carcinoma cells and an increase of IL-8 expression was associated with an enhanced metastatic potential in colon carcinoma cells (Csiszar et al., 2004; Li et al., 2001). Therefore, IL-8 seems to represent an important player in several diseases.

1.5 Green tea (*Camellia sinensis* (L.) O. KUNTZE, fam. theaceae)

1.5.1 General aspects

Tea, beside water, is one of the most consumed beverages in the world. According to the different manufacturing processes, teas can be classified into three types: green tea (non-fermented tea), black tea (fermented tea), and oolong tea (semi-fermented tea). The raw material of all of these teas are the leaves of the tea plant *Camellia sinensis* and its varieties. During fermentation, a series of complex chemical reactions takes place; the most important one representing the oxidation of polyphenols. This results in the formation of theaflavins, thearubigins, and other oxidized-polymerised compounds, which are responsible for the characteristic colour and flavour of black tea (Balentine et al., 1997). Unfortunately, this process was misleadingly termed “fermentation”, although it is mediated by cellular enzymes like phenol oxidase without the involvement of bacteria. For the production of green tea, the fermentation is prevented by heat treatment of the fresh leaves of *Camellia sinensis* (L.) O. KUNTZE.

In the past, green tea had an official state of medicine in several European Pharmacopoeias, including Ph.Port. IV, Ph.Franç. VIII-X, BP Edition 1968 and Ph.Helv. V, whereas nowadays, green tea is not listed in the Ph.Eur. (Netsch and Numao, 2001). Independently, in France green tea pharmaceuticals have been approved as drugs on the basis of the Ph. Franç. and the French Gazette for Marketing Authorisation of Plant Medicines (1990). Recently, cases of hepatic attack after the use of hydroalcoholic extracts of green tea in complement of reducing diets have been reported in France and Spain (Seddik et al., 2001). These observations have led to a suspension of the marketing authorisation of these products in both countries (www.who.int/medicines/library/pnewslet/3news2003.pdf). No marketing authorisation for medicinal use is available in other EC countries, but there exist preparations on the base of green tea as supplementary food.

The French Gazette for Marketing Authorisation of Plant Medicines (1990) listed some traditional indications of green tea: For oral use, 1) diuresis, 2) mild diarrhoea, 3) recovery from fatigue, and 4) dietary supplement for weight reduction and for external use, 1) calm for itching of skin ailment and 2) treatment of cracks, grazes, and insect bites, etc. (Ministère des affaires sociales et de la solidarité, 1990).

The composition of constituents of a green tea infusion is highly dependent on the amount of used tea leaves, on the extraction time, and on the quality of water used for extraction. Therefore, the composition can be subject to a strong variance within a certain range (Figure 1.1). The traditional preparation of green tea as an infusion contains a broad spectrum of components of the drug. In general, green tea contains about 30% (w/w) of catechins in the dry leaves (Graham, 1992). The major catechins are epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin (EC), and (-)-epicatechin-3-gallate (ECG), which comprise more than 60% of the total catechins (Figure 1.2) (Yang and Koo, 1997). Other green tea constituents are the flavonols (quercetin, kaempferol, and rutin), caffeine, phenolic acids, the green tea specific amino acid theanine, and flavour compounds such as (Z)-3-hexenols and its esters (Graham, 1992).

After oral administration green tea catechins are well absorbed (Yang et al., 1998a). Catechins are then biotransformed in the liver, and presumably already in the intestine (Vaidyanathan et al., 2002; Zhang et al., 2004), to conjugated metabolites such as glucuronidated, methylated, sulfated derivatives. While EGC and EC are predominantly conjugated, EGCG is usually present in the free form in human plasma (Chow et al., 2001; Lee et al., 2002). Conjugates pass into the bile through enterohepatic circulation and might therefore reach the colon as glucuronides or other metabolites via this route (Scalbert et al., 2000). In the colon deconjugation may occur due to tissue β-glucuronidases and microflora (Aura et al., 2002; Kroon et al., 2004). After absorption the catechins are widely distributed to the different tissues with concentrations presumably not exceeding the lower micromolar to nanomolar range (0.1-

1.0 μM) as indicated by EGCG plasma concentrations (Yang, 1997; Lee et al., 2002). However, due to the direct contact to the tea infusion, the gastrointestinal tract is likely to be exposed to higher concentrations of green tea constituents, irrespective of whether they are absorbed, retained, or recirculated to the gut tissues.

Components	Dry weight (%)
Soluble in water	
Flavonols	18-32
(-)-EGCG	9-14
(-)-EGC	4-7
(-)-ECG	2-4
(-)-EC	1-3
(+)-GC	1-2
(+)-C	0.5-1
minor catechins	0.4-1
Flavonol glucosides	3-4
Proanthocyanidins	2-3
Caffeine	3-4
Amino acids	2-4
Carbohydrates	3-5
Organic acids	0.5-2
Saponins	0.04-0.07
Pigments	0.5-0.8
Vitamins	0.6-1
Soluble minerals	2-4
Insoluble or slightly soluble in water	
Cellulose	6-8
Lignin	4-6
Polysaccharides	4-10
Lipids	2-4
Insoluble pigments	0.5
Insoluble minerals	1.5-3
Volatiles	0.01-0.02

Figure 1.1: Composition of constituents in green tea leaves (Zhen, 2002)

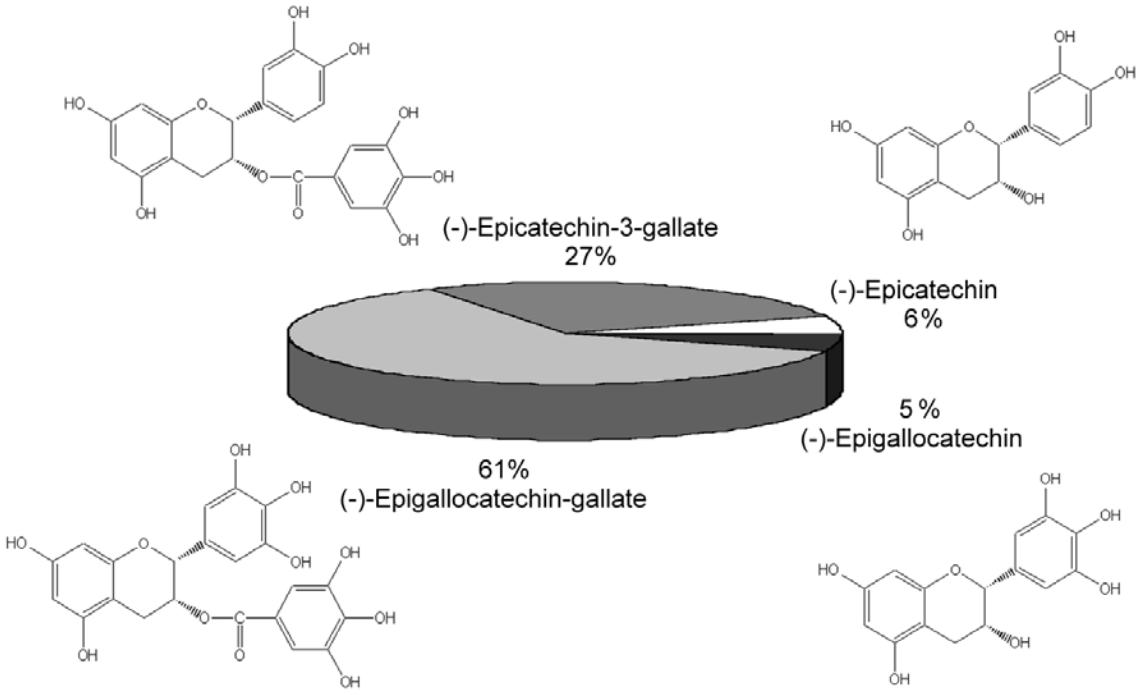

Figure 1.2: Structure and composition of the major catechins in green tea

1.5.2 Pharmacological activities

In recent time green tea and its components have moved into focus of scientific interest. Main reasons are several epidemiological observations of significantly reduced incidence of several human cancers and cardiovascular diseases in populations with a high consumption of green tea (Imai et al., 1995; Imai et al., 1997). Due to the high transition into the green tea infusion, the good bioavailability, as well as the strong antioxidative properties, the main focus of research has been laid on the major catechins (Figure 1.2). Nevertheless, it has been shown that the effects of the multicomponent system green tea seem to be superior to those of single green tea components (see Chapters 3, 4, 6), what might be explained by cumulative and/or complementary effects of single components. Besides the catechins, the green tea components theanine and caffeine have been of specific interest in anti-tumour research (Huang et al., 1997; Sugiyama et al., 1999).

Various population studies have shown that consumption of green tea reduces the risk of cardiovascular diseases (Kono et al., 1992; Imai et al., 1995). This suggests that green tea components exert an important role in protecting cells and tissues against oxidative damage mediated by free oxygen radicals. Accordingly, in a study on oxidative stress in smokers and non-smokers a reduction of oxidative DNA damage, lipidperoxidation, and free radical generation has been reported after consumption of green tea (Klaunig et al., 1999). Several in vitro studies have designated the green tea catechins as mainly responsible due to their strong

antioxidant and metal-chelating properties (Kondo et al., 1999; Kostyuk et al., 2000; Hider et al., 2001). Additionally, observations in vivo and in vitro give evidence that green tea may enhance endogenous antioxidant defences of the cell (Khan et al., 1992; Benzie et al., 1999; Mueller-Klieser et al., 2002).

Cancer is one of the most widespread causes of death in the industrialized countries. Therefore, epidemiologic reports of a significantly reduced incidence of different cancer types in populations with a large frequent green tea consumption have indicated green tea as a potential candidate for cancer chemoprevention and anticancer therapy (Nakachi et al., 1999; Fujiki, 2000). As oxidants can induce cell division and DNA mutations (Ames et al., 1990; Cerutti, 1991), the antioxidative potential of green tea represents an important protective mechanism. Moreover, green tea may act via inhibition of enzyme expression and activity and/or modulation of signal transduction pathways. Recently, EGCG was shown to be an inhibitor of dihydrofolate reductase activity in vitro, which may represent a mechanism of the proapoptotic activity of green tea in cancer cells against normal cells (Navarro-Peran et al., 2005). Another mechanism may be the reported differential inhibition of the antiapoptotic transcription factor NF-κB by EGCG in cancer versus normal cells (Ahmad et al., 2000). However, the inhibition of the activation of several onco-transcription factors, including NF-κB and AP-1, by EGCG has been reported (Bode et al., 2004). Additionally, EGCG seems to inhibit cell cycle progression in tumour cell lines and angiogenesis (Yang et al., 1998b; Mueller-Klieser et al., 2002; Cao et al., 1999; Garbisa et al., 1999). Taking these effects into consideration, evidence is given that green tea may act on the different stages of tumourgenesis, namely initiation, promotion, and progression, which emphasises its potential chemopreventive and anticancer activity.

Besides cancer, cardiovascular diseases represent the main fatal diseases of humans worldwide. Of the cardiovascular diseases, atherosclerosis is one of the most prevalent. As oxidation plays a central role in the development of atherosclerosis much attention has been laid on antioxidants including green tea. While an inverse relation between tea consumption and cardiovascular risk has been described by several epidemiologic studies (Hertog et al., 1993; Sesso et al., 1993; Kono et al., 1992; Imai et al., 1995), other studies suggested no beneficial effect (Vita, 2003). Green tea may exert protective effects via an inhibition of lipid peroxidation due to its radical scavenging activity (Terao et al., 1994; Yang et al., 1999). Beside lipid peroxidation, the development of thrombosis is promoted by proteases secreted by macrophages such as matrix metalloproteinases (Libby et al., 1999). An inhibition of matrix metalloproteinases by green tea has been observed, which may result in an increased plaque stability (Garbisa et al., 1999; Kim et al., 2005).

Although numerous health benefits have been proposed for the consumption of green tea, its effectiveness in humans remains unclear. Animal models and cell culture systems may differ

from the human situation as for example the used doses or concentrations of green tea often exceeded those consumed by humans or the plasma concentration, respectively. Mechanisms based on the use of such high concentrations may be relevant for cancers or chronic inflammations in the gastrointestinal tract but not for sites, which depend on systemic bioavailability.

2. Effect of green tea extract on reactive oxygen species (ROS) concentration under cell culture conditions

2.1 Introduction

Green tea polyphenols, particularly EGCG, are naturally occurring strong antioxidants (Tanaka, 2000; Higdon and Frei, 2003), but they also exhibit prooxidant properties. It has been suggested that prooxidant activities may be an important mechanism of the anticancer activities found for green tea (Malik et al., 2003; Nakagawa et al., 2002). Observations of a recent study showed that the addition of EGCG and other green tea constituents to tissue culture medium generated high levels of hydrogen peroxide (H_2O_2) and it was postulated that this might represent an artefact of cell culture (Long et al., 2000). An other study showed similar results, although discriminating between different EGCG concentrations, while the presence of cells decreased H_2O_2 concentrations (Dashwood et al., 2002). In contrast, it was also reported that at high concentrations EGCG functions as a ROS producer (100 μM and above), whereas at concentrations below 10 μM it exerts radical scavenging activity (Saeki et al., 2002). Therefore, it is important to examine the possible influence of GTE on H_2O_2 concentrations in the medium under cell culture conditions. To determine whether potential effects of GTE in future experiments might be a direct effect of GTE or an indirect effect of H_2O_2 generated as an artefact, H_2O_2 concentrations were determined under various experimental conditions.

2.2 Materials and methods

Materials:

Standardized green tea special extract EFLA®942 (GTE) was from Frutarom Switzerland Ltd., Wädenswil, Switzerland. Brief manufacturing description: After addition of 0.02% m/m Acidum ascorbicum, the leaves of Camellia sinensis (L.) O. KUNTZE are continuously extracted (percolation) with 80% (m/m) ethanol at 20-35°C. After a patented ultra-filtration process the crude extract is dried. The drug to extract ratio (DER) is 5.5=1. Finally, 4.6% m/m maltodextrin is added for content adjustment of polyphenols (49.3 % m/m), caffeine (8.46% m/m), theobromine (0.59% m/m), and theanine (1.73% m/m).

Cell culture

The human colon adenocarcinoma cell lines LS-180 and Caco-2 were purchased from American Tissue Culture Collection (ATCC, Manassas, USA) and cultured in Dulbecco's MEM

(DMEM) with Glutamax-I, supplemented with 10% (v/v) fetal calf serum (FCS), 1% non-essential amino acids, 1% sodium pyruvate and 50 μg/ml gentamycin. Per cell culture well, 10^6 cells have been distributed. Only freshly prepared and filtered (0.2 μm) mixtures of GTE with medium were used. All cultures were maintained in a humidified 37°C incubator with 5% carbon dioxide in air atmosphere. All cells used in this study were between passages 40 and 70.

Measurement of H_2O_2

The Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes, Leiden, The Netherlands) was used. This kit is based on a one-step fluorimetric method, which detects H_2O_2 when the reagent, 10-acetyl-3,7-dihydroxyphenoxazine, reacts with H_2O_2 in a 1:1 stoichiometry in the presence of horseradish peroxidase. Medium in the presence or absence of 0.01 mg/ml GTE was incubated for 1, 15, 30, 60, 90, or 150 min at 37°C. Caco-2 or LS-180 cells at confluence were incubated with or without 0.01, 0.1, or 1 mg/ml GTE for 1, 15, 30, 60, 90, or 150 min at 37°C. Samples were either diluted 1:4 with reaction buffer according to the recommendation of the manufacturer or used without dilution. A 50 μl aliquot of the sample was incubated with the reagent mixture for 30 min at room temperature in the dark. Absorption was measured at 560 nm. (Spectra MAX 250, Microplate Spectrophotometer, Molecular Devices Corporation, California, USA).

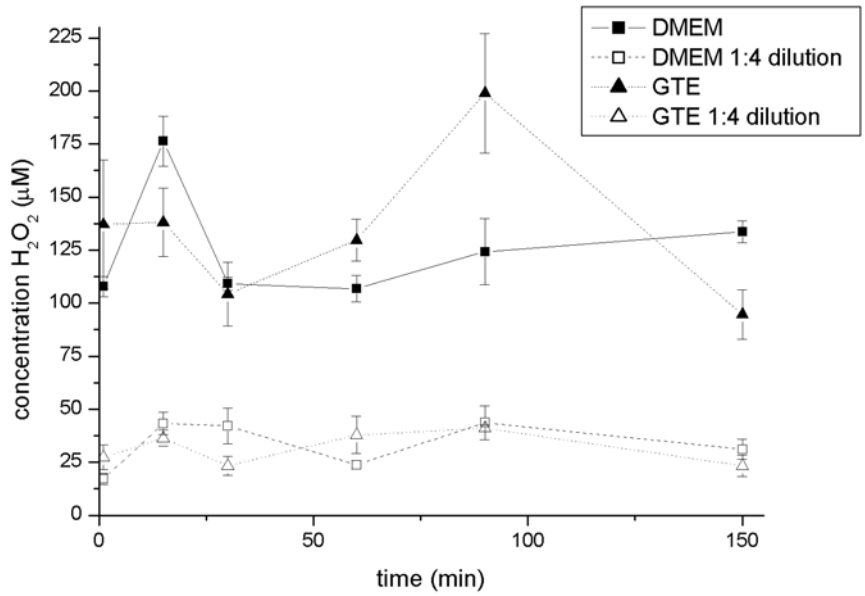

Figure 2.1: Time-dependent generation of H_2O_2 in the medium with or without 0.01 mg/ml GTE in the absence of cells. Samples have been analysed with or without previous 1:4 dilution. This figure is a representative of three

independent replications of the experiment, each with similar results. Data represents the mean values (±SEM) of one experiment (n=3).

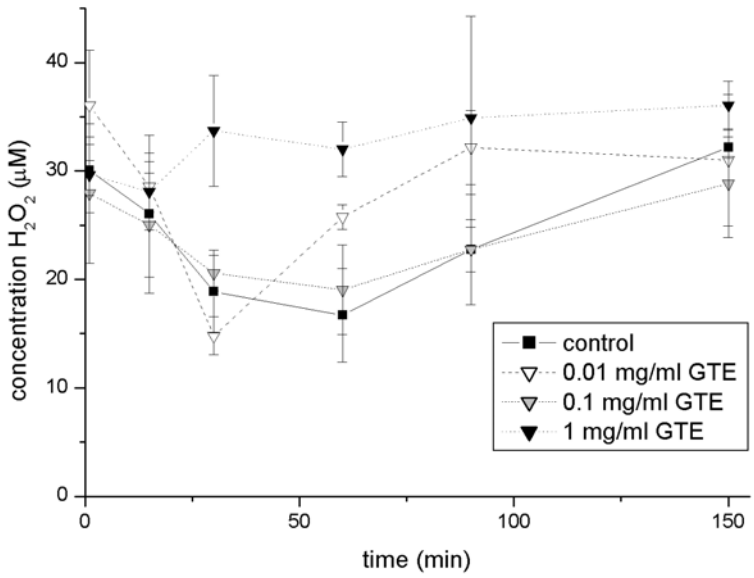

Figure 2.2: Time-dependent effect of different GTE concentrations on the generation of H_2O_2 in the medium in the presence of Caco-2 cells. This figure is a representative of three independent replications of the experiment, each with similar results. Data represents the mean values (±SEM) of one experiment (n=3).

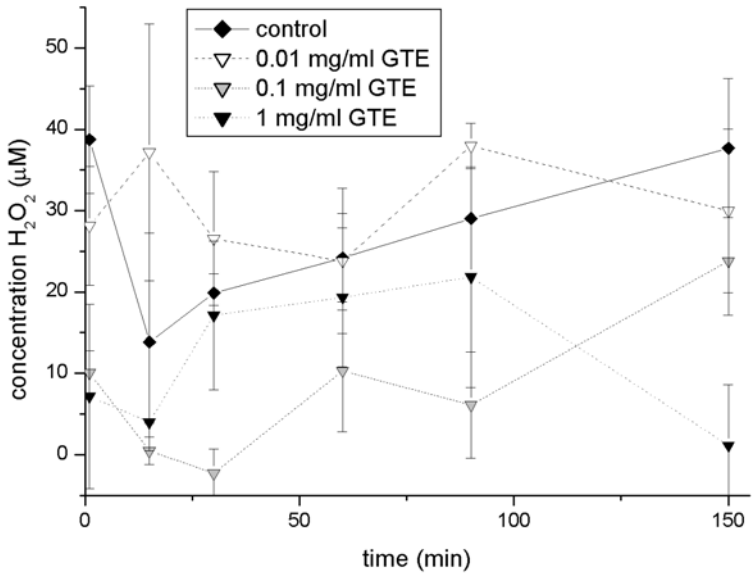

Figure 2.3: Time-dependent effect of different GTE concentrations on the generation of H_2O_2 in the medium in the presence of LS-180 cells. This figure is a representative of three independent replications of the experiment, each with similar results. Data represents the mean values (±SEM) of one experiment (n=3).

2.3 Results and discussion

Our present findings indicate that GTE in a concentration range of 0.01 to 1 mg/ml does not significantly increase H_2O_2 concentrations in DMEM in the presence of Caco-2 (Figure 2.2) or LS-180 (Figure 2.3) cells over a time range of 150 min. The results differ from recent publications, where the green tea component EGCG was shown to generate H_2O_2 in cell culture (Yang et al., 2000; Dashwood et al., 2002). However, it has to be noted that human bronchial epithelial cells grown in LHC-9 medium or HEK293 cells grown in DMEM were used in these studies, whereas in our study Caco-2 or LS-180 cells grown in DMEM were used. Importantly, GTE did not significantly modulate H_2O_2 concentrations in the absence of cells in a time range of 150 min (Figure 2.1), which was in accordance to results observed for EGCG in the same medium (Dashwood et al., 2002).

