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# DIPLOMARBEIT

Titel der Diplomarbeit

## The anti-mutagenic and antioxidative potential of bilirubin dimethyl ester and biliverdin dimethyl ester in the Ames Salmonella assay


Verfasserin

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# I. Index of tables, figures, pictures and diagrams

## *Tables:*



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# II. List of abbreviations

| | |
|---|---|
| **DMSO** | Dimethylsulfoxide |
| **his+** | Histidine independence |
| **NADP** | Nicotinamideadenindinucleotide Phosphate |
| **NB** | Nutrient Broth |
| **OVNC** | Overnight culture |
| **pH** | Potential hydrogenii |
| ***TA102*** | Histidine auxotrophe *salmonella* typhimurium tester strain *102* |
| ***TA98*** | Histidine auxotrophe *salmonella* typhimurium tester strain *98* |
| **$AFB_1$** | Aflatoxin $B_1$ |
| **PhIP** | 2-Amino-1-Methyl-6-Phenylimidazo[4,5-*b*]Pyridine |
| ***t*-BuOOH** | Tertiary-Butyl-hydroperoxide |
| **TNFone** | 2,4,7-Trinitro-9-Fluoroenone |
| **SOD** | Superoxide dismutase |
| **CAT** | Catalase |
| **GPx** | Glutathione peroxidase |
| **UCB** | Unconjugated bilirubin |
| **2-AF** | 2-aminofluorene |
| **L-DOPA-Cu(II)** | The amino acid L-Dopamin in the presence of Cu(II) |

# 1. Introduction

Bile pigments, such as unconjugated bilirubin (BR) and biliverdin (BV), are tetrapyrrolic, dicarboxylic acids belonging to the 'porphyrin' class of molecules and occur naturally in the body.

In subcutaneous bruises, tissue macrophages engulf the extravasated red cells and convert their haemoglobin to bile pigments, which is the reason for the characteristic progressive colour change from dark purple (heme) to blue-green (biliverdin) and eventually to yellow (bilirubin) (PIMSTONE *et al.*, 1971).

The physiological plasma bilirubin concentrations in the human body range from 5 to 17 µmol/L, predominantly bound to albumin, its carrier protein (STOCKER 2004).

The idea that bile pigments might possess medicative properties trace back to the traditional Chinese medicine thousands of years ago.

The pigments were applied in the form of powdered or whole gall stones to combat miscellaneous disorders underpinned by inflammation (READ, 1976).

Unlike in the conventional western medicine: bilirubin was primarily considered a toxic by-product associated with the potentially fatal conditions of neonatal hyperbilirubinemia, hepatic cirrhosis and viral hepatitis (DOLPHIN, 1979).

A reversal commenced in the 1900s, when the presence of bilirubin spared Vitamin A from oxidation in the intestinal lumen, *in vivo* (BERNHARD *et al.*, 1954). This indication was the activator for further research and in succession a flurry of publications on possible beneficial effects emerged.

In the past 20 years the evidence of the physiological relevance of bile pigments could be fortified with indication to suggest that bilirubin and biliverdin possess powerful antioxidant capacity towards the oxidation of lipids (FREI *et al.*, 1988) and proteins (NEUZIL *et al.*, 1993). And, more specifically, bile pigments are potent peroxyl radical scavengers and inhibit the mutagenic effects of a number of classes of mutagens (polycyclic aromatic hydrocarbons, heterocyclic amines, oxidants).

Nowadays, consolidated findings concerning the anti-mutagenic and antioxidative effects of bile pigments exist and research is still on the rise, since these compounds might play a key role in the treatment of cardiovascular diseases and cancer, which account for the major cause of death in industrialised countries.

Most of the experiments are based on *in vitro* studies, most frequently using the Ames *Salmonella microsome* assay as a test model. But further research investigating the mechanisms of the transactions, as well as cell culture studies and finally *in vivo* research probing are essential to explore the therapeutic potential of these naturally occurring compounds (BULMER *et al*., 2007).

The aim of this thesis was to explore the potential anti-mutagenic and antioxidative effects of bilirubin dimethyl ester and biliverdin dimethyl ester in the Ames *Salmonella* assay. The terms “bilirubin dimethyl ester” and ”biliverdin dimethyl ester” are used since the natural compounds were esterified to enhance the solubility and thereby facilitate the working processes.

The Ames test is always carried out in pairs. My workmate Andrea Steyrer and me tested 4 bile pigments – bilirubin dimethyl ester, biliverdin dimethyl ester, stercobilin and urobilin - and split these up between the two of us.

This study was part of the FWF project “Bile Pigments - Physiological Relevance of Bile Pigments”, in which the physiological relevance of the bile pigments, their possible transmission of *in vitro* to *in vivo* evidence, the compound’s anti-mutagenic and anti-carcinogenic potential and their mechanisms of action were investigated.
Besides the bile pigments bilirubin- and biliverdin dimethyl ester presented in this study, stercobilin and urobilin are discussed in the diploma thesis of my work-mate Andrea Steyrer.
Four further compounds, namely bilirubin, biliverdin, bilirubin-ditaurate and protoporphyrin were explored by Mag. Christine Mölzer and Hedwig Huber.

# 2. Literature survey

## *2.1. The Ames test*

In terms of health risk assessment the Ames test illustrates a relatively quick and competitive method to test substances, as for instance new chemicals, drugs or biocides for their mutagenic or genotoxic potential. Over the years its value as such has been acknowledged by the scientific community, by government agencies and corporations. For that reason the test is in use world-wide as an initial screen.
In the following, a prevailing review referring to this will be presented.

### 2.1.1. Principle of the test procedure

The Ames *Salmonella/microsome* mutagenicity assay is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can induce genetic damage, leading to gene mutations.
The test uses a number of *Salmonella* strains with pre-existing mutations that leave the bacteria unable to synthesize the required amino acid histidine. This explains why the bacteria are not able to grow and form colonies.
If a mutagenic substance is added, the gene's function can be restored and allows the cells to synthesize histidine again. These newly mutated cells illustrate the wild type of the *Salmonella* strain which is able to grow and form colonies without histidine. On this account the test is often called a "reversion" assay. The intensity of the increase of the so called $his^+$ revertants is an indicator for the mutagenic potential of the tested substance (MORTELMANS and ZEIGER, 2000). In short, the higher the quantity of the revertants, the stronger is the mutagen.
Generally, a compound is classed "pro-mutagenic" if the relative mutagenic response increased beyond 200%, relative to the positive control (BULMER, 2007).
Some carcinogenic chemicals, such as aromatic amines or polycyclic aromatic hydrocarbons, are biologically inactive unless they are metabolized to active forms. Since bacteria do not possess a cytochrome-based P450 metabolic

activation system, an exogenous mammalian organ activation system can be added instead of the buffer solution.

#### *2.1.1.1 Historical background*

The original Ames test dates back to 1964, when Bruce N. Ames, an American biochemist and geneticist developed an assay for the initial screening of a number of histidine mutants which led to the selection of mutants that were highly sensitive to reversion by a variety of bacterial strains (AMES, 1971). This was the pioneering finding for the Ames test and the cornerstone for the assay in the way it is performed today.
The original development took place in 1973, when mutagenic effects on bacteria, caused by potent carcinogens, were studied.
In 1966 the spot test was applied for the first time by Ames and Whitfield, previously used with an *E.Coli* strain. A number of histidine mutant bacteria were used to screen chemicals to detect their mutagenic potential by applying a small amount of the test chemical directly to the centre of a selective agar medium plate which was inoculated with the tester strain. The chemical diffused into the agar and built a concentration gradient. If revertant colonies increased this was an indication for a mutagenic chemical, whereas total growth inhibition was observed if the chemical was toxic.
It was in 1973 when Ames *et al.* evolved a more quantitative assay than the spot test, namely the plate incorporation assay.