Further investigation of the effect of EGCG on H_2O_2 generation in our experimental system would have been interesting for a determination of the impact of different cell types, no matter if derived from the same tissue (Hong et al., 2002) or not (Yamamoto et al., 2003), on the pro-oxidative activity of EGCG. Additionally, this might have clarified if, in case of a prooxidative effect of EGCG as described in the literature, other green tea components in GTE might scavenge H_2O_2 generated by EGCG, what would have given an explanation on our findings. However, basal H_2O_2 levels found in the cell culture medium in the absence or presence of Caco-2 or LS-180 cells were relatively high with concentrations about 120 μM.

In summary, we did not observe an impact of GTE, in the concentration range used, on H_2O_2 concentrations in the cell culture medium in the presence or absence of Caco-2 or LS-180 cells. In accordance to chemopreventive effects of EGCG described in the literature (Yamamoto et al., 2004), this excludes that the effects of GTE described in Chapters 3-6 are based on H_2O_2 generation by GTE.

3. Inhibitory activity of a green tea extract and some of its constituents on multidrug resistance-associated protein 2 functionality

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3.1 Abstract

Green tea extracts (GTE) might modulate ABC transporter gene expression or function. This may be relevant in treatment of cancer or in influencing intestinal drug permeability. To gain more insight on the influence of a GTE on secretory transport proteins we investigated the influence of GTE and several green tea components on the mRNA expression level of P-glycoprotein (P-gp) and multidrug resistance-associated protein 2 (MRP2) in human gastrointestinal epithelial LS-180 cells. Furthermore, functional activity of MRP2, using glutathione methylfluorescein (GS-MF) or [^{3}H]methotrexate (MTX) as substrate, was investigated in canine kidney cells stably overexpressing human MRP2 (MDCK-MRP2). GTE, at a concentration of 0.01 mg/ml, did not increase mRNA expression of P-gp or MRP2 in LS-180 cells. Functional assays in MDCK-MRP2 cells using GS-MF did not show any effect of 0.01 mg/ml GTE on MRP2 activity. In the same cell line the cellular accumulation of MTX (a specific substrate of MRP2) was significantly increased with the MRP-specific inhibitor MK-571 or with 1 mg/ml GTE, but not with 0.1 mg/ml. The green tea components (-)-epigallocatechin gallate, (-)-epigallocatechin, theanine, or caffeine, each in corresponding concentrations to the respective concentration of GTE, did not show any effect on MRP2 function. These data demonstrated that the mRNA expression patterns of P-gp and MRP2 in LS-180 cells are not altered by 0.01 mg/ml of GTE. However, MRP2 function was inhibited by 1 mg/ml GTE, whereas none of the green tea components tested were responsible for this effect.

3.2 Introduction

Transmembrane transport proteins play a crucial role for the maintenance of the barrier function of the intestinal epithelium, which is critical for the disposition as well as the cytotoxicity of xenobiotics. Accordingly, enterocyte derived cell lines, such as LS-180 cells, are provided with different transport systems including the ATP-binding cassette (ABC) transporters P-glycoprotein (P-gp) and the multidrug resistance protein (MRP) family (Gottesman and Pastan, 1993; Ambudkar et al., 1999; Borst et al., 2000; Keppler et al., 1997). P-gp, which is encoded by the MDR1 gene, is located in the apical membrane of enterocytes and acts as an efflux pump that extrudes many clinically important drugs thereby limiting their oral bioavailability (Kim et al., 1998; Greiner et al., 1999). Multidrug resistance-associated protein 2 (MRP2) is localized in the apical membrane of polarized cells and transports various glutathione S-conjugates and several anticancer drugs including methotrexate (König et al., 1999; Evers et al., 1998; Cui et al., 1999; Hooijberg et al., 1999; Fujiki, 1999).

Green tea is one of the most popular beverages worldwide and several beneficial/protective effects on life-style related diseases, including anticarcinogenic activities, are being attributed to its consumption (Fujiki, 1999; Kostyuk et al., 2000). Due to the increasing use of green tea

preparations as food supplements or drugs, possible influences on drug transporter systems have to be assessed. Recently, some green tea polyphenols, namely (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate, and (-)-catechin gallate, have been shown to interact with P-gp and to inhibit its function (Jodoin et al., 2002), whereas for (-)-epicatechin a concentration-dependent functional activation of P-gp was reported (Wang et al., 2002). Other green tea components are able to inhibit the efflux of the anticancer drug doxorubicin (DOX), a substrate of P-gp and MRP2 (Ambudkar et al., 1999; Borst et al., 2000; Sadzuka et al., 1998; Sadzuka et al., 2000).

Thus, GTE might modulate ABC transporter gene expression or function. This may be relevant in treatment of cancer or in influencing intestinal drug permeability. Therefore, this study focused on the effect of GTE on P-gp or MRP2 mRNA expression in cultured intestinal LS-180 cells and on the influence of GTE or different green tea components (Figure 3.1) on MRP2 functional activity.

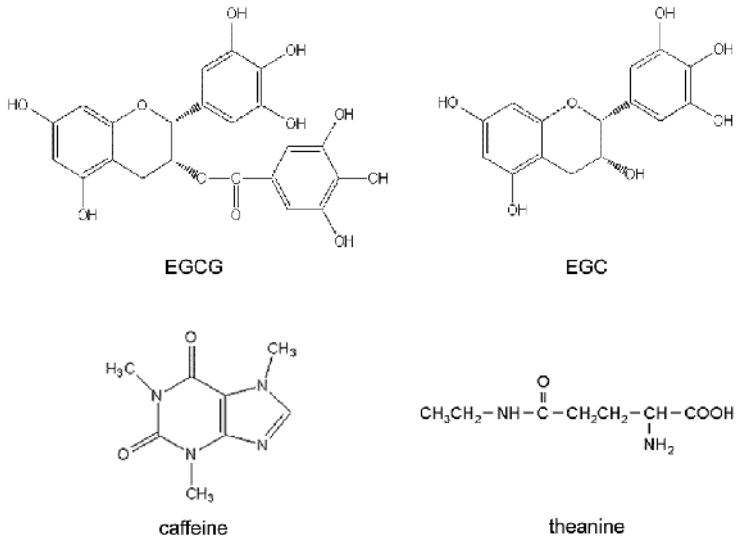

Figure 3.1: Chemical structures of GTE components epigallocatechin gallate (EGCG), epigallocatechin (EGC), caffeine, and theanine.

3.3 Materials and Methods

Materials

Standardized green tea special extract EFLA®942 (GTE) was from Frutarom Switzerland Ltd., Wädenswil, Switzerland. Brief manufacturing description: leaves of *Camellia sinensis* (L.) O. KUNTZE are continuously extracted (percolation) with 80% (m/m) ethanol. After a patented filtration process (US 6024998) the crude extract is dried. Finally, 5% m/m maltodextrin is added as carrier. The drug to extract ratio (DER) is 5.5=1. Characteristic components in the extract are polyphenols (47.5-52.5% m/m), caffeine (5.0-10.0% m/m), theobromine (0.30-1.20% m/m), and theanine (1.0-3.0% m/m). (-)-Epigallocatechin gallate (EGCG) was from CHEMOS GmbH, Regenstauf, Germany, (-)-epigallocatechin (EGC) was from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. In the batch used the following concentrations of constituents in 0.01 mg/ml GTE were determined: 4.02 μM EGCG; 2.27 μM EGC; 4.36 μM caffeine; 0.99 μM theanine. MK-571 was from Biomol, Plymouth Meeting, PA, USA. Chloromethylfluorescein-diacetate (CMFDA) was from Molecular Probes, Eugene, OR, USA; [^{3}H]methotrexate (MTX) was from Movarek Biochemicals, CA, USA; [^{14}C]sucrose was from Amersham, UK. All other chemicals were obtained from commercial sources in the highest quality available.

Cell cultures

The human colon adenocarcinoma cell line LS-180 was purchased from American Tissue Culture Collection (ATCC, Manassas, USA) and cultured in Dulbecco's MEM with Glutamax-I, supplemented with 10% (v/v) fetal calf serum (FCS), 1% non-essential amino acids, 1% sodium pyruvate and 50 μg/ml gentamycin; Madin Darby canine kidney (MDCK) cells stably overexpressing human MRP2 (MDCK-MRP2) were a kind gift from Dr. Evers (The Netherlands Cancer Institute, Amsterdam, Netherlands) and cultured in Dulbecco's MEM with Glutamax-I, in the presence of 50 μg/ml gentamycin. Per cell culture well, 10^{6} cells have been distributed. All cultures were maintained in a humidified 37°C incubator with 5% carbon dioxide in air atmosphere.

Colorimetric cytotoxicity assay

Cytotoxicity of GTE was screened in LS-180 or MDCK-MRP2 cells at confluence. LS-180 cells were incubated for 72 hours in the absence or presence of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, and 3 mg/ml GTE, respectively. MDCK-MRP-2 cells were incubated for 30 minutes with or without 0.01, 0.1, and 1 mg/ml GTE, or corresponding concentrations (4.02, 40.2, and 402 μM) of EGCG. Replacement with medium containing the compound of interest was done every 24 h. Only freshly prepared and filtered (0.2 μm) mixtures of GTE with medium were used.

Cultures fixed with trichloroacetic acid were stained for 30 minutes with 0.4 % sulforhodamine B (SRB) dissolved in 1 % acetic acid. Unbound dye was removed by washing four times with 1% acetic acid and protein bound dye was extracted with 10 mM Tris buffer. Absorption was measured at 540 nm. (Spectra MAX 250, Microplate Spectrophotometer, Molecular Devices Corporation, California, USA) (Skehan et al., 1990). SRB absorption intensity correlates linearly with the number of living cells.

Real time polymerase chain reaction (TaqMan assay)

For mRNA induction experiments LS-180 cells at confluence were incubated either with or without 10 μM rifampicin or with 0.01 mg/ml GTE for 72 h. Replacement with freshly prepared medium containing the compound of interest took place every 24 hours.

After removal of the medium at the end of the culture period, the cells were treated with lysis buffer RLT (Qiagen) and 1 % β-mercaptoethanol (Sigma, St. Louis, USA) was added. The total amount of RNA was extracted by using the RNeasy™ Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentration was measured with a Gene Quant photometer (Pharmacia, Uppsala, Sweden). The purity of the RNA preparations was high, as demonstrated by the 260/280 nm ratio (range 1.7 to 2.0). DNA was digested by using the DNA-Digestion Kit with DNase I Amplification Grade, 10x DNase I Reaction Buffer and 25mM EDTA (Invitrogen, Life technologies Basel, Switzerland) according to the manufacturers' protocol.

2 μg of total RNA was reverse transcribed to cDNA by using SuperScript ^{TM} II RT, 0.1 M DTT, 5x Strand Buffer, oligonucleotides (Invitrogen, Life technologies) and Random Hexamer (Applied Biosystems, Rotkreuz, Switzerland). Reverse transcription was done according to the manufacturers' protocol.

For DNA-Digestion as well as for reverse transcription the Eppendorf Mastercycler Personal heating cyclometer was used.

All RNA isolation procedures and reverse transcriptions were performed under RNase free conditions (RNeasy Erase Spray, ICN Biochemicals, Inc. Ohio, USA), freshly prepared water from highest quality was used (Milli Q, 18.2 mOhm, Kantonsspital Basel, Switzerland).

25 ng of complementary DNA was used as a template for real time quantitative PCR analysis. The cDNA was amplified in a 25 μl volume containing Master-Mix (TaqMan®Universal PCR Master Mix, Applied Biosystems, USA), water, forward primer, reverse primer (Eurogentec, Seraing, Belgium) and probe (Invitrogen, Basel, Switzerland) mixed according to the manufacturers' protocol.

The assay was performed using a Gene Amp 5700 Sequence Detector (Applied Biosystems, Rotkreuz, Switzerland), which combines a thermocycler and a fluorescence detector. Each sample of the reaction mixture was amplified during 40 cycles (15 sec at 90°C, 1 min at 60°C).

As a negative control not-transcribed total RNA was used. For P-gp detection the following primers and probe were used: 5`-AAGCTGTCAAGGAAGCC-AATGCCTATGACTT-3` (probe), 5`-CTGTATTGTTTGCC ACCACGA-3` (forward), and 5`-AGGGTGTCAAATTTATGAGGCAGT-3` (reverse). For MRP2 detection the following primers and probes were used: 5`-CTCAATATCACACAAACCCTGAACTG-GCTG-3` (probe), 5`-ACTGTTGGCTTTGTT CTGTCCA-3` (forward), and 5`-CAACA-GCCACAATGTTGGTCTCTA-3` (reverse).

A relative standard curve was generated by serial dilutions of cDNA. Fragments of cDNA corresponding to MDR1 and MRP2 that covered the TaqMan primer/probe area were obtained by PCR amplification. All of the DNA standards were quantified using the Pico Green reagent (Molecular Probes). For absolute quantification, Ct values of standards were plotted against the log of the respective dilution factors. Slope and y-intercept of the standard curve line were then calculated by linear regression. Each standard curve was generated from a known amount of corresponding cDNA and was then used to calculate the input amount for unknown samples for respective genes. Absolute quantification was done for every experiment.

Autofluorescence and Quenching

Changes of fluorescence intensity of the transport buffer Hank's Balanced Salt Solution (HBSS, Gibco, Basel, Switzerland) were analysed without cells in the presence of 0.01, 0.1 or 1 mg/ml GTE or the corresponding concentrations of EGCG, EGC, theanine or caffeine.

Changes in fluorescence of a fixed methylfluorescein (MF) concentration of 50nM were analysed in the presence of 0.01, 0.1 or 1 mg/ml GTE or the corresponding concentrations of EGCG, EGC, theanine or caffeine. Experiments were performed at room temperature. Fluorescence was measured with a HTS 7000 Plus Bio Assay Reader (Perkin Elmer Ltd., Buckinghamshire, UK).

Functional Assays

Functional assays for MRP2 transport were performed at 37°C using confluent monolayers of MDCK-MRP2 cells, which were grown in 24-well cell-culture plates. Cells were washed twice with HBSS.

In the assays using CMFDA as substrate, cells were preincubated with 10 µmol/l CMFDA for 1 hour at 10 °C. Thereafter cells were washed twice with ice-cold HBSS and kept at 10°C in HBSS with 10 % sodium pyruvate. CMFDA is metabolised in the cells to glutathione-methylfluorescein (GS-MF). Then, GS-MF efflux from the cells was measured at 37°C by incubating with medium containing 10% sodium pyruvate in the absence or presence of 20 µM MK-571, 0.01 mg/ml GTE, or corresponding concentrations of EGCG, EGC, theanine, or caffeine in concentrations corresponding to 0.01, 0.1, or 1 mg/ml GTE. After 5, 10, 15, 20, 25

and 30 minutes, 200µl samples were removed and fluorescence was measured with a HTS 7000 Plus Bio Assay Reader (Perkin Elmer Ltd., Buckinghamshire, UK). Cellular accumulation after 30 minutes was assessed by lysing the cells with 1% Triton X-100 in PBS and fluorescence determination of the cell homogenate.

Using MTX as substrate, cells were washed twice with HBSS and preincubated without tracers in the presence or absence of 20 µM MK-571, 0.1 or 1 mg/ml GTE, or the corresponding concentrations of EGCG for 10 minutes at 37°C. Then cells were incubated under the same conditions in the presence of 11.8 µM MTX (0.3 µCi) and 488 µM [^{14}C]sucrose (0.3 µCi) for 30 minutes at 37°C. For the quantification of the cellular accumulation of MTX and [^{14}C]sucrose the cells were lysed with trypsin-EDTA and transferred to a vial containing 3 ml of scintillation cocktail (Insta-Gel, Packard Instrument B.V., NL). Finally, radioactivity was measured.

Data analysis

For statistical comparison, data of groups were compared by analysis of variance (ANOVA). The level of significance was P = 0.05. If this analysis revealed significant differences, pairwise comparisons within groups were performed by two-sided unpaired t-tests. P-values were adjusted by Bonferroni's correction for multiple comparisons.

3.4 Results

Cytotoxicity assay with LS-180 cells was performed to determine the concentration range of GTE without toxic effects (Figure 3.2a). Significant toxic effects were observed at 0.3 mg/ml GTE. Since toxicity itself might influence the expression level of transport proteins, it is important, that GTE concentrations used in mRNA expression studies exhibit no cellular toxicity. None of the GTE or EGCG concentrations exhibited toxicity on MDCK-MRP2 cells after incubation for 30 minutes (Figure 3.2b).

At confluence, LS-180 cells were incubated for 72 h with medium only, 10 µM rifampicin or with 0.01 mg/ml GTE. Quantitative real time PCR was performed to determine the mRNA expression of MDR1 and MRP2. Rifampicin showed a significant induction of MDR1 mRNA expression, whereas GTE did not alter MDR1 mRNA levels. MRP2 mRNA expression was not influenced by GTE. Unfortunately, rifampicin did not serve as a positive control and elevated MRP2 mRNA expression only slightly but not significantly (Figure 3.3).

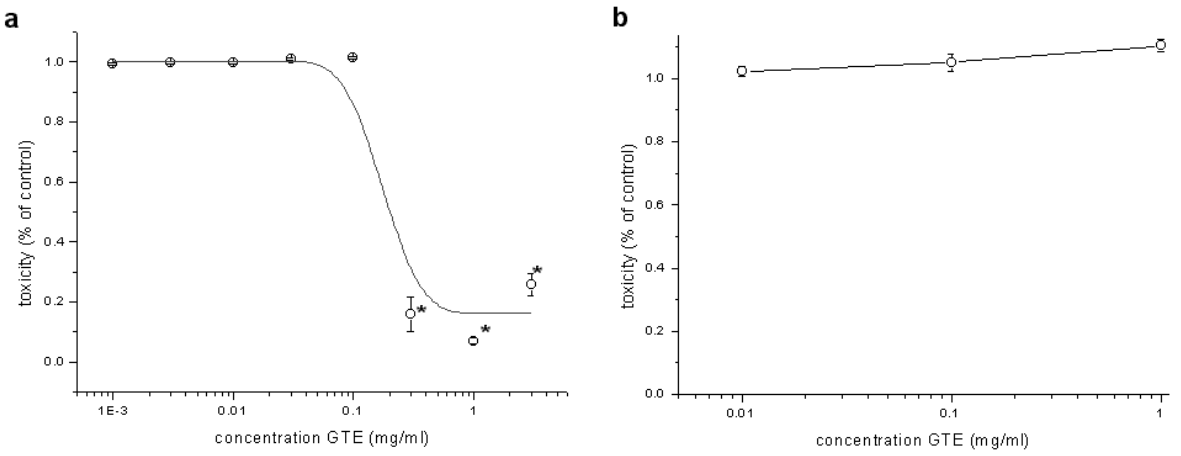

Figure 3.2: Dose-dependent toxicity of GTE in LS180 cells over 72 h (a) and MDCK-MRP2 cells over 30 min (b) using sulforhodamine B assay. Data represent mean values (± SEM) of 5 experiments. (* statistically significant different from control values, $p < 0.05$).

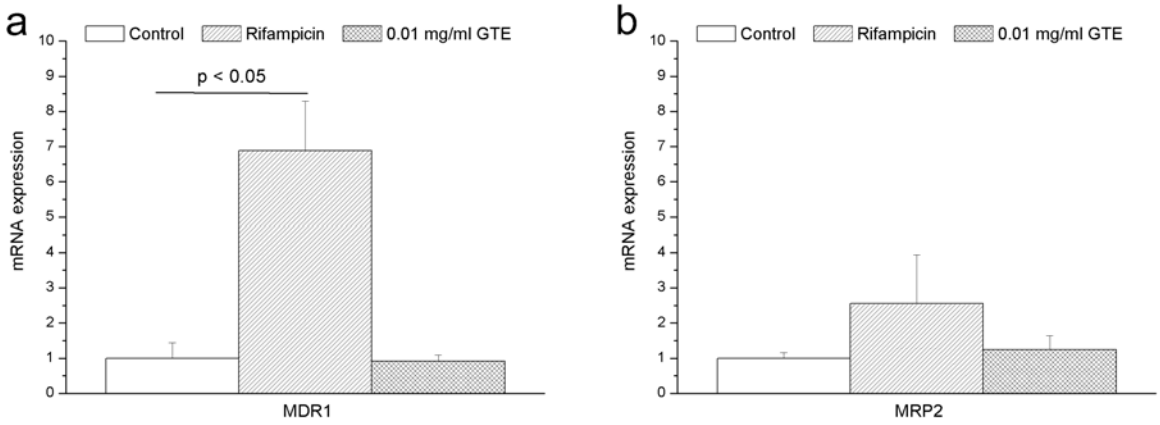

Figure 3.3: Relative mRNA expression of MDR1 (a) and MRP2 (b). Transcriptional expression was determined in LS-180 cells by quantitative real-time PCR. Cells were treated for 72h with medium only (black bars), or with either 10 μM rifampicin (dark grey bars) or with 0.01 mg/ml GTE (light grey bars). mRNA expression was relative to the respective control. MDR1 (n=3) data were generated in triplicate, MRP2 data represents the pooled results of 3 separate experiments (n=3). (* statistically significant difference, $p < 0.05$). Data represent means ± SEM.

To investigate the influence of GTE or the green tea components EGCG, EGC, theanine, or caffeine on the transport activity of MRP2, kinetic assays were performed in MDCK-MRP2 cells overexpressing MRP2. The cells were loaded with CMFDA, which is metabolised to glutathione-methylfluorescein (GS-MF), a specific substrate of MRP2. MK-571, a specific MRP-inhibitor, significantly increased the cellular accumulation of GS-MF, indicating a blockage of MRP2 function by MK-571. The cellular accumulation of GS-MF was not altered

by 0.01 mg/ml GTE or corresponding concentration of EGCG (Figure 3.4a-b). These results were confirmed by measurement of the extrusion of GS-MF, which was significantly decreased after treatment with MK-571, whereas 0.01 mg/ml GTE or the corresponding concentration of EGCG did not alter the extrusion of GS-MF in a relevant manner (Figure 3.4c-d). Concentrations of EGC, theanine or caffeine corresponding to 0.01, 0.1 and 1 mg/ml GTE showed no effect on GS-MF accumulation (Figure 3.5a-c).

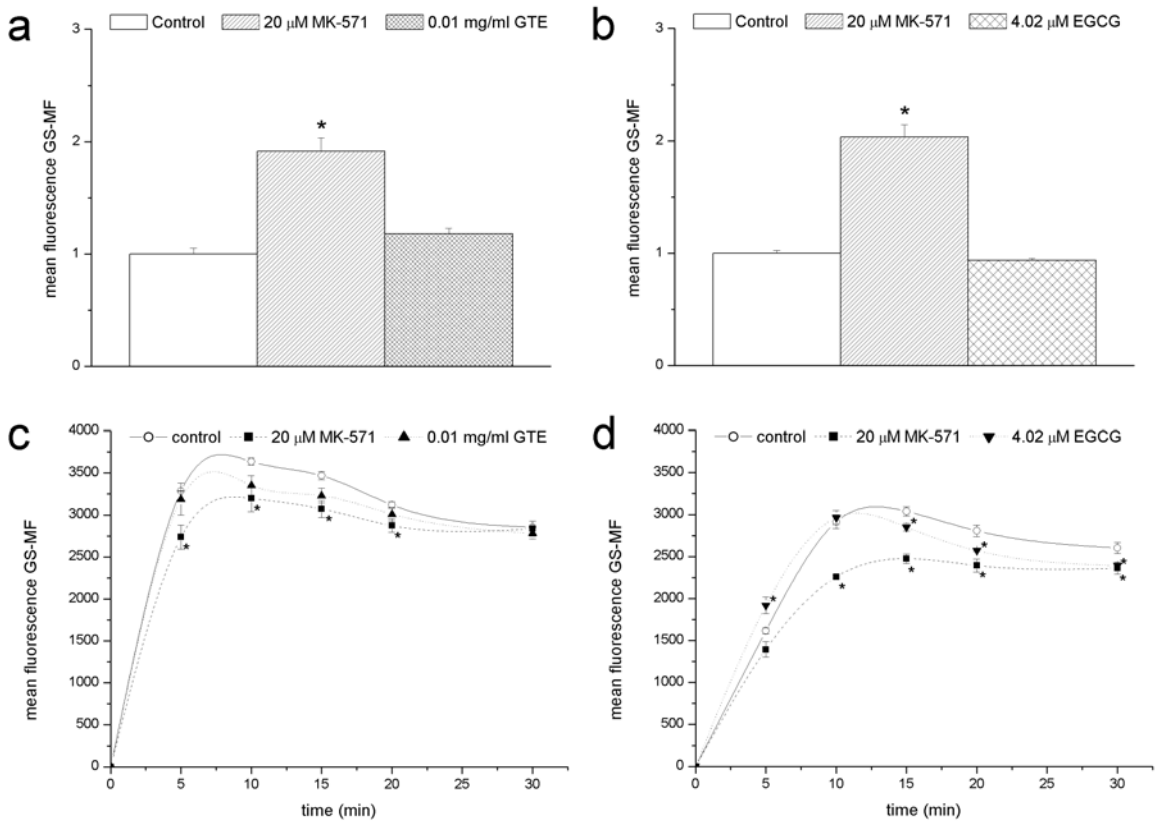

Figure 3.4: Accumulation of GS-MF in MDCK-MRP2 cells after incubation for 30 minutes at 37°C with medium only, with 20 μM MK-571, or either with GTE or corresponding concentration of EGCG (a-b). Time-dependent efflux at 37°C of GS-MF by MDCK-MRP2 cells during incubation with medium only, with 20 μM MK-571, or either with GTE or corresponding concentration of EGCG (c-d). (* statistically significant difference, $p < 0.05$). Data represent means ± SEM.

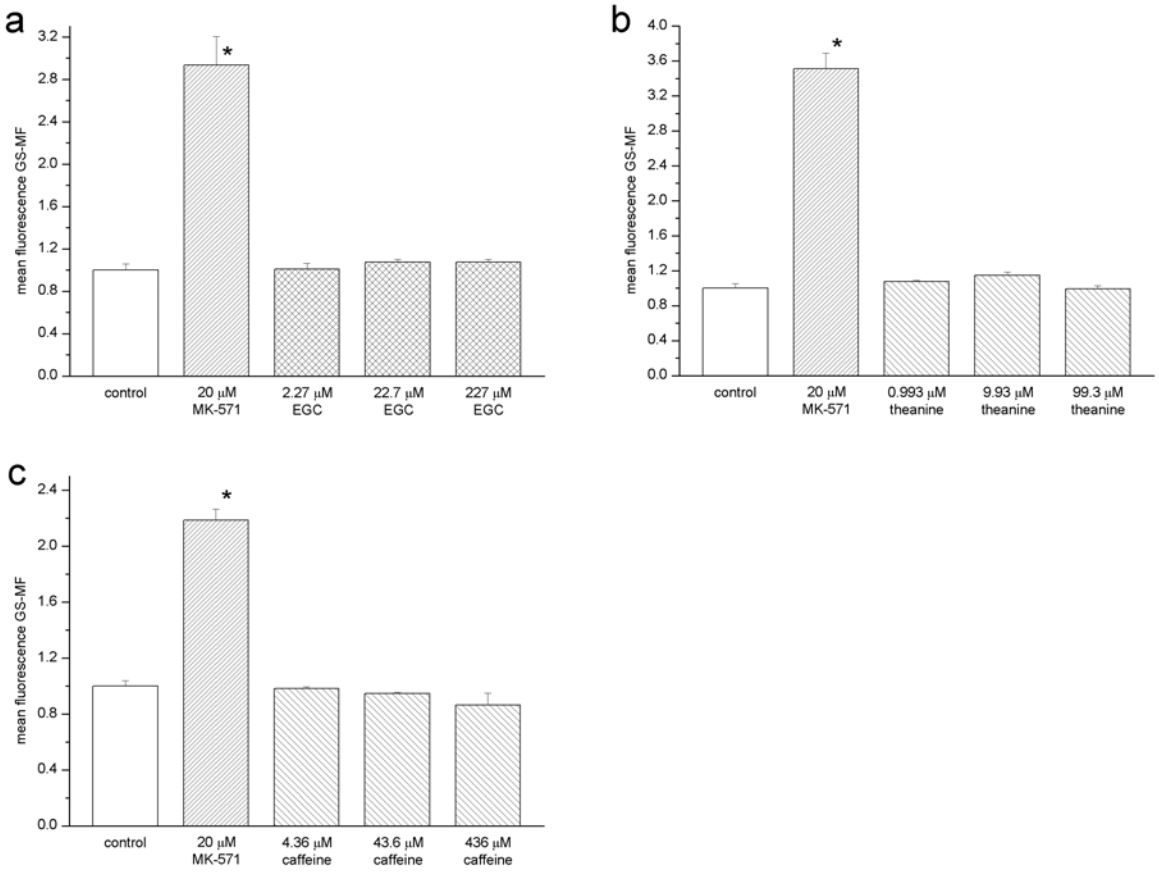

Figure 3.5: Accumulation of GS-MF in MDCK-MRP2 cells after incubation for 30 minutes at 37°C with medium only, with 20 μM MK-571, or either with EGC (a), theanine (b) or caffeine (c) in corresponding concentrations as in 0.01, 0.1, or 1 mg/ml GTE. (* statistically significant difference, $p < 0.05$). Data represent means ± SEM.

Due to significant autofluorescent activity of GTE at concentrations 0.1 and 1 mg/ml as well as significant quenching activities of 0.1 and 1 mg/ml GTE or corresponding EGCG concentrations with GS-MF (data not shown), functional assays in MDCK-MRP2 cells with these concentrations were performed with MTX as substrate (Figure 3.6). Therefore, cells were incubated with MTX, which is transported out of the cell via MRP2 but not via P-gp. MK-571 significantly decreased the efflux of MTX. GTE, at a concentration of 1 mg/ml, significantly increased the cellular accumulation of MTX by a factor of 1.7, suggesting a functional inhibition of MRP-2. Neither the corresponding concentration of EGCG nor the lower concentration of GTE or EGCG exerted an influence on MRP2 activity. The integrity of the monolayer barrier was not affected in these experiments as demonstrated by the measurement of [^{14}C]sucrose transport.

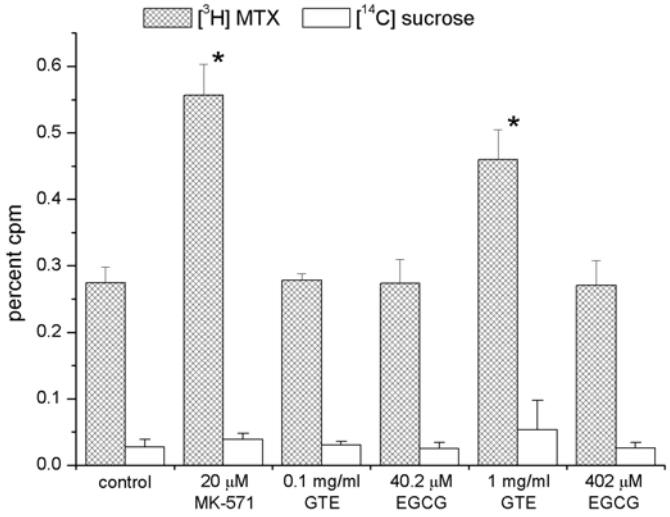

Figure 3.6: Cellular accumulation of MTX and [^{14}C]sucrose in MDCK-MRP2 cells after incubation for 30 minutes at 37°C with medium only, with 20 μM MK-571, or either with GTE or corresponding concentrations of EGCG. (* statistically significant difference, $p < 0.01$). Data represent means ± SEM.

3.5 Discussion

Intestinal cellular drug availability might be strongly modulated by P-gp and MRP2 function. Both are abundantly expressed in intestinal epithelial cells and show partly overlapping substrate specificities (Luo et al., 2002; Marbeuf-Gueye et al., 1998). The antibiotic rifampicin has been shown to significantly increase intestinal MDR1 and MRP2 expression (Greiner et al., 1999; Fromm et al., 2000). To investigate induction of MDR1 mRNA expression, LS-180 cell line has been shown to be a suitable model (Schuetz et al., 1996; Pfrunder et al., 2003). However, induction of MRP2 has not been demonstrated yet. Recently, inhibitory effects on P-gp function by green tea components have been reported in the literature (Jodoin et al., 2002; Sadzuka et al., 2000), whereas no data about the influence of green tea on MRP2 is available yet. Therefore, this study was designed to investigate the influence of GTE on the expression pattern of MDR1 and MRP2 as well as to examine the potential effect of GTE and several green tea components on the functional activity of MRP2.

Cytotoxicity assay demonstrated that 0.01 mg/ml GTE was in the non-toxic range for LS-180 cells after an incubation for 72h. Thus, artefacts of mRNA induction experiments due to cellular detoxification activities were prevented. No significant induction of MDR1 or MRP2 mRNA expression by GTE was observed in LS-180 cells. Regarding the influence on MRP2 protein functionality in MDCK-MRP2, neither 0.01 to 0.1 mg/ml GTE nor the green tea components EGCG, EGC, caffeine, or theanine, each in corresponding concentrations to GTE, exerted any appreciable influence on the MRP2-mediated export in comparison to the MRP-specific

inhibitor MK-571. At a concentration of 1 mg/ml, GTE significantly inhibited MRP2 activity, whereas the green tea components used above did not show any effect. All GTE concentrations used for MDCK-MRP2 cells were in the non-toxic range.

In brief, 0.01 mg/ml GTE did not modulate mRNA expression of MRP2 or MDR1 in intestinal epithelial LS-180 cells. Therefore, our results indicate, that neither the intestinal absorption through MRP2 nor through P-gp is altered by a change in gene transcription by 0.01 mg/ml GTE. The efflux of MRP2-substrates including GS-MF and MTX was inhibited only at a high concentration of 1 mg/ml GTE. EGCG is the principal catechin in green tea and most of the effects associated with green tea might be mediated by this catechin. It is tempting to speculate, that other green tea components except EGCG, EGC, caffeine, or theanine are responsible for the demonstrated inhibition of MRP2 function.

Acknowledgements

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4. Induction of CYP1A by green tea extract in human intestinal cell lines

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4.1 Abstract

In this study the influence of green tea extract (GTE) or its component epigallocatechin gallate (EGCG) on the expression of different cytochrome P450 (CYP) isoenzymes was investigated in the human gastrointestinal epithelial cell lines LS-180 and Caco-2. Additionally, the effect of GTE or EGCG on functional activity of different CYP isoenzymes was investigated in vitro. mRNA expression levels were determined by quantitative RT-PCR and compared with protein levels. In LS-180 cells GTE, but not EGCG, significantly induced CYP1A2 mRNA expression, whereas neither CYP1A1 nor CYP3A4 mRNA expression was modulated by GTE or EGCG. In Caco-2 cells CYP1A1 as well as CYP1A2 mRNA expression was significantly increased in a dose-dependent manner by GTE and EGCG. However, EGCG alone was about 3-5 fold less effective than GTE. mRNA expression of CYP1A1 or CYP1A2 induced by the promutagen benzo[a]pyrene was significantly down-regulated by EGCG but not by GTE. CYP1A protein levels in response to GTE in Caco-2 and LS-180 cells confirmed the mRNA expression results. CYP activity was measured with CYP1A2 or CYP3A4 expressed in insect cell membranes using a luminescent method. GTE or EGCG significantly inhibited CYP1A2 and CYP3A4 function in a dose-dependent manner. Therefore, it appears that green tea moderately modulates the expression of drug-metabolising enzymes but non-specifically inhibits the function of human CYPs. Since CYP enzymes play an important role in detoxification processes, these results might be of relevance concerning chemopreventive strategies using GTE.

4.2 Introduction

Cytochrome P450 (CYP) enzymes play a pivotal role in detoxification processes of the cell by metabolising xenobiotics. The cellular detoxification potential can be modulated by xenobiotics through induction of CYP expression or their inhibition. Hence, to assess the potential of a drug to interact with detoxification processes, early in vitro experiments are desirable. The mucosa of the small intestine represents an important determinant of drug metabolism due to the capability of metabolising orally ingested xenobiotics prior to systemic uptake (Kaminsky and Zhang, 2003). Accordingly, widely used intestinal in-vitro models, such as Caco-2 or LS-180, are equipped with different cytochrome P450 (CYP) isoforms including CYP1A1, CYP1A2, and CYP3A4 (Borlak and Zwadlo, 2003; Schmiedlin-Ren et al., 1997; Li et al., 1998; Schuetz et al., 1996). The principal CYP occurring in enterocytes, CYP3A4, has been associated with the metabolic elimination of many drugs, thereby substantially contributing to the poor oral bioavailability of some of these drugs (Hebert et al., 1992; Thummel et al., 1996). In humans extensive interindividual variability has been found for the intestinal expression of CYP1A1 and CYP1A2 (McDonnell et al., 1992; Lindell et al., 2003). Carcinogens like

polyaromatic hydrocarbons present in cigarette smoke and cooked meat, such as benzo[a]pyrene (B[a]P), as well as heterocyclic amines found in fried or grilled meats and fish are metabolised by CYP1A (Knize et al., 1999; Gautier et al., 1996).

The role of green tea in cancer chemoprevention, meaning the ability to delay the onset of the carcinogenic process, has become an intense area of research over the past years (Weisburger and Chung, 2002). Protection against polyaromatic hydrocarbon-induced cancers by green tea has been demonstrated in different animal models (Katiyar et al., 1993; Xu et al., 1996).

In this study, the two colon carcinoma-derived cell lines Caco-2 and LS-180 were used. In both cell lines induction of xenobiotic metabolising enzymes by possibly carcinogenic xenobiotics has been shown (Borlak and Zwadlo, 2003; Li et al., 1998). LS-180 cells represent a more suitable model to investigate the inducibility of CYP3A4 by rifampicin than Caco-2 cells (Pfrunder et al., 2003).

The present study was undertaken to investigate whether green tea extract (GTE), or its major constituent epigallocatechin gallate (EGCG) (Figure 3.1), modulate the mRNA expression of intestinal CYP1A or CYP3A4. This was conducted by quantitative RT-PCR. Western blot analysis was performed to correlate CYP1A protein expression with the results from mRNA expression experiments. For comparative reasons two different human intestinal cell lines were used. Additionally, the implication of simultaneous treatment with B[a]P and GTE or EGCG on CYP1A mRNA expression was examined in Caco-2 cells. Finally, the influence of GTE or EGCG on CYP1A2 or CYP3A4 function was investigated using a luminescent method. Insight into the inducibility and functional inhibition of different human intestinal CYP isoforms by GTE might provide important information concerning dietary interactions with the human intestinal xenobiotic metabolism.

4.3 Materials and Methods

Materials

Standardized green tea special extract EFLA®942 (GTE) produced of the leaves of *Camellia sinensis* (L.) O. KUNTZE was from Frutarom Switzerland Ltd., Wädenswil, Switzerland. The manufacturing process has been described before (Chapter 2.2). (-)-Epigallocatechin gallate was from CHEMOS GmbH, Regenstauf, Germany. Benzo[a]pyrene and rifampicin were from Sigma, Switzerland. Furafylline and ketoconazole were from Becton Dickinson Ltd. Allschwil, Switzerland. All other chemicals were obtained from commercial sources in the highest quality available.

Cell cultures

The human colon adenocarcinoma cell lines LS-180 and Caco-2 were purchased from American Tissue Culture Collection (ATCC, Manassas, USA) and cultured in Dulbecco's MEM with Glutamax-I, supplemented with 10% (v/v) fetal calf serum (FCS), 1% non-essential amino acids, 1% sodium pyruvate and 50 μg/ml gentamycin. Per cell culture well, 10^6 cells have been distributed. At confluence, cell cultures were used for experiments. Replacement with medium containing the compound of interest was done every 24h. Only freshly prepared and filtered (0.2 μm) mixtures of GTE with medium were used. All cultures were maintained in a humidified 37°C incubator with 5% carbon dioxide in air atmosphere.

Colorimetric cytotoxicity assay

Cytotoxicity of GTE was screened in LS-180 or Caco-2 cells. LS-180 or Caco-2 cells were incubated for 72 hours in the absence or presence of 0.01, 0.05, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, and 1 mg/ml GTE, or corresponding concentrations (4.02, 20.1, 40.2, 60.3, 80.4, 100.5, 120.6, 140.7, 160.8, 180.9, 201, and 402 μM) of EGCG. The sulforhodamine B assay used for the measurement of cytotoxicity has been described before (Chapter 3.1.3).

Real time polymerase chain reaction (TaqMan assay)

For mRNA induction experiments, LS-180 cells at confluence were incubated either with or without 10 μM rifampicin or 10 μM B[a]P, or with 0.1, 0.25, or 0.4 mg/ml GTE, or with 40.2 or 100 μM EGCG for 72 h. For mRNA induction experiments Caco-2 cells at confluence were incubated either with or without 10 μM B[a]P, with or without 0.01, 0.1, 0.25, or 0.4 mg/ml GTE, or with or without 4.02, 40.2 or 100 μM EGCG for 24 h. RNA extraction, reverse transcription, DNA digestion, and PCR amplification was performed as described elsewhere (Chapter 3.1.3). For CYP1A1 detection the following primers and probe were used: 5`-CGCTATGACCACAACCACCAAGAACT-3` (probe), 5`-GTCATCTGTGCCATTTGCTTTG-3` (forward), and 5`-CAACCACCTCCCCGAAATTATT-3` (reverse). For CYP1A2 detection the following primers and probes were used: 5`-CACAGCCATCTCCTGGAGCCTCATGTA-3` (probe), 5`-CAATGACGTCTTTGGAGCAGGAT-3` (forward), and 5`-CAATCACAGTGTCCAGCTCCTTC-3` (reverse). For CYP3A4 detection the following primers and probes were used: 5`-TTCTCCTGGCTGTCAGCCTGGTGC-3` (probe), 5`-TCTCATCCCAGACTTGGCCA-3` (forward), and 5`-CATGTGAATGAGTTCCATATAGATAGA-3` (reverse).

All samples were run in triplicates and were quantified using a standard curve. Standards were generated by a serial dilution of PCR products of the appropriate gene. Fragments of cDNA corresponding to CYP1A1, CYP1A2 and CYP3A4 that covered the TaqMan primer/probe area were obtained by PCR amplification. To standardize the amount of sample cDNA added to the

reaction the calculated amount of the gene of interest was divided by the calculated amount of the constitutively expressed ribosomal 18S gene in the sample. These normalized amounts were then used to compare the relative amount of target in different samples. The expression level of the endogenous control gene 18S did not alter under any of the different treatments.

Protein extraction

Proteins were extracted by lysing a frozen (-20°C) cell pellet in 1ml protein extraction buffer (pH 7.4), containing 20 mM Tris-HCl, 0.5 mM Na_3VO_4 and 1 % (v/v) Igepal CA-630. Proteases were inhibited with 1mM PMSF, 8 μM leupeptin, 5 μM bestatin, 2 μg/ml aprotinin, 6 μM E-64 and 1 μM pepstatin. The suspension was centrifuged at 15000 rpm for 10 min at 4°C and supernatant was frozen at –70°C. Protein quantification was performed with the BCA Protein Assay (Pierce, Rockford, Il, USA). Samples were transferred into a 96-well microtiter plate and UV-detection was performed with a Spectra Max 250 (Molecular Devices Corp., Sunnivale, CA, USA) at 562 nm and compared to a heat-shock inactivated bovine serum albumin (BSA) standard.