### 2.1.2 Bacterial strains

The concept of carcinogenesis as an expression of mutation in DNA has been revived over the past years. Mutations are of different types: base substitutions, frame shifts, and larger lesions. For the classification of mutations induced by chemical carcinogens, Ames and his co-workers have been developing tester systems with the bacterium *Salmonella* (AMES, 1971).
The principle of their approach is scoring carcinogen-induced reversion to histidine independence of histidine-requiring tester strains.

In addition to the genetic histidine dependence, the standard tester strains contain other mutations that highly increase their ability to detect mutagenic substances (MARON and AMES, 1984).
These mutations act as hot spots for mutagens that cause DNA damage via various mechanisms.

### *2.1.2.1 Genotype of the tester strains adopted within the assays*

| *Bacterial strain* | *His mutation* | *uvrB-bio* | *LPS Defect* | *Plasmid* | *Reversion by* |
|---|---|---|---|---|---|
| TA98 | hisD3052 | Yes | Rfa | pKM 101 | Frame shift mutation |
| TA102 | hisG428 | No | Rfa | pKM 101, pAQ 1 | Oxidative damage |

***Table 1:*** *Mutation forms of the in the study used Salmonella tested strains*

*TA98* contains the hisD3052 mutation, *TA102* carries the hisG428 mutation. Both strains are histidine dependent by virtue of a mutation in the histidine operon. Additional mutations/genetic alterations that have made the tester strains more sensitive to chemical mutagens are listed below.
*TA98* exhibits a deletion mutation through the uvrB-bio genes. The uvrB deletion mutation eliminates the accurate excision repair mechanisms, thereby allowing more DNA lesions to be repaired by the error-prone DNA repair mechanism. The deletion through the biotin gene results in the bacteria's dependency on biotin (AMES and MC CANN, 1976).
*TA102*, not showing the uvrB-bio deletion mutations, is primarily included into the set of tester strains for detecting mutagens that require the conventional excision repair mechanism (MOERTELMANS and ZEIGER, 2000).
Rfa-mutation occurs in both strains. This type leads to a defective lipopolysaccharide (LPS) layer that coats the bacterial surface, making the bacteria more permeable to bulky chemicals (AMES *et al.*, 1976).
The plasmid pKM101 has been introduced in *TA98* and *TA102* in order to increase the error-prone DNA repair pathway and similarly enhance mutagenic changes caused by chemicals.
*TA102* also contains the hisG428 mutation on plasmid pAQ1 to enlarge the number of target sites.

As a consequence of these mutations and genetic alterations *TA98* is typically sensitive to frame shift mutations, *TA102* is prone to oxidative mutagens (MORTELMANS and ZEIGER, 2000; MARON and AMES, 1983).

## *2.2 Chemistry of the bile pigments*

### *2.2.1 Chemical basics*

Bilirubin and biliverdin, the oxidised product of unconjugated bilirubin, are tetrapyrrolic, dicarboxylic acids belonging to the “porphyrin” class of molecules. They possess unique, divergent, three dimensional structures (Fig. 1) (BULMER *et al.*, 2007) and show intense colours.

Although bilirubin is a polar molecule Brodersen *et al.* explored that the solubility of bilirubin in aqueous buffers and under exclusion of light amounted to 7 nM at pH=7.4, temperature 37°C, increasing with higher pH, approximately in inverse proportion with the squared hydrogen ion concentration.

Nevertheless, the bile pigment poorly dissolves in most lipid solvents, except for asymmetrical chloralkanes. Hydrogen bond-breaking solvents, in particular dimethyl sulfoxide, are most effective in solubilizing bilirubin. These internal hydrogen bounds in the compound suppress the ionization of the carboxyl groups and monopolize all of the polar groups in the bilirubin molecule, limiting their interaction with water (BRODERSEN, 1979).

The very low aqueous solubility of unconjugated bilirubin is less than 0.5% of the normal total UCB concentration in the plasma. This explains why the majority of UCB is carried in the circulation as water-soluble complexes with plasma proteins. 90% of UCB in the adult circulation is bound to albumin, the remaining 10% mainly to the apolipoprotein D in high density lipoprotein (HDL) (SUZUKI *et al.*, 1988).