Western blot

Samples of 150 μg protein from LS-180 cells or 100 μg protein from Caco-2 cells were diluted 1:1 with Laemmle dilution buffer and loaded on a 4% stacking and 7.5% separating acrylamide / bisacrylamide gel. Electrophoresis was performed in a Mini-Protean 3 cell (BioRad Laboratories AG, Reinach, Switzerland) with 80 V in the stacking gel and constant 120 V in the separating gel for 1 h. Running buffer (pH 8.5) contained 0.25 M Tris-HCl, 1.93 M glycine and 1% (w/v) SDS. Proteins were electrophoretically transferred to a 0.45 μm pore size pure nitrocellulose membrane using a Mini Trans-Blot Transfer Cell (BioRad Laboratories AG, Reinach, Switzerland). Transfer buffer (pH 8.3) contained 0.025 M Tris-HCl, 0.193 M glycine and 20 % (m/v) methanol. Blotting was performed with a constant current of 250 mA for 2.5 h and was controlled by staining the membrane with 0.1% ponceau S in 3% acetic acid. The membrane was blocked overnight at 4°C with 5% (w/v) skimmed milk powder in PBS containing 0.05% (v/v) Tween 20 (PBS-T).

Immunodetection of CYP1A2

Membranes were washed three times for 15 min in PBS-T and then incubated for 24 h at 4°C with the mouse anti-CYP1A2 monoclonal antibody (Gentest, Woburn, MA, US), diluted 1:1000 in PBS-T containing 1% (w/v) skimmed milk powder and 1% (m/v) BSA. After washing the membrane as mentioned above, incubation for 1 h at room temperature was performed with the secondary, horse-radish peroxidase conjugated rabbit-anti-mouse IgG antibody, diluted 1:500 in PBS-T containing 1% (w/v) skimmed milk powder and 1% (m/v) BSA. After washing

the membrane as mentioned above, immunodetection was performed using enhanced chemiluminescence reagent ECL (Amersham Biosciences, Buckinghamshire, UK) on a hyperfilm ECL with various light exposure times. Molecular weights were determined in comparison to a commercially available protein standard.

Enzyme assays

A standard incubation mix consistent of 100 mM (CYP1A2) or 200 mM (CYP3A4) potassium phosphate buffer (pH 7.4), a NADPH-regeneration system (2.6 mM NADP+, 6.6 mM $MgCl_2$, 6.6 mM glucose-6-phosphate, and 0.4 U/ml glucose-6-phosphate dehydrogenase) and the insect cell membrane fraction containing human CYP1A2 (0.5 pmol) or human CYP3A4 (1 pmol) together with NADPH-CYP reductase and cytochrome B5 were used in a final volume of 50 µl (Becton Dickinson Ltd., Allschwil Switzerland). For measurement of CYP1A2 demethylation activity 100 µM luciferin 6' methyl ether was used (Catalys Ltd. Wallisellen, Switzerland). For measurement of CYP3A4 debenzylation activity 50 µM luciferin 6' benzyl ether was used (Catalys Ltd. Wallisellen, Switzerland). Incubations were performed at room temperature for 30 minutes. To stop the reaction 50 µl luciferin detection reagent (Catalys Ltd. Wallisellen, Switzerland) was added. Control reactions to exclude an interaction of GTE or EGCG with membrane proteins were performed by preincubation of the substance of interest with insect membranes not harbouring the respective CYP isoenzyme. After incubation at room temperature for 20 minutes the formation of the luminogenic metabolite was quantified by recording luminescence with a HTS 7000 Plus Bio Assay Reader (Perkin Elmer Ltd., Buckinghamshire, UK).

Data analysis

For statistical comparison, data of groups were compared by analysis of variance (ANOVA). The level of significance was $P \leq 0.05$. If this analysis revealed significant differences, pairwise comparisons within groups were performed by two-sided unpaired t-tests. P-values were adjusted by Bonferroni's correction for multiple comparisons.

4.4 Results

Colorimetric cytotoxicity assays were performed to determine the non-toxic range of GTE and EGCG in LS-180 or Caco-2 cells. Figure 4.1 shows that concentrations of GTE ≤0.4 mg/ml or EGCG ≤200 µM did neither exert dramatic toxic effects in LS-180 cells nor in Caco-2 cells.

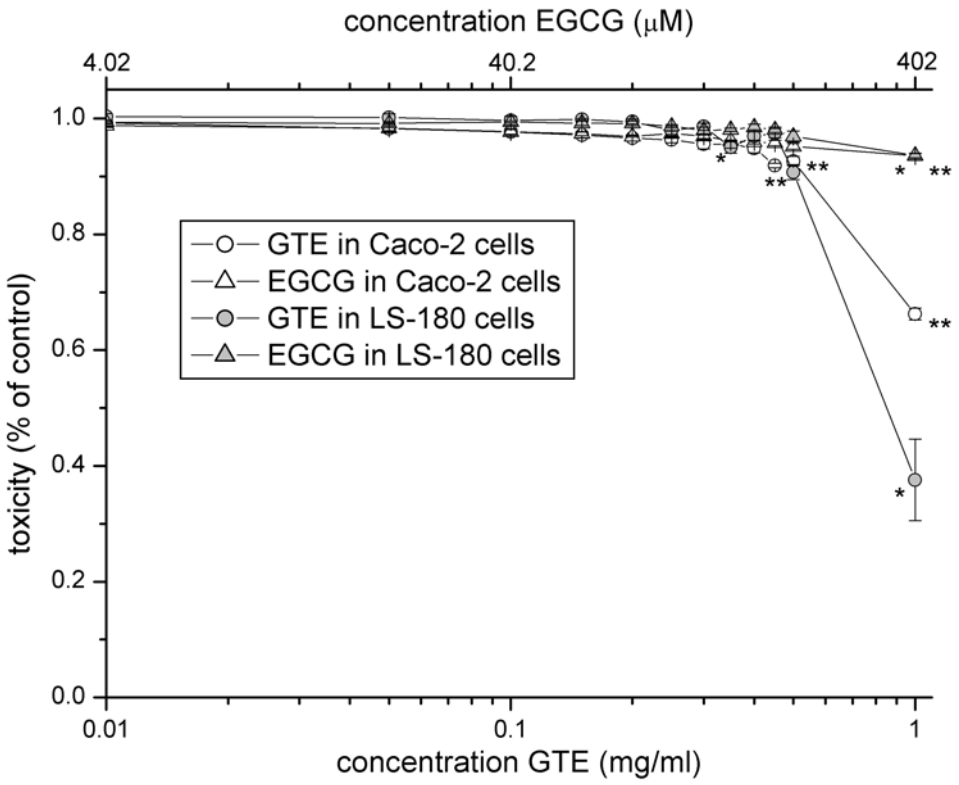

Figure 4.1: Dose-dependent toxicity of GTE and EGCG in LS-180 cells or Caco-2 cells over 72h using the sulforhodamine B assay. Data represents the mean values (±SEM) of 3 experiments. LS-180 cells: (*) statistically significant different from control values of ($p<0.005$); Caco-2 cells: (**) statistically significant different from control values ($p<0.0001$).

Quantitative real-time PCR was used to monitor mRNA expression of the different CYP isoforms. The average Ct values of the different cytochromes were 28.5 (CYP1A1), 34.0 (CYP1A2), and 35.4 (CYP3A4) in LS-180 cells and 27.9 (CYP1A1) and 33.8 (CYP1A2) in Caco-2 cells. For each single experiment, a standard curve was generated. Linearity was assumed, when the coefficient of correlation was greater than $r = 0.9$.

In LS-180 cells CYP1A2 mRNA expression was significantly induced about 7-fold by GTE concentrations of 0.25 and 0.4 mg/ml, whereas 0.1 mg/ml GTE or EGCG did not modulate mRNA expression (Figure 4.2). Neither GTE nor EGCG did alter CYP1A1 mRNA expression. B[a]P showed a significant induction of CYP1A1 and CYP1A2 mRNA expression. Rifampicin significantly induced CYP3A4 mRNA expression, whereas none of the GTE or EGCG concentrations did alter CYP3A4 mRNA levels.

In Caco-2 cells, GTE significantly induced CYP1A1 and CYP1A2 mRNA expression in a concentration-dependent manner up to 25-fold and 6-fold, respectively (Figure 4.3). Only the highest concentration of 100 μM EGCG significantly increased mRNA expression of CYP1A1 about 5-fold and CYP1A2 mRNA expression about 3-fold. B[a]P significantly increased CYP1A1 and CYP1A2 mRNA expression. The effect of B[a]P on mRNA levels of CYP1A1

was not modulated by coincubation with GTE or EGCG. Induction of CYP1A2 mRNA expression by B[a]P was significantly inhibited by 100 μM EGCG, whereas GTE or 4.02 to 40.2 μM EGCG had no effect (Figure 4.4). Due to the low levels of CYP3A4 mRNA expression in Caco-2 cells (Pfrunder et al., 2003) the possible influence on CYP3A4 mRNA expression by GTE was not investigated in this cell line.

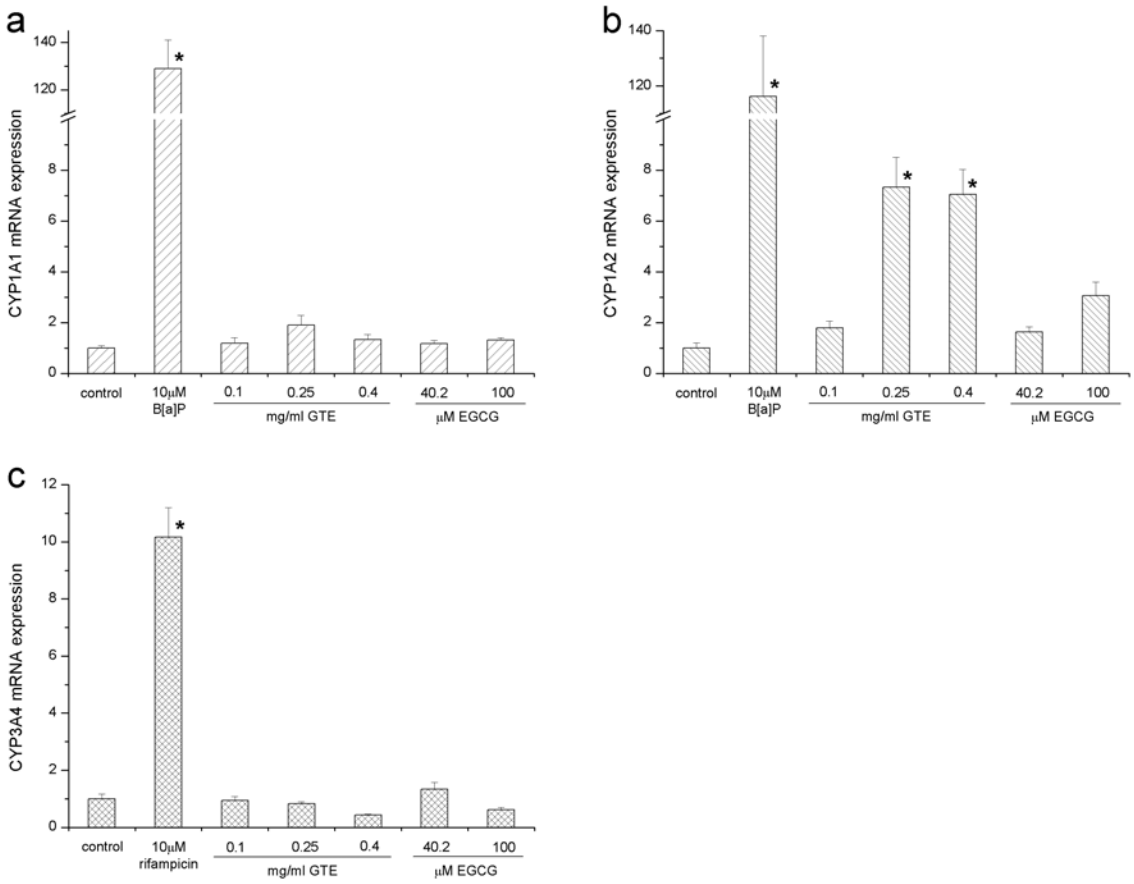

Figure 4.2: Relative mRNA expression of **(a)** CYP1A1, **(b)** CYP1A2 and **(c)** CYP3A4 in LS-180 cells. Transcriptional expression was determined by quantitative real-time PCR. Cells were treated for 72h with medium only, or with either 10 μM rifampicin or B[a]P, or with different concentrations of GTE or EGCG. mRNA expression was relative to the respective control. Data represents the pooled results of 3 separate experiments (n=3). (* statistically significant difference, $p < 0.01$). Data represent means ± SEM

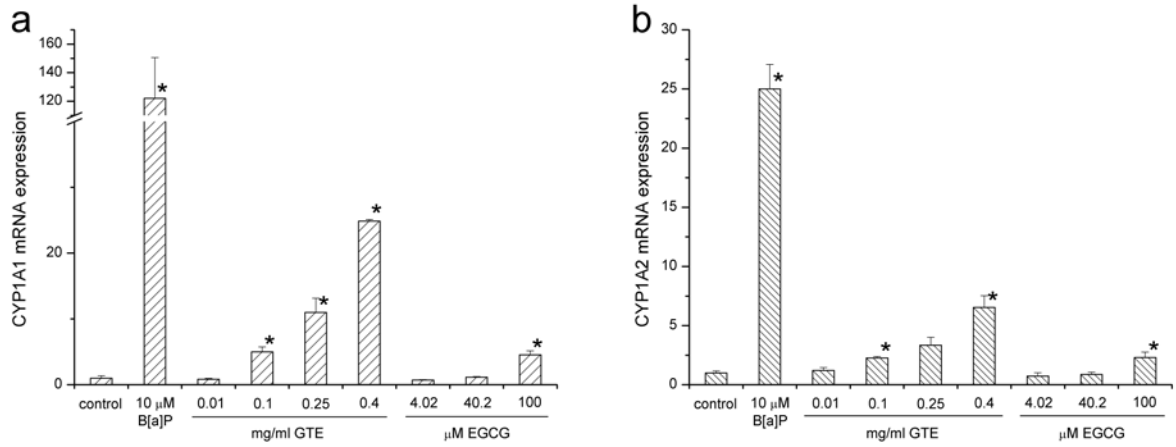

Figure 4.3: Relative mRNA expression of **(a)** CYP1A1 and **(b)** CYP1A2 in Caco-2 cells. Transcriptional expression was determined by quantitative real-time PCR. Cells were treated for 24h with medium only, or with either 10 μM B[a]P, or with different concentrations of GTE or EGCG. mRNA expression was relative to the respective control. Data represents the pooled results of 3 separate experiments (n=3). (* statistically significant difference, $p < 0.05$). Data represent means ± SEM

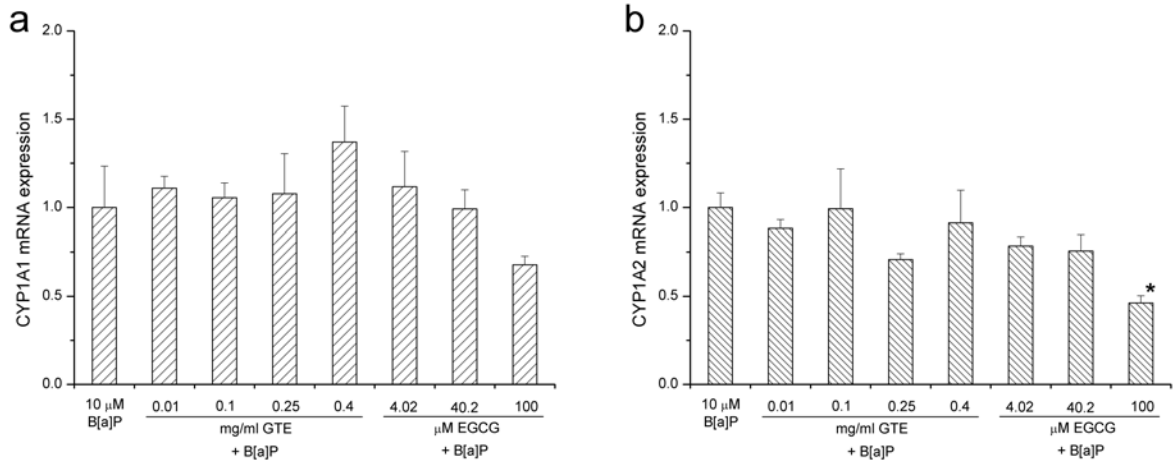

Figure 4.4: Relative mRNA expression of **(a)** CYP1A1 and **(b)** CYP1A2. Transcriptional expression was determined in Caco-2 cells by quantitative real-time PCR. Cells were treated for 24h with 10 μM B[a]P and with different concentrations of GTE or EGCG. mRNA expression was relative to the respective expression induced by B[a]P. Data represents the pooled results of 3 separate experiments (n=3). (* statistically significant difference, $p<0.05$). Data represent means ± SEM

Western blot analysis of total protein content from LS-180 or Caco-2 cells was performed to qualitatively determine whether the results of CYP1A1 and CYP1A2 mRNA expression experiments correlate with the respective levels of protein. Two major immunoreactive bands appeared (Figure 4.5), which are in accordance to literature data (Yang and Raner, 2005), indicated a cross-reactivity of the CYP1A2-antibody against CYP1A1. At confluence, LS-180 cells or Caco-2 cells were incubated with or without 10 μM B[a]P or 0.4 mg/ml GTE for 72h or

24h, respectively. Western blot results were analogue to mRNA induction experiments. Only CYP1A2 protein detected in LS-180 cells did not correlate to the observed mRNA induction by B[a]P and GTE.

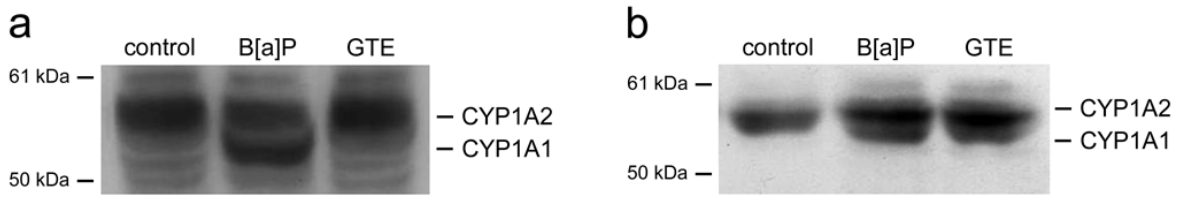

Figure 4.5: Western blot analysis of CYP1A1 (58.3 kDa) and CYP1A2 (58.2 kDa) expression in **(a)** LS-180 cells and **(b)** Caco-2 cells. Two bands are shown corresponding to CYP1A2 (upper band) and CYP1A1 (lower band). Total cell protein, preincubated for 24h with or without 10 μM B[a]P or 0.4 mg/ml GTE, was electrophoretically separated and examined by western blotting with anti-CYP1A2 antibody at 1:1000 dilution. Protein from LS-180 cells was applied at 150 μg in each lane, protein from Caco-2 cells was applied at 100 μg in each lane.

The effect of 0.01, 0.1, or 1 mg/ml GTE, or corresponding EGCG concentrations on CYP1A2 function was investigated (Figure 4.6a). GTE dose-dependently inhibited CYP1A2 activity. A reduction of activity of about 50% was observed for 0.1 mg/ml GTE and 402 μM EGCG. The highest concentration of GTE inhibited CYP1A2 activity at a similar extent as 100 μM furafylline. The influence of different GTE (0.01, 0.025, 0.05, 0.1, or 1 mg/ml) or EGCG (4.02, 40.2, 402 μM) concentrations on CYP3A4 activity was examined (Figure 4.6b). GTE and EGCG significantly inhibited CYP3A4 activity in a dose-dependent manner. The extent of inhibition was similar or higher than the inhibition found for 2.5 μM ketoconazole (KTZ).

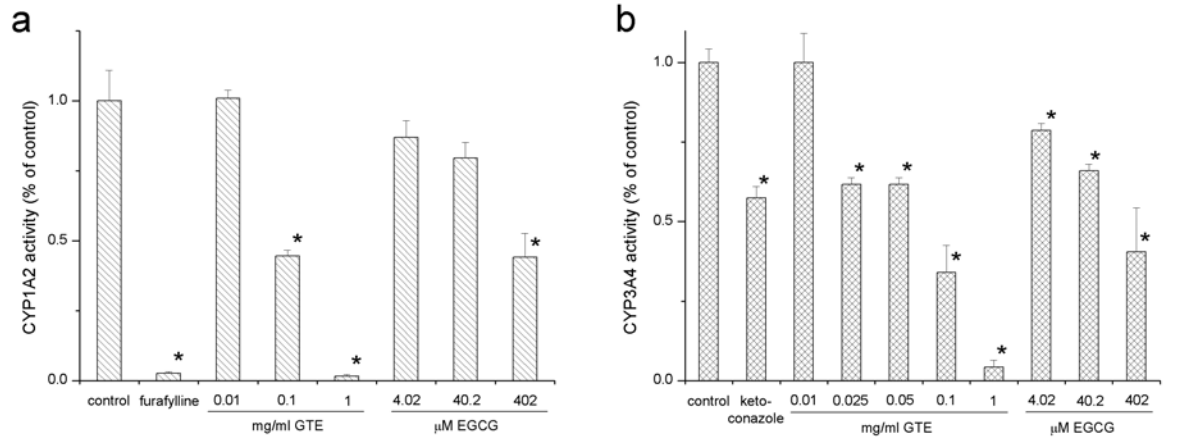

Figure 4.6: Dose-dependent functional inhibition of CYP1A2 and CYP3A4 by GTE or EGCG. **(a)** Luciferin 6' methyl ether (100 μM) was incubated with 0.5 pmol of human CYP1A2; **(b)** luciferin 6' benzyl ether (50 μM) was incubated with 1 pmol of human CYP3A4. Assays were performed at room temperature for 30 minutes. Insect cell membrane

harbouring each form of human CYP together with NADPH-CYP reductase and cytochrome B5 were used as enzyme sources. The luminogenic metabolite was quantified by recording luminescence. Data represents the pooled results of 3 separate experiments (n=3). (* statistically significant difference, $p<0.05$). Data represent means ± SEM

4.5 Discussion

Small intestinal CYP metabolism has the potential to substantially affect the toxicity and therapeutic efficacy of orally administered xenobiotics by limiting systemic uptake (Watkins, 1997). Therefore, our results might be of interest considering dietary interactions with medical therapies. However, in this study we showed that the mRNA expression patterns of CYP1A1 and CYP3A4 in LS-180 cells were neither altered by GTE up to a concentration of 0.4 mg/ml nor by EGCG up to 100 μM. In contrast, GTE significantly induced CYP1A2 mRNA expression up to 7-fold in LS-180 cells at concentrations above 0.25 mg/ml. Interestingly, in Caco-2 cells GTE significantly increased mRNA expression of CYP1A1 as well as of CYP1A2 in a concentration-dependent manner. Similar effects have been shown recently in human liver and tongue cells (Allen et al., 2001; Yang and Raner, 2005). In Caco-2 cells EGCG induced CYP1A1 and CYP1A2 mRNA expression at a concentration of 100 μM, although less extensive than GTE. Importantly, it has to be considered that even the highest GTE concentration exhibited about 5-fold less inductive potential on CYP1A mRNA expression than B[a]P.

Recently, it has been reported that GTE-mediated induction of CYP1A1 mRNA expression in the human hepatocyte derived cell line HepG2 is regulated via the aryl hydrocarbon receptor (AhR) pathway (Williams et al., 2000; Whitlock Jr., 1999). In this study significant B[a]P-mediated induction of CYP1A1 mRNA expression was detected in both cell lines whereas GTE induced CYP1A1 mRNA only in Caco-2 cells. This suggests that there might be differences between Caco-2 and LS-180 cells concerning regulatory proteins involved in the induction of CYP1A1 by GTE. This is in accordance to our observation that Caco-2 cells differ from LS-180 cells concerning the inductive potential of B[a]P. In LS-180 cells B[a]P induced mRNA expression of both CYP1A isoenzymes at a similar level. In Caco-2 cells, CYP1A2 mRNA expression was about 5-fold less induced by B[a]P than CYP1A1 mRNA expression, which ranged in a similar extent as in LS-180 cells. Interestingly, this feature was also found for the induction of CYP1A1 and CYP1A2 mRNA expression by GTE. A possible explanation for these differences might be that Caco-2 cells rather display similarities to human jejunal tissue, whereas LS-180 cells have retained their colonic characteristics (Taipalensuu et al., 2001; Pfrunder et al., 2003). We suggest that xenobiotic inducers of CYP1A increase CYP1A1 expression more pronounced than CYP1A2 expression in human cells displaying jejunal characteristics. This is supported by a report where ingestion of omeprazole induced

predominantly CYP1A1 mRNA expression in the duodenum of healthy volunteers (McDonnell et al., 1992). Additionally, our results indicate that the induction of CYP1A mRNA expression by GTE cannot be reduced to the activity of EGCG, as EGCG alone was less effective than GTE. This is consistent to in vivo results, where the induction of CYP1A2 by green tea in the rat was ascribed to its caffeine content (Chen et al., 1996).

When Caco-2 cells incubated with B[a]P were coincubated with GTE no change in CYP1A1 or CYP1A2 mRNA expression could be observed. This is partly in contrast to results where GTE inhibited 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-mediated CYP1A1 and CYP1A2 mRNA accumulation in HepG2 cells, probably via AhR-agonist activities (Williams et al., 2000; Whitlock Jr., 1999; Williams et al., 2003). Partly in accordance with this study were our results that 100μM EGCG inhibited B[a]P-induced CYP1A1 mRNA expression, although not significantly, and CYP1A2 mRNA expression. As mentioned above differences of regulatory proteins involved in the induction of CYP1A1 by GTE in Caco-2 might be responsible for this observation.

Results from the literature remain inconclusive regarding effects of green tea on CYP1A and CYP3A4 activities. Stimulatory as well as inhibitory activities in different *in vivo* or *in vitro* models have been described (Maliakal et al., 2001; Nishikawa et al., 2004; Yang and Raner, 2005). To overcome autofluorescent and quenching activities of GTE observed before (Chapter 3.1), we used luminogenic substrates for measurement of CYP1A2 or CYP3A4 activities. In accordance with the literature, we could detect a strong inhibitory effect at higher concentrations of GTE on CYP1A2 as well as on CYP3A4 function. This shows the non-specific inhibitory activity of GTE against human CYP isoforms. As EGCG alone exerted only half of the inhibitory activities in concentrations corresponding to GTE containing the same amount of EGCG, other components in GTE may contribute to this effect. One possible candidate represents the CYP1A2 substrate caffeine. These effects were concentration dependent. Low concentrations of GTE did not significantly change CYP1A2 or CYP3A4 activities. This was in accordance to an *in vivo*-study where CYP3A4 activity was not modulated after ingestion of GTE (Donovan et al., 2004).

In summary, our findings demonstrate that GTE, although in a moderate manner in comparison to B[a]P, differentially induces CYP1A expression at the mRNA and protein level in the intestinal cell lines LS-180 and Caco-2. Whereas in LS-180 cells GTE induced only CYP1A2 expression, in Caco-2 cells GTE increased both CYP1A1 and CYP1A2 expression. These effects could not be reduced to the green tea component EGCG. Therefore, it appears that green tea moderately modulates the expression of drug-metabolising enzymes. A significant inhibition of CYP1A2 and CYP3A4 activity by GTE or EGCG at higher concentrations was demonstrated. This inhibitory activity might reflect the chemopreventive character of GTE. Cell-specific differences in the extent of induction of the expression of the

single CYP1A enzymes were observed, which we suggest to be related to tissue characteristics of the cell lines LS-180 and Caco-2. This might be of interest in the assessment of appropriate cell models for investigations on gastrointestinal drug metabolism.

Acknowledgements

We are thankful to U. Behrens for excellent technical assistance.

5. Effect of green tea extract on tightness of intestinal epithelia

5.1 Introduction

Green tea is one of the most consumed beverages worldwide. Recent insights into beneficial/protective effects on life-style related diseases have rendered green tea preparations attractive for medicinal applications (Fujiki, 1999; Klaunig et al., 1999; Haqqi et al., 1999). Concerning possible modification of passive drug absorption, the purpose of this study was to clarify the influence of a green tea extract (GTE) and the green tea component (-)-epigallocatechin gallate (EGCG) on the intestinal epithelial barrier function. Under normal conditions, the intestinal epithelium serves as a highly selective barrier that permits absorption of water, electrolytes, and various nutrients from the lumen while simultaneously restricting the passage of larger, potentially toxic compounds of microbial origin (Madara and Stafford, 1989). Increased permeability of this barrier has been implicated in the pathogenesis of several gastrointestinal disorders including inflammatory bowel disease (Farhadi et al., 2003).

In a model for murine colitis protective effects of green tea have been demonstrated recently (Varilek et al., 2001). As EGCG has been shown to prevent the increase of epithelial permeability induced by interferon-γ (IFNγ) we hypothesised a similar effect of GTE on intestinal epithelial cells (Watson et al., 2004).

To assess the effect of GTE and EGCG, after preincubation for 72 hours, on the intestinal permeability we measured the apical to basolateral transport of [^{3}H]sucrose or [^{14}C]sucrose through Caco-2 monolayers over 5 hours. Caco-2 monolayers, which reflect many of the features of normal human small intestinal absorptive epithelium, have been widely used for studies of intestinal permeability (Meunier et al., 1995). IFNγ was demonstrated to increase the permeability of monolayers of intestinal epithelial cells in previous studies (Adams et al., 1993; Madara and Stafford, 1989). Because IFNγ receptors have been shown to be located in the basolateral membrane of Caco-2 cells (Unno et al., 1995), IFNγ was applied to the basolateral compartment for 72 hours ahead of the transport experiments. To elucidate a possible inhibitory effect of GTE or EGCG on the IFNγ-induced hyperpermeability of Caco-2 monolayers, coincubation of IFNγ in the basolateral compartment and GTE or EGCG in the apical or basolateral compartment prior to transport studies was performed. With respect to the physiological situation the transwell insert system seems a suitable model to mimic the separation of the intestinal lumen from the submucosa through the intestinal cell monolayer.

5.2 Materials and methods

Chemicals:

(-)-Epigallocatechin gallate (EGCG) was from CHEMOS GmbH, Regenstauf, Germany, human recombinant Interferon-γ (IFNγ) was from Sigma-Aldrich CO, St. Louis, MO, USA, [^{3}H]sucrose and [^{14}C]sucrose were from Amersham Pharmacia, UK. All other chemicals were obtained from commercial sources in the highest quality available.

Materials:

Standardized green tea special extract EFLA®942 (GTE) was from Frutarom Switzerland Ltd., Wädenswil, Switzerland. A brief manufacturing description was given in Chapter 2.2.

Cell culture

Caco-2 cells from the American Tissue Culture Collection (ATCC, Manassas, USA) were cultured in Dulbecco's MEM with Glutamax-I, supplemented with 10% (v/v) fetal calf serum (FCS), 1% non-essential amino acids, 1% sodium pyruvate, and 50 μg/ml gentamycin at 37°C in 5% CO_2. Only freshly prepared and sterile filtered (0.2 μm) mixtures of GTE or EGCG with medium were used. All cells used in this study were between passages 40 and 68.

Transport assay

Confluent monolayers of Caco-2 cells, which were grown in 0.4 μm pore Transwell-Clear filter inserts (no. 3460, Corning Costar), were incubated for 72 hours at 37°C in the presence or absence of 0.01 mg/ml or 0.1 mg/ml GTE, or 4.02 μM or 100 μM EGCG in the apical compartment. Cell culture medium or 1000 U/ml IFNγ with or without 100 μM EGCG was applied to the basolateral compartment. Control monolayers were incubated with cell culture medium only. Medium was changed every day. Transport experiments were performed in HBSS (pH 7.4) containing 10 % sodium pyruvate in the presence of absence of the substance of interest. Cells were washed twice, followed by an incubation at 37°C in the presence of 0.3 μCi [^{3}H]sucrose in the apical compartment. After 5, 15, 25, 35, 45, 60, 90, 120, 150, 180, 210, 240, and 300 minutes, 100μl samples were removed from the basolateral compartment. When cells were incubated at 37°C in the presence of 0.3 μCi [^{14}C]sucrose, 100μl samples were removed from the basolateral compartment after 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, and 360 minutes For the quantification of the [^{3}H]sucrose or [^{14}C]sucrose concentration the samples were transferred to a vial containing 3 ml of scintillation cocktail (Insta-Gel, Packard Instrument B.V., NL). Finally, radioactivity was measured. All data are expressed as means ± S.D. of quadruple experiments and each experiment was performed twice.

Cytotoxicity:

Cytotoxicity of GTE and IFNγ was screened in Caco-2 cells at confluence. Cells were incubated for 72 hours in the absence or presence of 1-10'000 U/ml IFNγ, respectively. Replacement with medium containing the compound of interest was done every 24 h. Cultures fixed with trichloroacetic acid were stained for 30 minutes with 0.4% sulforhodamine B (SRB) dissolved in 1% acetic acid. Unbound dye was removed by washing four times with 1% acetic acid and protein bound dye was extracted with 10 mM Tris buffer. Absorption was measured at 540 nm. (Spectra MAX 250, Microplate Spectrophotometer, Molecular Devices Corporation, California, USA) [12]. SRB absorption intensity correlates linearly with the number of vital cells. Every experiment was performed in quintuplicate and all experiments have been repeated at least twice.

Statistics:

For statistical comparison, data of groups were compared by analysis of variance (ANOVA). The level of significance was $P \leq 0.05$. If this analysis revealed significant differences, pairwise comparisons within groups were performed by two-sided unpaired t-tests. P-values were adjusted by Bonferroni's correction for multiple comparisons.

5.3 Results

To exclude artefacts during transport experiments in respect of reduced cell viability, cytotoxicity of IFNγ or GTE on Caco-2 cells was determined preliminary. The tested IFNγ concentrations (1-10'000 U/ml) did not exert toxic effects on Caco-2 cells (data not shown). No dramatic toxic effects in Caco-2 cells were observed at concentrations of GTE ≤0.4 mg/ml (see chapter 4).

The concentration of 0.01 or 0.1 mg/ml GTE, or 4.02 μM EGCG, was applied during the preincubation period of the transport studies and during transport assay. Control monolayers were incubated with medium only. In contrast to the effect of IFNγ, no significant modulation of [^{3}H]sucrose transport through Caco-2 monolayers was found for GTE or EGCG (Figure 5.1). The significant increase of transcellular transport of [^{3}H]sucrose induced by IFNγ in the basolateral compartment was not prevented by apical application of GTE or EGCG (Figure 5.2).

For transport studies with [^{14}C]sucrose Caco-2 monolayers were preincubated with or without IFNγ in the basolateral compartment and with or without 100 μM EGCG in the apical or basolateral compartment. Control monolayers were incubated with medium only. IFNγ significantly increased transcellular permeability of [^{14}C]sucrose, whereas EGCG decreased transcellular permeability no matter if added to the apical (Figure 5.3) or basolateral (Figure

5.4) compartment. EGCG did abrogate the IFNγ-mediated effect when coincubated with IFNγ. In contrast to the logarithmic curve obtained for [^{3}H]sucrose, the curve observed with [^{14}C]sucrose showed an exponential growth (Figure 5.3).

5.4 Discussion

Various epithelial functions are directly modulated by cytokines like IFNγ as described in several in vitro studies (Madara and Stafford, 1989; McKay and Baird, 1999). Green tea has been shown to inhibit activation of IFNγ-induced signal transduction (Menegazzi et al., 2001; Watson et al., 2004). Increased epithelial permeability and a concomitant increase in tissue levels of cytokines including IFNγ is common in inflammatory enteropathies (Desreumaux et al., 1997; Inoue et al., 1999). Therefore, strategies to prevent this cytokine-mediated hyperpermeability would be of therapeutic benefit.

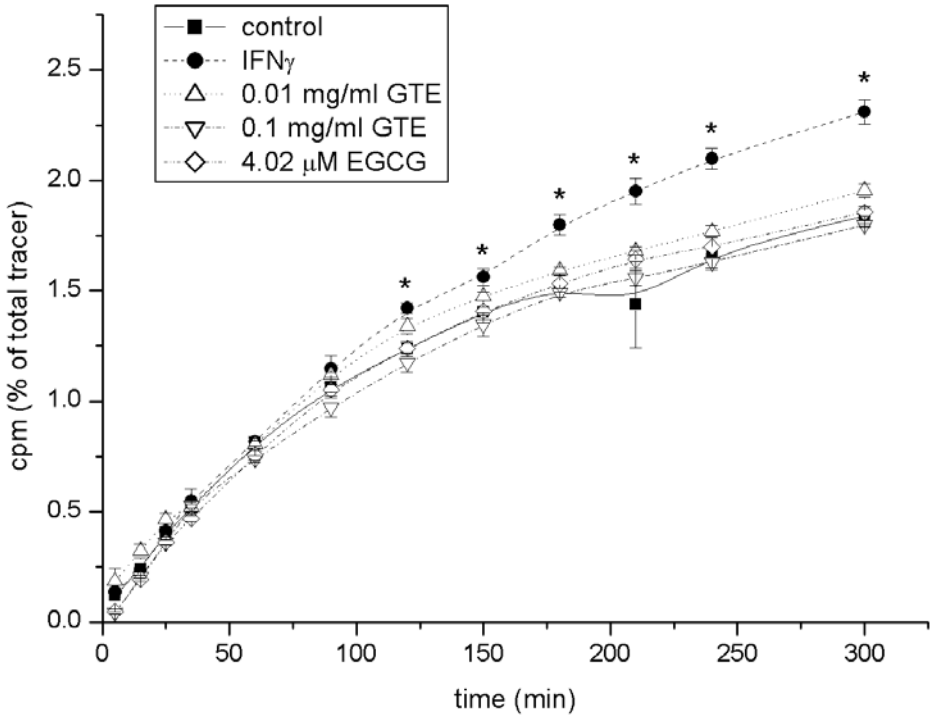

Figure 5.1: Time-dependent apical to basolateral transport of [^{3}H]sucrose after incubation with or without IFNγ basolaterally, or GTE or EGCG apically for 72 h.

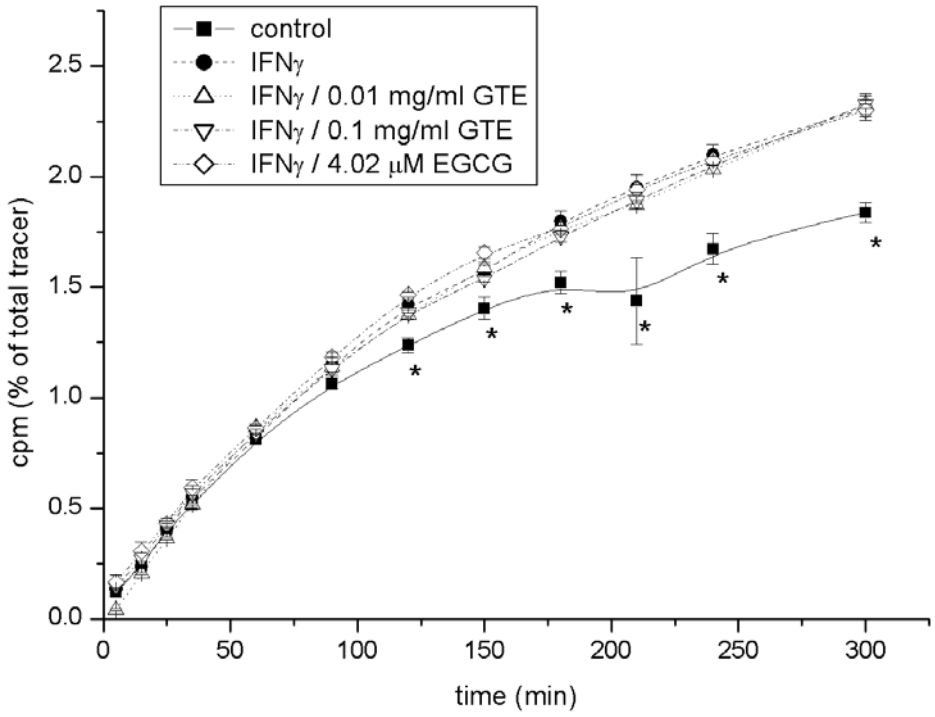

Figure 5.2: Time-dependent apical to basolateral transport of [^{3}H]sucrose after co-incubation of IFNγ basolaterally and GTE or EGCG apically for 72 h.

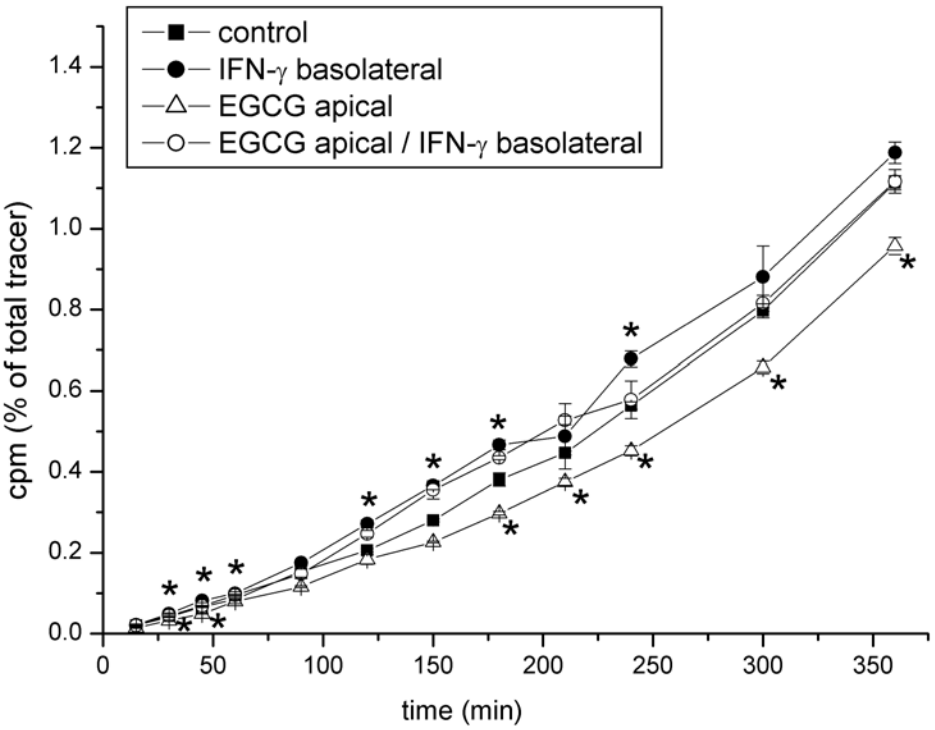

Figure 5.3: Time-dependent apical to basolateral transport of [^{14}C]sucrose after incubation with or without IFNγ and/or 100 μM EGCG for 72 h.