The antioxidant potency of unconjugated bilirubin is enhanced when binding to plasma albumin (STOCKER *et al.*, 1987), and possibly to cytosolic proteins (BRASS *et al.*, 1989). From this it follows that bound as well as unbound unconjugated bilirubin and conjugated bilirubins in plasma and the cytosol are available to protect cell membranes from lipid peroxidation. On the other hand,

only “free” unconjugated bilirubin, which is not protein bound, can enter cells by passive of facilitated diffusion and therefore detects its potential toxic effects intracellularly (OSTROW and TIRIBELLI, 2003).
Binding of bilirubin to albumin sequesters the molecule into a non-toxic form but also distributes the pigment throughout the entire circulation and extra vascular space (BRODERSEN, 1979).
Biliverdin, obtainable from bilirubin by abstraction of two hydrogens, is more soluble (MINETTI *et al*., 1997).
The hydrophobe qualities of bilirubin have both positive and adverse effects. As a negative aspect the toxic phenomenon (see chapter 4.2.4) as well as the affinity for the pathogenesis of gallstones hast to be mentioned, compared to the beneficial function of bilirubin as an effective antioxidant molecule for lipophilic substances (see chapter 4.2.2.3) (LÖFFLER and PETRIDES, 2007).
The fact, that bile pigments are highly photosensitive and additionally tend to oxidize at either very low or high pH, requires special attention when handling these substances.

### 2.2.2 Formation of bile pigments

In humans unconjugated bilirubin and biliverdin are formed via the catabolism of heme, which possesses the prosthetic group of hemoglobin and also other heme proteins. Hemoglobin, released from senescent red blood cells, and heme containing enzymes are the major source of heme for the synthesis of bile pigments (SCHMID and MC DONAGH, 1975).
The catabolism of heme takes place in the cells of the reticulo-endothelial system (*e.g.* liver, spleen), resulting in the excretion of about 300 mg of bile pigments from the body each day (STOCKER; 2004).
In Fig. 1 the metabolism of bile pigments in the human body is shown.
Free heme, which is toxic, is degraded by microsomal heme oxygenase (HO-1) and then converted forming biliverdin, carbon monoxide and iron by mediating stereospecific cleavage (always opening of the α-bridge) of the heme ring.
Actually this describes the initial rate-limiting step of heme degradation, since

one mole of heme is converted to one mole each of $Fe^{2+}$ and carbon monoxide and biliverdin (RYTER *et al*., 2006).

In succession, biliverdin is reduced to unconjugated bilirubin, by the cytosolic enzyme BVR (SCHMID and MC DONAGH, 1975). BVR offers a stereochemical preference for bilirubin IXα. It is most abundant in tissues with high HO activity, and is present abundantly, resulting in the absence of biliverdin in human plasma or bile (CRAWFORD *et al*., 1988).

At first sight, this metabolic event appears to be disadvantageous, since bilirubin elimination requires a considerable amount of energy (NADP) but it might be interpreted as a hint that bile pigments play a substantial role in physiological processes.

After the formation of bilirubin, this hydrophobic compound is bound to serum albumin, for which it has a strong affinity (BRODERSEN, 1979). Bilirubin is circulated to the liver where it is actively and passively absorbed into the hepatocyte.

Since bilirubin is toxic and insoluble in aqueous solutions, it must be glucuronidated before excretion in the bile (SEDLAK *et al*., 2008). Hence, intercellular glutathione-S-transferase transports bilirubin to the endoplasmic reticulum where glucuronic acid conjugates are formed by UDP glucuronosyl transferase (UGT1A1) (KAMISAKO, 2000).

Due to conjugation bilirubin becomes water soluble and is then actively transported into the bile caniculi by multidrug resistance protein 2 (MRP2) (KEPPLER and KONIG, 2000).

In the next step the bilirubin conjugates are delivered into the duodenum via the bile duct. When bilirubin glucuronides enter the gastrointestinal tract, bacterial enzymes including ß-glucuronidase, hydrolyse the bilirubin esters forming an unconjugated pigment (SKAR and SAXERHOLT, 1989).