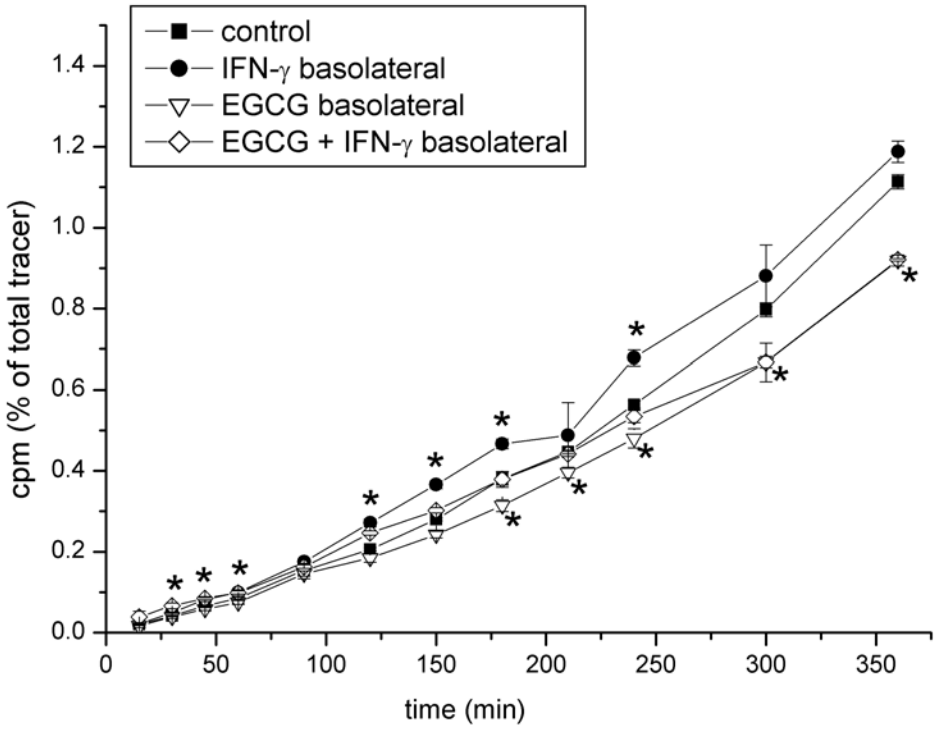

Figure 5.4: Time-dependent apical to basolateral transport of [^{14}C]sucrose after incubation with or without IFNγ and/or 100 μM EGCG for 72 h.

In our study using Caco-2 cells, 100 μM EGCG was shown to inhibit apical to basolateral [^{14}C]sucrose transport no matter if applied apical or basolateral. IFNγ-induced hyperpermeability was blocked by EGCG as described for T84 cells (Watson et al., 2004). Interestingly, while apical applied EGCG only reduced IFNγ-mediated leakiness to basal levels, basolateral co-incubation of EGCG along with IFNγ showed a decreased permeability as observed for EGCG alone. This discrepancy might be due to an apical efflux of EGCG mediated by MRP2 or P-gp as described in the literature (Hong et al., 2002; Jodoin et al., 2002). At lower concentrations, neither EGCG nor GTE did modulate basal or INFγ-mediated hyperpermeability when [^{3}H]sucrose was used as substrate. Remarkably, it has to be noted that the two substrates, [^{14}C]sucrose and [^{3}H]sucrose, showed different permeability characteristics through the Caco-2 cell monolayer. As this is not related to the sucrose molecule itself, we suggest as cause a decomposition of sucrose-bound tritium with protons of H_2O in the storage solution. The increase in the rate of decomposition during storage is characteristic of many tritium labelled compounds. As expected, a rapidly increasing radioactivity towards a limit of equal distribution was observed. However, this demonstrates the passive diffusion of water from the medium through the Caco-2 cell monolayer. In

accordance to the literature, in all experiments the overall penetration rate was very low, with about 1% of total tracer after 5 h of incubation (Watson et al., 2004).

In summary, higher EGCG concentrations reduced basal and IFNγ-induced hyperpermeability of Caco-2 cell monolayers. Nevertheless, regarding the low penetration rate of the marker substrate we suggest that this effect may not be of relevance *in vivo*. Therefore, these results suggest that ingestion of green tea or GTE does not influence absorption of low molecular weight constituents in the intestine.

6. Side project: Influence of green tea extract on intestinal cytokine expression and secretion

6.1 Green tea extract or its constituent (-)-epigallocatechin gallate induce interleukin-8 (IL-8) mRNA and protein expression but specifically inhibit IL-8 secretion in Caco-2 cells

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6.1.1 Abstract

The chemokine interleukin (IL)-8 is a cytokine involved in neutrophil attraction and activation and elevated levels have been observed in intestinal inflammation. Anti-inflammatory activities have been attributed to green tea or its major constituent (-)-epigallocatechin gallate (EGCG). In this study, we investigated the effects of a defined green tea extract (**1**) or EGCG (**2**) on basal or IL-1β-induced IL-8 expression and secretion in the human gastrointestinal epithelial cell line Caco-2. mRNA expression levels were determined by quantitative RT-PCR. **1** and **2** significantly induced IL-8 mRNA expression, which was not mediated indirectly via an induction of IL-1β mRNA expression. Intracellular and extracellular protein levels were analysed by enzyme-linked immunosorbent assay. **1** and **2** significantly decreased secreted IL-8 concentrations. Determination of intracellular and secreted IL-8 concentrations after 24 h, 48 h, and 72 h of incubation suggested that **1** specifically inhibited IL-8 secretion while inducing new synthesis of IL-8. IL-1β-mediated increase of IL-8 secretion was significantly inhibited by **1** in a dose-dependent manner. At highest concentration, **1** inhibited IL-1β-induced IL-8 secretion in a similar extent as found for brefeldin A, a fungal inhibitor of vesicular transport. These results suggest that **1** may exert an anti-inflammatory activity in enterocytes, which may be useful for the treatment of intestinal inflammation.

6.1.2 Introduction

Intestinal epithelial cells play an important role in the enteric immune system. This is achieved by forming a physical barrier against the microbial flora as well as by their ability to initiate a transient inflammatory response (Yang et al., 1997). Accordingly, intestinal epithelial cells secrete a wide array of inflammatory mediators such as cytokines, e.g. IL-1β, and chemokines, e.g. IL-8, that are able to induce an inflammatory state in intestinal cells or to attract inflammatory immune cells (Yang et al., 1997; Woywodt et al., 1994). An increase in the number and activity of inflammatory cells in the gut mucosa contributes, by their physical presence and secreted products, to the inflammation associated tissue damage and ulceration. Accordingly, intestinal inflammatory response needs to be tightly regulated as dysregulation of inflammation has been observed in inflammatory bowel diseases (IBD) including Crohn's disease and ulcerative colitis (Papadakis and Targan, 2000). This has been demonstrated by several studies where elevated levels of inflammatory chemokines such as interleukin (IL)-8 in intestinal tissues of IBD patients have been described (MacDermott et al., 1998; Puleston et al., 2005). IL-8 is a member of the CXC family of chemokines and plays a critical role in neutrophil recruitment and activation (Baggiolini et al., 1994; Baggiolini, 1998). IL-8 can be produced by different cell types, including the human gastrointestinal epithelial cell line Caco-2, and has been implicated in the initiation and maintenance of the inflammatory

response (Harada et al., 1994; Eckmann et al., 1993). Furthermore, the proinflammatory cytokine IL-1β represents a pivotal player in the intestinal inflammatory response (Ligumsky et al., 1990). Its significance in IBD has been demonstrated by the increased expression in affected tissues and the attenuation of its damaging properties by the IL-1 receptor antagonist (Cominelli et al., 1992; Casini-Raggi et al., 1995). Accordingly, the maintenance of a normal mucosal steady state is dependent on mechanisms that control the tissue concentrations of inflammatory chemokines and cytokines. The extracellular concentration of these inflammatory mediators can be, at least in part, regulated on two levels in the cell, the new synthesis of the mediators as well as the secretion of newly synthesized mediators.

The physiological close proximity of luminal nutrients to intestinal cells may favour the influence of these nutrients on biological processes within the intestinal mucosa. Natural compounds including green tea [dried leaves of the plant *Camellia sinensis* (L.) O. KUNTZE (Theaceae)] have been shown to modulate inflammation in vitro and in vivo (Chen et al., 2003; Varilek et al., 2001). Recently, inhibitory effects of green tea polyphenols on IL-8 expression and secretion have been reported in intestinal epithelial cells, respiratory epithelial cells, endothelial cells, and keratinocytes (Porath et al., 2005; Chen et al., 2002; Tang and Meydani, 2001; Trompezinski et al., 2003).

In the present study we examine whether a defined commercially available green tea extract (**1**), or its main constituent EGCG (**2**), are capable to modulate basal or IL-1β-induced mRNA expression of the chemokine IL-8 in the human colonic adenocarcinoma cell line Caco-2. Furthermore, we assessed the effect of **1** and **2** on the secretion of IL-8 protein in Caco-2 cells. Due to the inhibition of IL-8 secretion by **1** we investigated the time-dependent effect of **1** on the concentration of newly synthesized IL-8 protein in the cell and the extracellular concentration of secreted IL-8. Finally, we evaluated the influence of **1** on IL-1β-induced IL-8 mRNA expression and IL-8 protein secretion in Caco-2 cells. The observed inhibition of IL-1β-induced IL-8 secretion was compared with the effect of a strong inhibitor of secretion, the fungal derivative brefeldin A (BFA).

6.1.3 Results and discussion

Effect of GTE (1) or EGCG (2) on basal or IL-1β-induced IL-8 mRNA expression in Caco-2 cells. As shown in Figure 6.1, IL-8 mRNA expression was significantly induced by **1** in a dose-dependent manner up to 6-fold after an incubation period of 24 h. A significant increase in IL-8 mRNA expression (about 2-fold) occurred with **2** only at the highest concentration. This was in contrast to a recently reported EGCG-mediated reduction of IL-8 mRNA levels in two intestinal cell lines, HT-29 and T84 (Porath et al., 2005). In accordance to the literature, the positive control IL-1β showed a significant induction of IL-8 mRNA expression (Haller et al.,

2000). The induction of IL-8 mRNA expression by IL-1β was in the same magnitude as found for the highest concentration of **1**.

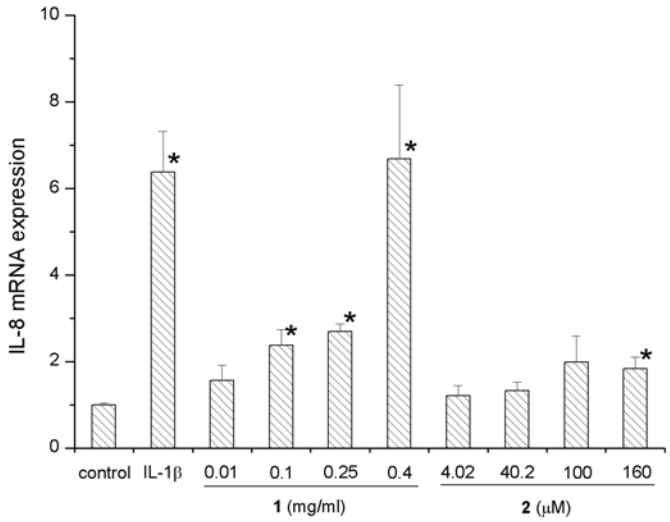

Figure 6.1: Effect of compound **1** or **2** on IL-8 mRNA expression in Caco-2 cells after incubation for 24 h. Transcriptional expression was determined by quantitative real-time PCR. Each data value is expressed as a percentage of the control. Data represent the pooled results of 3 separate experiments (n=3) and the bars represent means ± SEM. An asterisk (*) indicates $p < 0.05$ when compared to the control.

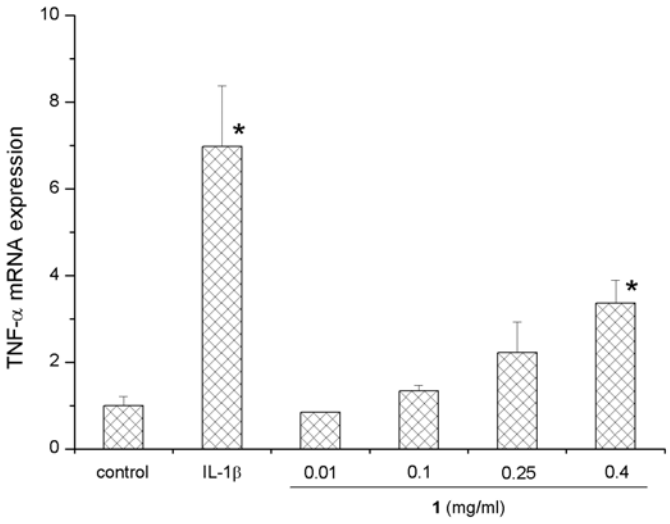

Figure 6.2: Effect of compound **1** on TNF-α mRNA expression in Caco-2 cells. Transcriptional expression was determined by quantitative real-time PCR. Compound treatment time and data presentation are the same as described in Figure 6.1.

In a next step the induction of tumour necrosis factor (TNF)-α mRNA expression was investigated. A significantly induction of TNF-α mRNA expression was observed for the positive control IL-1β (about 7-fold), which has been reported before, while only the highest concentration of **1** significantly induced TNF-α mRNA expression about 3-fold (Figure 6.2) (Vallee et al., 2004). TNF-α mRNA expression levels were not significantly altered by **2** up to 100 μM (data not shown).

To exclude that the observed induction of IL-8 and TNF-α mRNA expression by **1** is mediated by an induction of IL-1β expression, the influence of **1** on IL-1β mRNA expression levels were determined. While **1** did not alter IL-1β mRNA expression levels, IL-1β significantly induced its own mRNA expression (about 2-fold) as described in the literature (data not shown) (Jobin et al., 1997). Besides, under the same experimental conditions **2** also did not change IL-1β mRNA levels (data not shown).

The stability of IL-8 mRNA has been described to be dependent on the presence of IL-1β in fibroblast cells (Stoeckle, 1990). By measurement of the effect of **1** on IL-8 mRNA stability we wanted to assessed if **1** post-transcriptionally regulates IL-8 mRNA in Caco-2 cells. Therefore, cells were incubated with 0.4 mg/ml of **1** for 24 h in order to obtain an induction of IL-8 mRNA expression. In a next step transcription was blocked with actinomycin D and the effect of **1** on the amount of IL-8 mRNA was detected after 7 h by quantitative real-time PCR. Our results showed that **1** does not significantly modulate IL-8 mRNA decay in Caco-2 cells (data not shown). Therefore, the observed induction of IL-8 mRNA expression by **1** was not related to an increase of IL-8 mRNA stability by **1**.

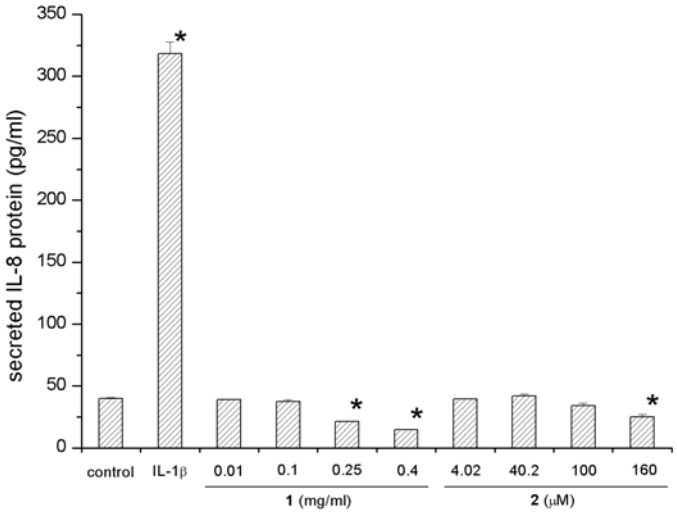

Figure 6.3: Effect of compound **1** or **2** on IL-8 protein secretion in Caco-2 cells after incubation for 24 h. IL-8 concentrations in the supernatants were determined by EIA. Data represent the pooled results of 3 separate

experiments (n=3) and the bars represent means ± SEM. An asterisk (*) indicates $p < 0.05$ when compared to the control.

Effect of GTE (1) on de novo synthesis and secretion of IL-8 in Caco-2 cells. Secreted IL-8 protein levels were significantly reduced by **1** at 0.25 mg/ml and 0.4 mg/ml after an incubation of 24 h as determined by enzyme-linked immunosorbent assay (EIA) (Figure 6.3). Basal concentrations of secreted IL-8 protein were about 40 pg/ml, which was significantly reduced by **1** to about 15 pg/ml. At highest concentration, **2** also reduced secreted IL-8 concentration, although to a lower extend than **1**. The positive control IL-1β significantly increased secreted IL-8 concentration to about 320 pg/ml, which is probably due to the observed induction of IL-8 mRNA expression. Therefore, the overall effect of **1** on basal IL-8 secretion seems to be of temperate manner in Caco-2 cells.

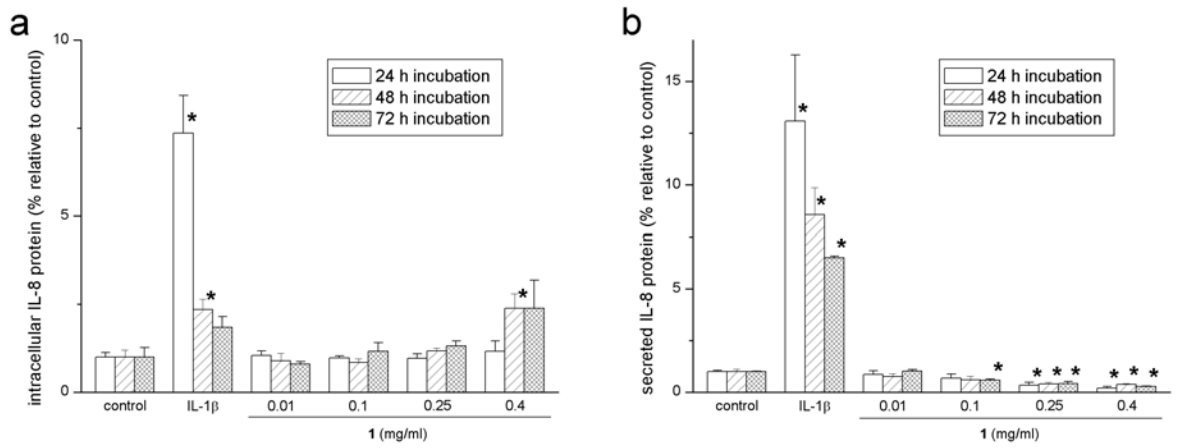

Figure 6.4: Time-dependent effect of compound **1** on intracellular and secreted IL-8 protein concentration in Caco-2 cells. Medium with or without the substance of interest was changed every 24 h. IL-8 concentrations in the (a) cell lysates or the (b) supernatants were determined by EIA. Data represents the pooled results of 3 separate experiments (n=3). Data presentations are the same as described in Figure 6.3.

As this inhibition of IL-8 protein secretion by **1** was in contrast to the observed induction of IL-8 mRNA expression, we wanted to clarify the effect of **1** on the IL-8 protein expression and the IL-8 protein secretion in Caco-2 cells. We therefore assessed the time-dependent effect of **1** on the intracellular and extracellular IL-8 concentration over a time period of 72 h. At highest concentration, **1** significantly increased intracellular IL-8 protein concentration after 48 h of incubation in Caco-2 cells (Figure 6.4a). This confirms the induction of IL-8 mRNA expression by **1** at the protein level and suggests a time lag of 24 h to 48 h between induction of transcription and resulting translation into protein. The significant inhibition of IL-8 protein

secretion by **1** was constant over 72 h (Figure 6.4b). However, incubations with IL-1β demonstrated that the increase of secreted IL-8 protein concentration after an incubation period of 24 h was related to an early phase induction of IL-8 expression. After a first increase, we found a significant time-dependent decrease of IL-8 protein expression during incubation with IL-1β, which accordingly resulted in decreased levels of secreted IL-8. The time-dependent down-regulation of IL-1β-mediated IL-8 expression has to be confirmed at the mRNA level. This desensitisation of IL-8 expression to IL-1β may be explained by an IL-1β-mediated induction of IL-1 receptor antagonist expression, which inhibit IL-1β-induced IL-8 production in Caco-2 cells as reported in the literature (Bocker et al., 1998; Garat and Arend, 2003).

To assess the specificity of the inhibition of IL-8 secretion by **1**, we additionally investigated the effect of **1** on secreted TNF-α protein levels in Caco-2 after an incubation time of 24 h (data not shown). According to the results from the mRNA induction experiments, **1**, at the highest concentration of 0.4 mg/ml, significantly increased secreted IL-8 protein concentrations about 2-fold and seems not to inhibit secretion. Therefore we suggest that **1** inhibits the secretion of IL-8 in Caco-2 cells specifically. However, it has to be noted that basal levels of secreted TNF-α protein were already relatively high (about 2 pg/ml). Therefore, the small increase from 2 pg/ml to 4 pg/ml mediated by **1** might not account for a proinflammatory activity of **1** via TNF-α induction (Komatsu et al., 2001).

Effect of GTE (1) on IL-1β-induced expression and secretion of IL-8 in Caco-2 cells. Finally, we wanted to investigate the effect of **1** on IL-1β-induced IL-8 secretion in Caco-2 cells. After a preincubation of the cells with IL-1β for 1 h, **1** was added to the medium for 23 h. We observed a significant decrease of IL-1β-induced extracellular IL-8 protein levels by **1** in a concentration-dependent manner (Figure 6.5). Similar results were obtained when IL-1β was co-incubated with **1** for 24 h without a preincubation phase (data not shown). As **1** did not significantly alter IL-1β-induced IL-8 mRNA expression (data not shown) or IL-1β-induced intracellular IL-8 protein concentration (Figure 6.6) after an incubation of 24 h, evidence is given that **1** inhibits the secretion of IL-1β-induced newly synthesized IL-8 protein. To estimate the strength of inhibition of IL-8 secretion by **1**, we compared the effects of **1**, at the highest concentration of 0.4 mg/ml, and brefeldin A (BFA). The lipophilic fungal antibiotic BFA is a potent inhibitor of protein secretion, which it inhibits at an early step in the secretory pathway (Klausner et al., 1992). **1** and BFA significantly inhibited IL-8 secretion induced by IL-1β in a similar extent in Caco-2 cells (Figure 6.6). Noteworthy, BFA seemed to induce IL-8 expression in Caco-2 cells as intracellular IL-8 protein concentrations were significantly increased.