A part of the unconjugated bilirubin is reabsorbed (GILBERSEN *et al*., 1962) and re-excreted. The remains of the pigments are reduced by the intestinal bacterial flora to urobilins and stercobilins, which provide the distinctive colouration of faeces (WATSON *et al*., 1969).

Overall, the decomposition of heme is an observable process since the several steps of the process are colourfully represented in the time course of bruising where the blue-green colour of biliverdin is followed by the yellow colouration of bilirubin. Interestingly, in birds and reptiles, which lack the enzyme biliverdin reductase, the sequence of bile pigment formation stops at the level of non-toxic biliverdin (NEUZIL and STOCKER, 1993).

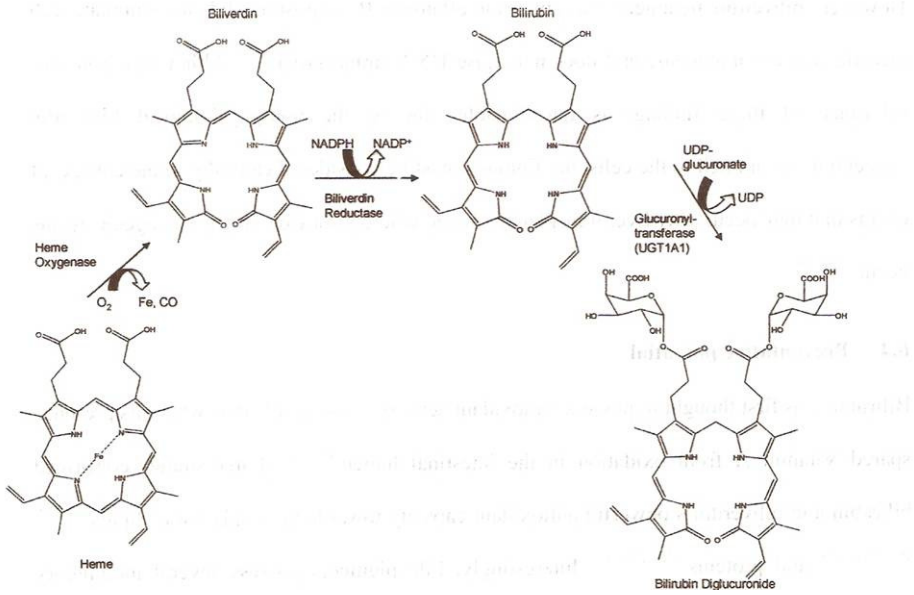



***Figure 1:*** *Schematic representation of the metabolism of heme to biliverdin, bilirubin and bilirubin glucuronide (BULMER et al., 2008).*

## 2.2.2 Antioxidative properties of bile pigments

### *2.2.2.1 General aspects*

Prior to the 1980s bilirubin had been considered a toxic by-product closely connected with the possibly fatal conditions of neonatal hyperbilirubinemia, hepatic cirrhosis and viral hepatitis. But after the increase of perceptive research, bilirubin was identified as an active antioxidant with several modulatory properties including anti-viral (BERNHARD and RITZEL, 1953), anti-apoptotic (BERNHARD *et al*., 1954), anti-inflammatory (LAFARGE-FRAYSSINET, 1983), and anti-compliment (STRAUSS *et al*., 2006) activities.

All these encouraging findings provided new and additional impulse for further analysis of the bile pigments.

Antioxidants are a group of compounds that protect the body's cells from oxidation by quenching reactive oxygen species (ROS). They are seperated into the classes simple and enzymatic. Glutathione, vitamin E (tocopherols), vitamin C (ascorbate), carotenoids, uric acid and bilirubin are typically small molecules belonging to the simple group whereas the enzymatic type of antioxidants is larger in size and occurs within and outside biological cells. Enzymatic scavengers, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) work together in order to transform one free radical to another, ultimately reducing them to less harmful molecules like water ($H_2O$) and oxygen ($O_2$).