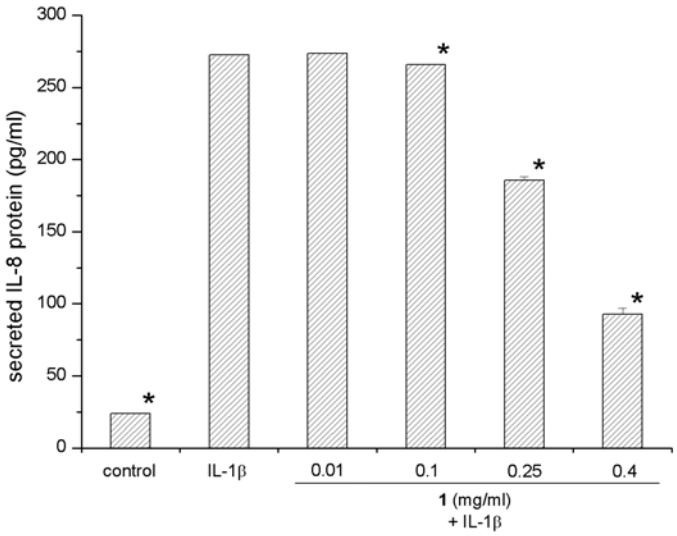

Figure 6.5: Concentration-dependent effect of compound **1** on IL-1β-induced IL-8 protein secretion in Caco-2 cells. Cells were pre-incubated at 37°C for 1 h with or without 10 ng/ml IL-1β. Subsequently, GTE was added to the medium followed by an incubation for 23 h. IL-8 concentrations in the supernatants were determined by EIA. Data represent the pooled results of 3 separate experiments (n=3) and the bars represent means ± SEM. An asterisk (*) indicates $p < 0.05$ when compared to IL-1β.

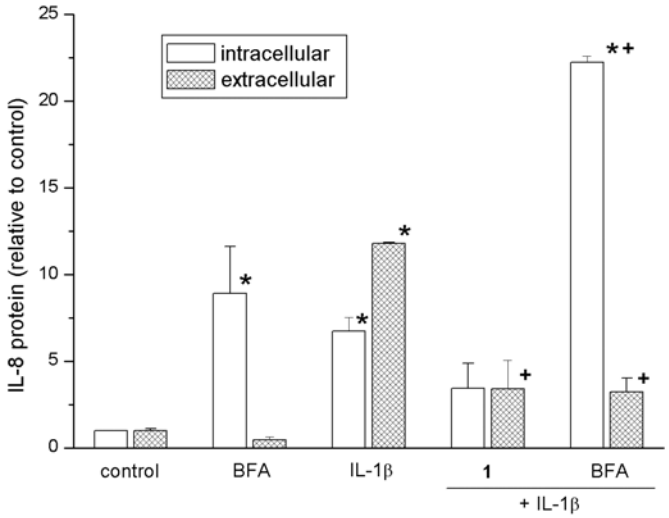

Figure 6.6: Comparison of the effect of compound **1** or brefeldin A (BFA) on basal or IL-1β-induced IL-8 protein expression and secretion in Caco-2 cells after an incubation for 24 h. IL-8 concentrations in the cell lysates or supernatants were determined by EIA. Data represent the pooled results of 3 separate experiments (n=3) and the bars represent means ± SEM. An asterisk (*) indicates $p < 0.05$ when compared to the control, a cross ($^{+}$) indicates $p < 0.05$ when compared to IL-1β.

While low plasma concentrations of green tea catechins have been reported, the gastrointestinal tract is likely to be exposed to higher concentrations of green tea constituents, irrespective of whether they are absorbed, retained, or recirculated to the gut tissues (Chow et al., 2001; Lee et al., 2002). This suggests that our results concerning the specific inhibition of IL-8 secretion found for **1** in the intestinal cell line Caco-2 might be of relevance *in vivo*. As **1** also inhibited IL-8 secretion in enterocytes activated with IL-1β, evidence is given for a chemopreventive potential of **1**. The production of chemokines, including IL-8, by epithelial cells and leukocytes establishes a chemotactic gradient within the intestine. This enables a migration of activated lymphocytes, macrophages, and granulocytes from the circulation into the mucosa as observed during intestinal inflammation (MacDermott, 1999). Therefore, the specific inhibition of IL-8 secretion by **1** in Caco-2 cells may be, at least in part, responsible for an anti-inflammatory activity of **1**. An attenuation of intestinal inflammation after orally ingestion of green tea polyphenols has been reported recently in a murine model of IBD (Varilek et al., 2001). Regarding the moderate effects of **2**, it is tempting to speculate that other green tea components are responsible for the demonstrated inhibition of IL-8 secretion by **1**. The results of this study may point to a beneficial effect of green tea in the prevention or treatment of intestinal inflammation. However, more preclinical and clinical studies are necessary to judge the relevance of our findings.

6.1.4 Experimental section

Experimental biologic materials:

Standardized green tea special extract EFLA®942 (**1**) was from Frutarom Switzerland Ltd., Wädenswil, Switzerland. Brief manufacturing description: leaves of *Camellia sinensis* (L.) O. KUNTZE are continuously extracted (percolation) with 80% (w w^{-1}) ethanol. After a patented filtration process (US 6024998) the crude extract is dried. Finally, 5% w w^{-1} maltodextrin is added as carrier. The drug to extract ratio (DER) is 5.5=1. Characteristic components in the extract are polyphenols (47.5-52.5% w w^{-1}), caffeine (5.0-10.0% w w^{-1}), theobromine (0.30-1.20% w w^{-1}), and theanine (1.0-3.0% w w^{-1}).

Cell culture and general experimental procedures

The human colon adenocarcinoma cell line Caco-2 was purchased from American Tissue Culture Collection (ATCC, Manassas, USA) and cultured in Dulbecco's MEM (DMEM) with Glutamax-I, supplemented with 10% (v v^{-1}) fetal calf serum (FCS), 1% non-essential amino acids, 1% sodium pyruvate and 50 μg/ml gentamycin. All cultures were maintained in a humidified 37°C incubator with 5% carbon dioxide in air atmosphere. All cells used in this study were between passages 40 and 68. EGCG (**2**) was from CHEMOS GmbH, Regenstauf,

Germany. IL-1β was from Becton Dickinson Ltd., Wallisellen, Switzerland. Actinomycin D and BFA were from Sigma, Switzerland. All other chemicals were obtained from commercial sources in the highest quality available.

Real-time polymerase chain reaction (TaqMan assay):

For mRNA induction experiments Caco-2 cells, at confluence, were incubated for 24 h with four different concentrations of **1** (0.01, 0.1, 0.25, or 0.4 mg/ml) or **2** (4.02, 40.2, 100, or 160 μM) with or without 10 ng/ml IL-1β. Control cells were incubated for 24 h with medium only. In RNA stability experiments, cells were washed twice with phosphate-buffered saline (PBS) after induction with 0.4 mg/ml of compound **1** for 24h, and were then placed for 1 h or 8 h in serum-free medium with or without 5 μg/ml ActD or 5 μg/ml ActD in combination with 0.4 mg/ml of compound **1**.

RNA extraction, reverse transcription, DNA digestion, and PCR amplification was performed as described before (Chapter 3.1.3).

For IL-8 detection the following primers and probe (Acc.No. BT007067.1) were used: 5`-CTTGGCAAAACTGCACCTTCACACAGA-3` (probe), 5`-CTCTTGGCAGCCTTCCTGATT-3` (forward), and 5`-TATGCACTGACATCTAAGTTCTTTAGCA-3` (reverse). For IL-1β detection the following primers and probe (Acc.No. NM_000576) were used: 5`-TTCCAGGACCTGGACCTCTGCCCTC-3` (probe), 5`- CTGATGGCCCTAAACAGATGAAG -3` (forward), and 5`- GGTGGTCGGAGATTCGTAGC -3` (reverse). For TNF-α detection the following primers and probe (Acc.No. X02910) were used: 5`-TAGCCCATGTTGTAGCAAACCCTCAAGCT -3` (probe), 5`- TCTTCTCGAACCCCGAGTGA -3` (forward), and 5`- CCTCTGATGGCACCACCAG -3` (reverse).

A relative standard curve was generated by serial dilutions of cDNA. Fragments of cDNA corresponding to IL-8, IL-1β, or TNF-α that covered the TaqMan primer/probe area were obtained by PCR amplification. To standardize the amount of sample cDNA added to the reaction the calculated amount of the gene of interest was divided by the calculated amount of the constitutively expressed ribosomal 18S gene in the sample. These normalized amounts were then used to compare the relative amount of target in different samples. The expression level of the endogenous control gene 18S did not alter under any of the different treatments.

The Ct values of the controls were 31.3, 33.2, and 28.2 for IL-8, TNF-α, and IL-1β, respectively. Standard curves of the respective mRNA were generated separately for each experiment and displayed linearity (R=0.9).

Cytokine expression assays:

To assess the effect on basal cytokine expression or secretion, Caco-2 cells, at confluence, were incubated for 24 h, 48 h, or 72 h with medium only, 10 ng/ml IL-1β, four different concentrations of **1** (0.01, 0.1, 0.25, or 0.4 mg/ml), or **2** (4.02, 40.2, 100, or 160 μM).

To investigate the effect of compound **1** on IL-1β-induced IL-8 secretion, Caco-2 monolayers were preincubated with 10 ng/ml IL-1β for 1 h. Subsequently, cells were exposed to 10 ng/ml IL-1β with or without different concentrations of **1** (0.01, 0.1, 0.25, or 0.4 mg/ml) for 23 h. Control cells were incubated for 24 h with medium alone.

To compare the effect on IL-8 secretion mediated by compound **1** with the effect of BFA on IL-8 secretion, Caco-2 cells were incubated for 24 h with or without 0.4 mg/ml of **1**, 5 μg/ml BFA, or 10 ng/ml IL-1β with or without 0.4 mg/ml of **1** or 5 μg/ml BFA.

After incubation the supernatants were collected and, in some experiments, intracellular proteins were extracted by lysing a cell pellet in 1ml protein extraction buffer (pH 7.4), containing 20 mM Tris-HCl, 0.5 mM Na_3VO_4 and 1 % (v v^{-1}) Igepal CA-630. Proteases were inhibited with 1mM PMSF, 8 μM leupeptin, 5 μM bestatin, 2 μg/ml aprotinin, 6 μM E-64 and 1 μM pepstatin. IL-8 or TNF-α protein concentration was assayed using OptEIA enzyme-linked immunosorbent assay set (Becton Dickinson Ltd., Wallisellen, Switzerland) and detected using a HTS 7000 Plus Bio Assay Reader (Perkin Elmer Ltd., Buckinghamshire, UK).

Statistical analysis:

For statistical comparison, data of groups were compared by analysis of variance (ANOVA). The level of significance was $P \leq 0.05$. If this analysis revealed significant differences, pair wise comparisons within groups were performed by two-sided unpaired t-tests. *P*-values were adjusted by Bonferroni's correction for multiple comparisons.

Acknowledgement

We thank U. Behrens for excellent technical assistance. This work was supported by an unconditional research grant of Frutarom Switzerland Ltd., 8820 Waedenswil, Switzerland.

6.2 Evaluation of the effect of green tea extract on intracellular IL-8 protein stability

6.2.1 Introduction

Proteasome inhibitors have been shown to induce IL-8 mRNA expression and protein secretion in different human cell types including epithelial, endothelial, and monocytic cells (Hipp et al., 2002; Gerber et al., 2004). As green tea catechins were recently reported to inhibit proteasome activity in vitro as well as in tumour cells (Nam et al., 2001; Smith et al., 2004), we investigated the influence of GTE on intracellular IL-8 protein stability in Caco-2 cells. Assuming that GTE may also act as a proteasome inhibitor, the GTE-mediated induction of IL-8 mRNA expression in Caco-2 cells described in Chapter 6.1 would be in accordance to the literature. In contrast to the proteasome inhibitors used in the literature such as lactacystin, GTE additionally exerted secretory inhibitor activity leading to increased intracellular IL-8 protein concentrations (Chapter 6.1).

For the assessment of the influence of GTE on IL-8 protein stability we used the classic pulse-chase assay. In a first step, Caco-2 cells at confluence were starved with medium lacking cysteine. This leads to an attenuation of cellular protein synthesis as soon as the residing intracellular cysteine has been used up. Then the cells were incubated with medium containing radioactively labelled cysteine (pulse), which results in metabolically labelled proteins. Finally, the cells were incubated for different time periods with medium containing non-labelled cysteine with or without GTE (chase). After harvest, intracellular IL-8 proteins were immunoprecipitated from the cell lysates and analysed on SDS polyacrylamide gels by autoradiography. In comparison to Coomassie blue protein staining, the use of radioactive metabolic labelling has the advantage that after immunoprecipitation only IL-8 expressed during the defined pulse period is detected, which may be used for quantification of the results. An influence of GTE on intracellular IL-8 protein stability would result in quantitatively different radioactivity in comparison to the control. To balance the secretory inhibition described for GTE in Chapter 6.1, some control cells were additionally incubated during the chase with BFA, which inhibits the vesicular transport of the Golgi apparatus (Misumi et al., 1986). For control reasons, some cells were incubated with GTE and BFA simultaneously during chase. The inhibition of IL-8 secretion by BFA or GTE was confirmed by analysis of supernatants using EIA.

6.2.2 Materials and methods

Materials and cell culture

Caco-2 cells were seeded in 60 mm dishes and grown in DMEM with Glutamax-I, supplemented with 10% heat-inactivated FBS, 1% non-essential amino acids, 1% sodium pyruvate and 50 μg/ml gentamycin (chase medium). All cultures were maintained in a humidified 37°C incubator with 5% carbon dioxide in air atmosphere. Standardized green tea special extract EFLA®942 (GTE) was from Frutarom Switzerland Ltd., Wädenswil, Switzerland. A brief manufacturing description was given in Chapter 2.2. [^{35}S]-L-cysteine (370 MBq/ml, 10 mCi/ml) was from Amersham Pharmacia, UK. N-ethylmaleimide (NEM) was from Sigma, Switzerland.

Metabolic labelling

After washing three times with prewarmed PBS cells at confluence were starved for 30 minutes in DMEM with Glutamax-I lacking cysteine (GIBCO BRL Life Technologies), supplemented with 10% heat-inactivated FBS, 1% non-essential amino acids, 1% sodium pyruvate and 50 μg/ml gentamycin (starvation medium). The cells were then incubated in starvation medium containing [^{35}S]-L-cysteine (0.5-5 μCi/well) (pulse medium) for 1 h followed by an incubation for 1 h or 6 h in chase medium. In some experiments 0.4 mg/ml GTE, 5 μg/ml BFA, or a combination of GTE and BFA was added to the chase medium. Then the reaction was stopped by adding 20 mM NEM in PBS to the cells. Supernatants were collected for the determination of IL-8 concentration. Then the cells were solubilised for 30 min on ice in buffer containing 20 mM Tris-HCl, 0.5 mM Na_3VO_4 and 1 % (v/v) Igepal CA-630. Proteases were inhibited with 1mM PMSF, 8 μM leupeptin, 5 μM bestatin, 2 μg/ml aprotinin, 6 μM E-64 and 1 μM pepstatin.

Immunoprecipitation

Cell lysates were incubated at 4°C for 16-24 h with protein A-Sepharose plus rabbit anti-IL-8 polyclonal antibody (Abcam Ltd., Cambridge, UK) or anti-IL-8 monoclonal capture antibody of OptEIA enzyme-linked immunosorbent assay set (Becton Dickinson Ltd., Wallisellen, Switzerland). After recovery by centrifugation, the protein A-Sepharose beads were washed two times with HBSS containing 0.25% (v/v) Igepal CA-630 and two times in HBSS only. Bound proteins were eluted by the addition of 3X Laemmli sample buffer and analysed on 15% SDS polyacrylamide gels under reducing conditions. The gels were vacuum dried with or without a prestaining with Coomassie blue followed by destaining in 10 % acetic acid, 40 % methanol. The dried gels were exposed to hyperfilm ECL with various light exposure times.

Determination of secreted IL-8 concentrations was described in Chapter 6.1.4.

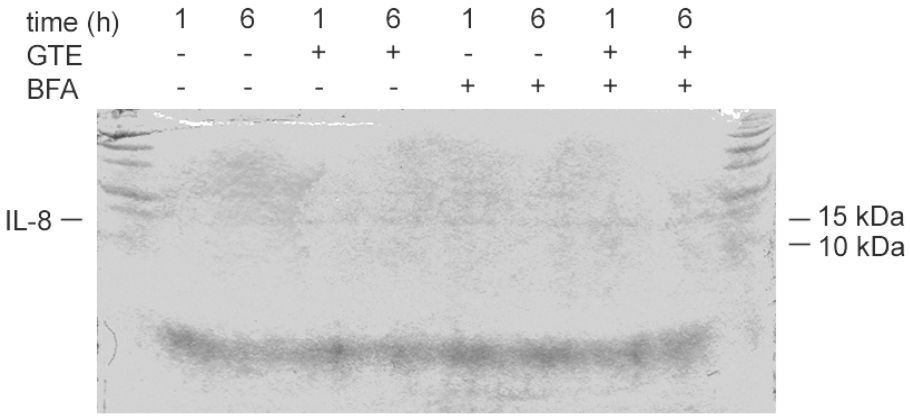

Figure 6.7: Coomassie blue staining of intracellular IL-8 immunoprecipitated with rabbit anti-IL-8 polyclonal antibody.

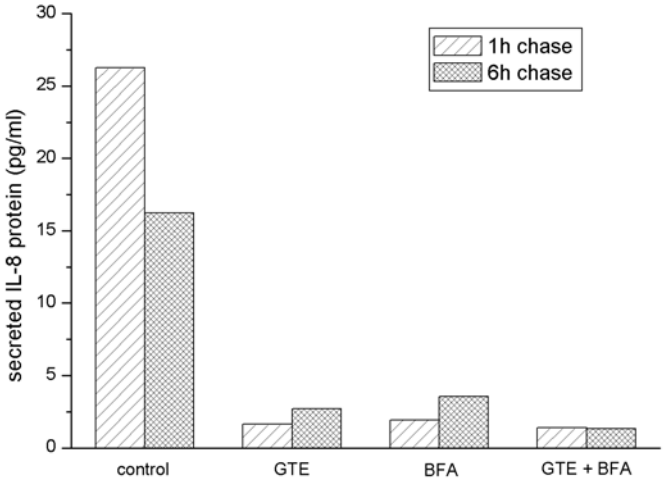

Figure 6.8: Concentration of extracellular IL-8 concentration in the chase medium 1 or 6 h after pulse. This figure shows one experiment.

6.2.3 Results and discussion

Two independent pulse-chase experiments were performed, while the extracellular concentration of IL-8 was only determined once by EIA. All the results of the immunoprecipitation showed a band of about 14 kDa and a faint band of about 20 kDa after

Coomassie blue staining (Figure 6.7), whereas no radioactive signal could be detected (data not shown). Secretion of IL-8 was inhibited in a similar extent by GTE or BFA (Figure 6.8).

The Coomassie blue staining indicates that immunoprecipitation of IL-8 was successful, although the faint band at about 20 kDa was not expected. As intracellular IL-8 still contains about 100 amino acids (Chapter 1.4.2) we expected a molecular weight of about 10-14 kDa. The significant inhibition of IL-8 secretion by BFA or GTE (Figure 6.8) was similar to results described in figure 6.6. The cells incubated with medium alone seem to contain less intracellular IL-8 protein than cells incubated with BFA (Figure 6.7). This is in accordance to the increase of intracellular IL-8 concentration induced by BFA shown in figure 6.6. Incubations for different time periods showed no visible alteration of the intracellular IL-8 protein content. However, radioactively labelled IL-8 could not be detected. Incubation with BFA showed that secretion of metabolically labelled IL-8 during incubation with or without GTE was not the reason for the observed loss of signal. An explanation might be that the intracellular pool of metabolically labelled IL-8 has been secreted concomitant to its generation during the pulse phase. To exclude this possibility further experiments with BFA already added to the pulse medium should be performed.

Additionally, intracellular IL-8 protein degradation might be that fast that after 1 h pulse and 1 h chase the amount of non-degraded labelled IL-8 was below the detection limit. Another possibility is that an excess of non-labelled IL-8 generated during the chase phase might compete with radioactively labelled IL-8 for the binding to IL-8 antibody for immunoprecipitation. Finally, the applied amount of 0.5-5 μCi/well [^{35}S]-L-cysteine might have been too low for a detectable specific incorporation into IL-8 protein.

Taken together, the classical pulse-chase method for the determination of protein degradation needs further validation for an establishment in our laboratory. As no signal of radioactive labelled IL-8 could be detected, it was not possible to determine in this experimental series the influence of GTE on intracellular IL-8 protein stability in Caco-2 cells.

6.3 Evaluation of the influence of green tea extract on the mRNA expression of different proinflammatory mediators

6.3.1 Introduction

During intestinal inflammation an array of different proinflammatory mediators is produced including cytokines, prostaglandins, and leukotriens. The proinflammatory cytokine IL-6 has been implicated to play a role in several intestinal diseases like IBD and colorectal carcinoma (Brown et al., 2002; Csiszar et al., 2004). In the intestinal mucosa IL-6 may regulate immunoglobulin A production in Peyer's patch B cells, thereby influencing intestinal immune function (Beagley et al., 1989). Additionally, evidence is given for a key role of IL-6 in the regulation of acute phase protein synthesis in enterocytes (Molmenti et al., 1993). Nevertheless, recent studies suggest that IL-6 may exert also anti-inflammatory effects beside its commonly considered proinflammatory activity (Xing et al., 1998). It has been shown that IL-1β upregulates IL-6 expression at the transcriptional level in the intestinal cell line Caco-2 (Parikh et al., 1997). In the human colon, arachidonic acid is metabolised primarily by cyclooxygenase (COX) and arachidonate lipoxygenase (ALOX) to prostaglandins and leukotriens, respectively, which are involved in a variety of physiological and pathological processes in the immune response and in inflammation. Cyclooxygenases (COX) enzymes catalyse the generation of prostaglandins. The inducible COX isoform, COX-2, has been implicated in colon cancer development and other sites of inflammation like IBD (Eberhart et al., 1994; Hendel and Nielsen, 1997). Lipoxygenase-5 (ALOX) is a key enzyme in the generation of leukotriens. It has been shown to play an important role in colonic tumorigenesis and experimental colitis (Ye et al., 2004; Cuzzocrea et al., 2005). However, no change of ALOX mRNA expression in the colon of patients with active inflammatory bowel disease has been found (Hendel et al., 2002). Nevertheless, substances modulating the production of IL-6, prostaglandins, or leukotriens in enterocytes may have important clinical implications.