However, the existing and auspicious results concerning the antioxidative capacity of bile pigments, which will be discussed in the following, apply to *in vitro* studies only. Since currently – to the best of our knowledge - there is no information available, the efficacy of their supplementation *in vivo* (HUANG *et al*., 2006) is still unsettled, since they are almost not absorbed into the body via anoral supplement.

#### *2.2.2.2 Oxidative stress definition*

Oxidative stress is the consequence of an imbalance between pro- and antioxidants in favour of the former.

Prooxidants (*e.g.* light, radiation, metallic ions) catalyse redox-reactions or homolytic cleavage and, as a result, produce free radicals. These molecules are very reactive due to an uneven pair of electrons and can damage biological tissues, by competing with these molecules for their electrons. Free radicals *in vivo* include amongst others the superoxide radical cation ($O_{2}^{\cdot -}$), hydroxyl radical ($OH^{\cdot -}$) and hydrogen peroxide ($H_2O_2$). The latter is an example of an oxidant that promotes free radical formation (BERGAMINI *et al*., 2004).

Oxidative damage of cell components and alterations of cellular functions are the results of continuous exposure to reactive oxygen species (ROS), emerging

from exogenous and endogenous sources. In series, excessive generation of ROS and other radicals can damage proteins, carbohydrates, polyunsaturated fatty acids, and DNA, and may thus lead to oxidative stress and various degenerative processes and diseases such as aging, immunodeficiencies, neuropathological disorders like Parkinson's and Alzheimer's disease, further inflammation, diabetes, rheumatoid arthritis and certain cancers (HALLIWELL and GUTTERRIDGE, 1999).
Besides, ROS are also involved in the occurrence of coronary heart disease, the major cause of death in industrialised countries.
By measuring the concentration of specific products, evolved from the damage of free radicals, oxidative stress can be quantified.
For this purpose various different techniques have been developed to monitor this process and its consequences. These approaches can also be used to identify dietary antioxidants and their mode of action.

#### *2.2.2.3 Bilirubin and its potent antioxidative forms*

Bilirubin displays an active antioxidant in the following three forms:

1. Free unconjugated bilirubin
2. Conjugated bilirubin
3. Albumin-bound bilirubin.

*Ad. 1. Free unconjugated bilirubin:*
In this form bilirubin is particularly effective due to distinctive reactivity with superoxyl- and peroxyl anions, as well as the ability to scavenge $O_2$-radicals (STOCKER and AMES, 1987).

*Ad. 2. Conjugated bilirubin*
Because of its higher water-solubility, conjugated bilirubin circulates free in the blood and shows unique efficiency in preventing lipids against oxidation. In

comparison, biliverdin displays a 2.5 higher potency in its protective properties per molecule (STOCKER *et al*., 1990).

*Ad. 3. Albumin-bound bilirubin*
The prevention of albumin ligands and albumin-bound fatty acids against oxidation is the predominant task of albumin-bound bilirubin (TOMARO and BATLLE, 2002).

### *2.2.2.4 Bile pigments in comparison to other antioxidants*

To provide a better overview, results of *in vitro* results dealing with the antioxidative capacity of bile pigments will be given foremost, followed by topical *in vivo* research findings.

In 1953 the antioxidant activity of bile pigments was proposed primarily by Bernhard *et al.* This study depicted a considerable impulse on further research since it was demonstrated that the oxidation of Vitamin A could be inhibited through co-incubation with unconjugated bilirubin or biliverdin respectively.
A further fundamental study was presented by Stocker *et al.* in 1987:
Bilirubin, bound to human albumin and present at normal concentrations in human plasma, protects albumin-bound linoleic acid from peroxyl radical-induced oxidation *in vitro*. Initially, albumin-bound bilirubin is oxidized at the same rate as peroxyl radicals are formed and, as a result, a stoichiometrical amount of biliverdin is produced as the oxidation product. In regard of other antioxidants it was shown that on an equimolar basis, albumin-bound bilirubin successfully competes with uric acid for peroxyl radicals but in comparison with vitamin C it is less efficient in scavenging these radicals.
This study verified that 1 mol of albumin-bound bilirubin could scavenge 2 mol of peroxyl radicals and that small amounts of plasma bilirubin were capable of preventing oxidation of albumin-bound fatty acids as well as of the protein itself. By this means the role for albumin-bound bilirubin as a physiological antioxidant in the plasma and the extra vascular space was confirmed again.