Green tea is widely consumed and it has been used medicinally in China for 5000 years. It was suggested that green tea possess anti-inflammatory as well as anticarcinogenic properties (Katiyar and Mukhtar, 1996). Green tea polyphenols have been reported to inhibit COX- and ALOX-dependent metabolism of arachidonic acid in human colon mucosa and colon tumour tissues (Hong et al., 2001). In intestinal cell lines, e.g. Caco-2, EGCG was shown to down-regulate COX-2 mRNA expression and prostaglandin production (Porath et al., 2005). A potential effect of green tea on intestinal IL-6 expression and secretion has not been investigated yet.

The important role of the ALOX and COX-2 in the metabolism of arachidonic acid makes these enzymes possible therapeutic targets in gut inflammatory conditions. A modulation of IL-6 production, and therefore of the mucosal immune function, might also be relevant in the management of intestinal inflammation. Several evidences have shown the benefits of green tea in a variety of inflammatory conditions (Hussain et al., 2005; Wheeler et al., 2004). Hence, we investigated the effect of different concentrations of GTE or its constituent EGCG on basal mRNA expression of IL-6, COX-2, and ALOX in Caco-2 cells. Additionally, the effect of GTE or EGCG on the influence of IL-1β on mRNA expression of these inflammatory mediators was investigated.

6.3.2 Materials and methods

Materials

Standardized green tea special extract EFLA®942 (GTE) was from Frutarom Switzerland Ltd., Wädenswil, Switzerland. A brief manufacturing description was given in Chapter 2.2. (-)-Epigallocatechin gallate (EGCG) was from CHEMOS GmbH, Regenstauf, Germany. IL-1β was from Becton Dickinson Ltd., Wallisellen, Switzerland. All other chemicals were obtained from commercial sources in the highest quality available.

Cell culture was described in Chapter 5.2.

Real-time polymerase chain reaction (TaqMan assay)

For mRNA induction experiments Caco-2 cells, at confluence, were incubated for 24 h with four different GTE concentrations (0.01, 0.1, 0.25, or 0.4 mg/ml) or EGCG concentrations (4.02, 40.2, 100 μM) with or without 10 ng/ml IL-1β. Control cells were incubated for 24 h with medium only. RNA extraction, reverse transcription, DNA digestion, and PCR amplification was performed as described elsewhere (Chapter 3.1.3).

For IL-6 detection the following primers and probe (Acc.No. M54894.1) were used: 5`-TTACTCTTGTTACATGTCTCCTTTCTCAGGGCTG -3` (probe), 5`-TGACAAACAAATTCGGTACATCCT-3` (forward), and 5`- AGTGCCTCTTTGCTGCTTTCAC -3` (reverse). For COX-2 detection the following primers and probe (Acc.No. BC013734.1) were used: 5`- TTCCTACCACCAGCAACCCTGCCA -3` (probe), 5`-GAATCATTCACCAGGCAAATT -3` (forward), and 5`- TTTCTGTACTGCGGGTGGAAC -3` (reverse). For ALOX detection the following primers and probe (Acc.No. NM_000698.1) were used: 5`- CTTCGAGCGTGGCGCGGTG -3` (probe), 5`- TGGACAAGCCCTTCTACAACG -3` (forward), and 5`- CTCGTCCACAGTCACGTCGT -3` (reverse).

A relative standard curve was generated by serial dilutions of cDNA. Fragments of cDNA corresponding to IL-6, COX-2, or ALOX that covered the TaqMan primer/probe area were obtained by PCR amplification. To standardize the amount of sample cDNA added to the reaction the calculated amount of the gene of interest was divided by the calculated amount of the constitutively expressed ribosomal 18S gene in the sample. These normalized amounts were then used to compare the relative amount of target in different samples. The expression level of the endogenous control gene 18S did not alter under any of the different treatments.

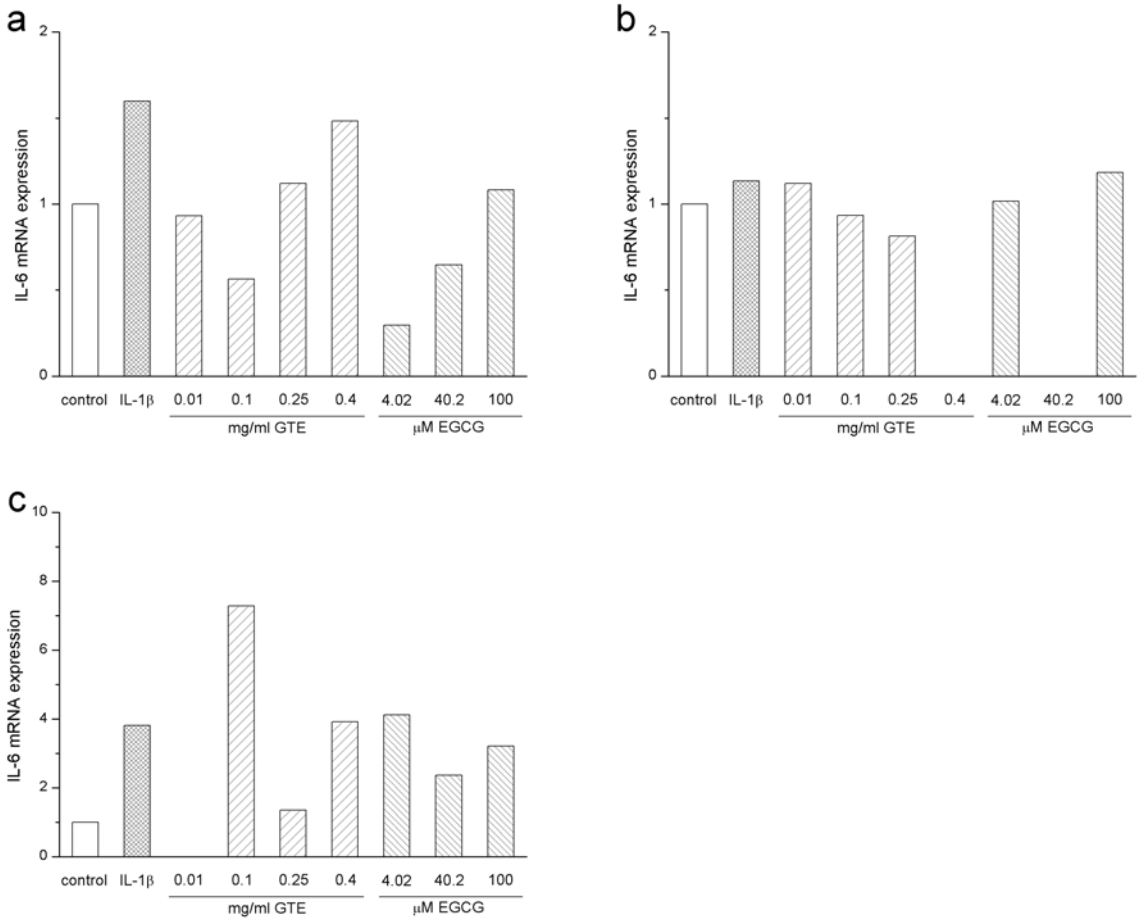

Figure 6.9: Effect of different concentrations of GTE or EGCG on basal IL-6 mRNA expression in Caco-2 cells after 24 h incubation. Transcriptional expression was determined by quantitative real-time PCR. Each data value is expressed as a percentage of the control. Data were not pooled and represent the results of 3 independent experiments (a-c).

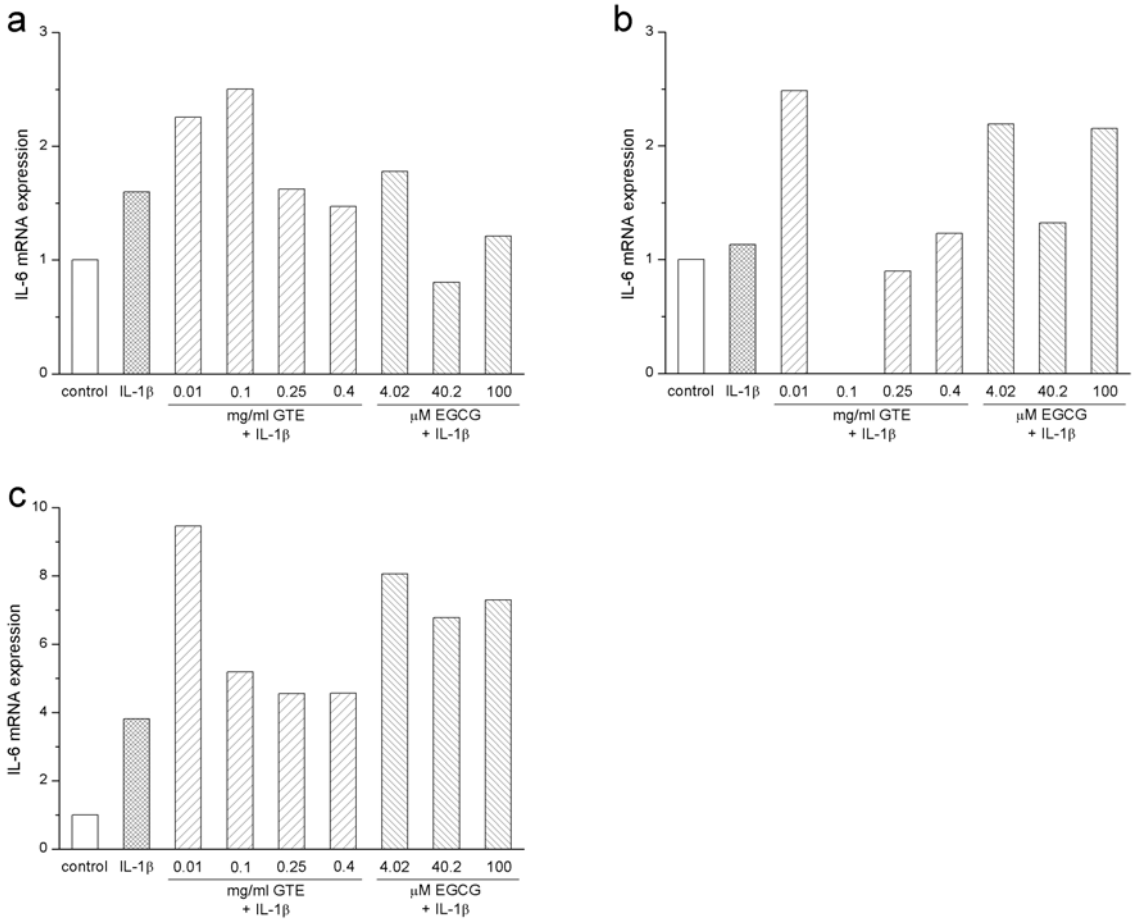

Figure 6.10: Effect of different concentrations of GTE or EGCG on IL-1β-influenced IL-6 mRNA expression in Caco-2 cells after 24 h incubation. Transcriptional expression was determined by quantitative real-time PCR. Each data value is expressed as a percentage of the control. Data were not pooled and represent the results of 3 independent experiments (a-c).

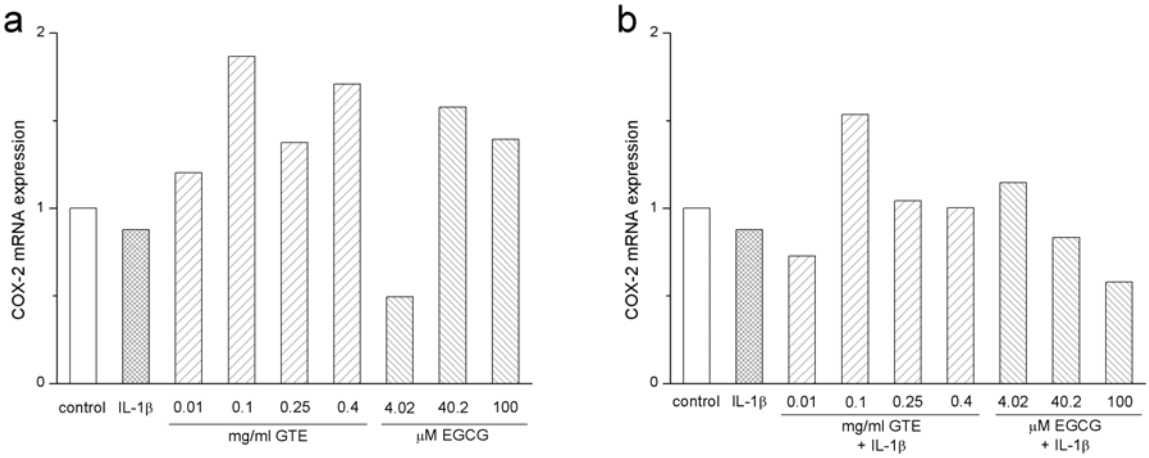

Figure 6.11: Effect of different concentrations of GTE or EGCG on (a) basal or (b) IL-1β-influenced COX-2 mRNA expression in Caco-2 cells after 24 h incubation. Transcriptional expression was determined by quantitative real-time PCR. Each data value is expressed as a percentage of the control. Data represent the results of one single experiment.

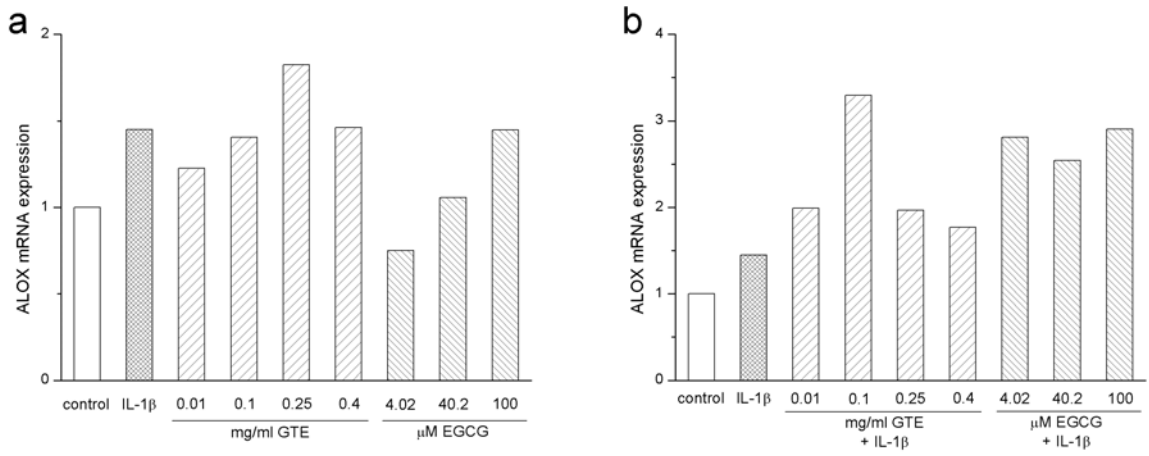

Figure 6.12: Effect of different concentrations of GTE or EGCG on (a) basal or (b) IL-1β-modulated ALOX mRNA expression in Caco-2 cells after 24 h incubation. Transcriptional expression was determined by quantitative real-time PCR. Each data value is expressed as a percentage of the control. Data represent the results of one single experiment.

6.3.3 Results and discussion

The data in figures 6.9 to 6.12 represent the results of single independent experiments. The results of the effect of GTE or EGCG on basal IL-6 mRNA expression in Caco-2 cells were inconclusive (Figure 6.9a-c). In two of three experiments IL-1β or 0.4 mg/ml GTE induced IL-6 mRNA expression 1.5- to 4-fold. In the literature IL-1β has been described as an inductor of IL-6 mRNA expression in Caco-2 cells (Parikh et al., 1997). The effect of GTE or EGCG on the IL-1β-influenced IL-6 mRNA expression found in these two experiments were also inconclusive (Figure 6.10a-c). However, at a concentration of 0.01 mg/ml GTE enhanced the effect of IL-1β-mediated IL-6 mRNA expression. In Caco-2 cells, the mRNA expression of COX-2 was not induced above 2-fold by GTE, EGCG, or IL-1β (Figure 6.11a). This was in contrast to results described in the literature (Porath et al., 2005). Also the effect of GTE or EGCG on IL-1β-influenced COX-2 mRNA expression could not be determined in Caco-2 cells (Figure 6.11b). Furthermore, also the effect of GTE, EGCG, or IL-1β on ALOX mRNA expression could not be clarified in Caco-2 cells (Figure 6.12). A slight induction of ALOX mRNA expression was observed for GTE or EGCG. Because of these discouraging results the experiments concerning the influence of GTE on COX-2 and ALOX mRNA expression were not repeated.
Taken together, due to the inconsistent results, the effect of GTE on IL-6 mRNA expression in the colon carcinoma cell line Caco-2 remains controversial and might be clarified using a different intestinal cell line.

8. Conclusions and outlook

Pharmacokinetic herb-drug interactions often occur as a result of activity changes of drug-metabolising and transporting proteins and have received much attention recently (Butterweck et al., 2004). These proteins seem to participate in the pathology as well as in therapy resistance of several diseases including cancer and IBD (Van Tellingen, 2001; Farell et al., 2000). Because of the recent increase of green tea consumption due to its potential health promoting effects there is a need for clarification of possible drug-interactions of green tea.

In this thesis the effect of a commercially available GTE on the expression and function of several intestinal transporters and drug-metabolising enzymes was investigated. We have shown that GTE, at low concentrations, does not alter P-gp or MRP2 mRNA expression in LS-180 cells. The functional activity of MRP2 was inhibited by GTE at a concentration found to exert toxic effects in intestinal cell lines. GTE did not induce CYP3A4 expression in LS-180 cells, but did dose-dependently inhibit CYP3A4 metabolic activity *in vitro*. Although CYP3A4 and P-gp have strikingly overlapping substrates and inhibitors, the ability to predict the potential of GTE-mediated P-gp functional inhibition is hindered by the complexity of the enzyme kinetics. Thus, despite literature data suggest a functional inhibitory activity of GTE against P-gp, it remains to be elucidated whether GTE inhibits P-gp function. However, GTE did dose-dependently induce CYP1A, although with cell line specific differences between the expression of the isoenzymes CYP1A1 and CYP1A2. The functional activity of CYP1A2 was inhibited by GTE, which is in accordance to its reported chemopreventive character.

These results may contribute to the prediction of possible effects of GTE on therapeutical effectiveness. Regarding the inhibitory activity on CYP3A4 function, GTE may represent a potential candidate for therapeutic use. Co-administration of GTE with chemotherapeutic or antiviral drugs may increase their cellular concentration, which may enhance the therapeutic effect. On the other hand, a dose-reduction with the desirable side effects of lower toxicity and a prolonged therapeutic window might become possible. Nevertheless, a general increase of a potential toxicity of drugs metabolised by CYP3A4 due to co-ingestion of green tea has to be considered. However, clinical studies in humans have to be performed to overcome the preliminary character of cell culture based observations.

The intestinal mucosa as a site of immune regulation has come into focus due to observed high titers of inflammatory mediators in diseases with chronic intestinal inflammation including IBD (Neutra et al., 2001; Sartor, 1994). Thus, the identification of herbal preparations inhibiting the activity or the production of these mediators is highly desirable for therapeutical

application. Green tea has been shown to represent such a potential candidate (Sueoka et al., 2001).

In this thesis we assessed the ability of GTE to interfere with the gut mucosal production of the chemokine IL-8, which is involved in neutrophil migration and activation. We found that GTE specifically inhibits IL-8 secretion of Caco-2 cells, while inducing its expression. GTE did not alter IL-1β mRNA expression in these cells, but slightly induced TNF-α production in theses cells. As IL-8 is a pivotal regulator of neutrophil recruitment and respiratory burst, this result may have implications for preventive or attenuating therapies for the treatment of IBD. Therefore, data on the effect of GTE on IL-8 in biopsies of IBD patients should be generated in a future step. Furthermore, the reported anticancer and chemopreventive potential of green tea might be, at least in part, based on the effects observed. This is supported by the use of the cancer cell line Caco-2 in our experiments.

However, as the effects of IL-8 are dependent on the binding to its receptors, a possible influence of GTE on the expression and function of these have to be elucidated in neutrophils. Because of the high redundancy of different chemokines and cytokines, possible effects of GTE on further inflammatory mediators need to be clarified. Due to the complex interplay of pro- and anti-inflammatory stimuli *in vivo*, the effect of GTE on the intestinal inflammatory process should be investigated in suitable animal models. Nevertheless, GTE has been shown to exhibit antiinflammatory activity in intestinal cells *in vitro*, which are likely to occur *in vivo* as higher concentrations of GTE after oral administration can be obtained in the intestine in comparison to other tissues.

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Original Papers:

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Congress-Contributions, Plenary Lectures, Posters:

2001 Workshop "Einsatz tierischer Zellkultursysteme in der Phytopharmaka-Forschung", Lecture, Berlin, Germany

2001 12th Food Design Show, Memorial Lecture, Tokyo, Japan

2001 49th Annual Meeting of the Society for Medicinal Plant Research, Poster, Erlangen, Germany

2004 Fruehjahrstagung der DGPT (Deutsche Gesellschaft für Pharmakologie und Toxikologie), Poster, Mainz, Germany

2004 International Congress on Natural Products Research (ICNPR), Poster, Phoenix, Arizona, USA.

2005 53rd Annual Congress of the Society for Medicinal Plant Research, Poster, Florence, Italy