In 1994 *in vitro* studies showed that the oxidation of lipids, in particular low density lipoproteins (LDL), could be reduced by unconjugated and conjugated bilirubins. Since oxidation of LDL is causally related to the pathogenesis of arterial diseases (STOCKER and KEANEY, 2004), these were very auspicious results, proposing bilirubin as a cardio-vascular protective compound. Concerning lipid peroxidation, bile pigments actually turned out to be the most effective free radical generating system amongst all the others. In addition, uconjugated bilirubin emerged as an even stronger protector of proteins from oxidation than vitamin E in further *in vitro* studies. As proteins are found throughout cells and include transporters and enzymes that are substantial for the cell function, this is another noteworthy aspect (NEUZIL *et al.* 1993).

Another experiment focused particularly on the differences between glutathione, a tripeptid which is part of a redox system in the organism, and bilirubin. Both compounds represent important endogenous antioxidants and act in a very similar way in terms of their metabolism. Both systems contain an oxidized and a reduced form, whereas the latter is predominant under healthy conditions. However, the hydrophobic bile pigment protects cell membranes from lipidoxidation, while the water-soluble glutathione mainly prevents cytoplasmic components from radical-mediated damage. In this study, hepatic cells were exposed to different concentrations of $H_2O_2$ to investigate whether biliverdin reductase is able to attenuate oxidative stress related events in the cells. When biliverdin reductase was absent, cell death rates increased obviously, for glutathione there was just a little effect at one concentration of the oxidant (200 µM). Bilirubin succeeded in the protection of cells from a 10 000-fold excess of $H_2O_2$. It is assumed that the biliverdin reductase system is at least as substantial as the glutathione system in physiologic cytoprotection (BARANANO *et al.*, 2002).
A comparison of the antioxidant properties of bilirubin and biliverdin was presented by Asad *et al.* in 2001.
For this purpose the inhibition of DNA cleavage, induced by L-DOPA-Cu(II) (HUSAIN and HADI, 1995) by both compounds was studied at different

concentrations. Bilirubin succeeded and therefore turned out to be the more potent antioxidant, since it inhibited the DNA cleavage to a greater extent than biliverdin at the same concentration rates.

Generally and according to research results in 1997 it was found that the antioxidant role of bilirubin was not that of a preventive antioxidant (antioxidant intercepting the initiator of the oxidative process), but appeared to be more similar to that of a chain-breaking antioxidant (antioxidant acting on secondary oxidants involved in the propagation of the damage) (MINETTI *et al.*, 1997). Referring to *in vivo* effects in humans, actually there do not exist any studies involving the oral supplementation of bile pigments. That is why the impact of elevated circulating bile pigment concentrations have been determined in models in which the increase was induced by exogenous bile pigment administration or underexpression of UGT1A1 (Gunn rat), resulting in an increased circulating bilirubin concentration. This is for example the case in the Gilbert's syndrome, a condition which appears at birth.
In 1995 Dennery *et al.* published the perhaps most widely known *in vivo* study of unconjugated bilirubin protection. It was shown that hyperbilirubinemic Gunn rats had reduced levels of plasma oxidative stress, when exposed to chronic hyperoxia, compared to control rats with normal serum bilirubin concentrations. There was no difference between the groups in regard of serum antioxidant concentrations (superoxid dismutase, catalase, glutathione, vitamins A, C, E and uric acid) and, therefore they could not account for the decreased concentration of oxidative metabolites. Another finding based on the results was the apparent negative correlation between serum bilirubin and thiobarbituric acid reactive substances, a product of lipid oxidation. In summary, the results suggested that a raise of the serum unconjugated bilirubin concentration from 0.7 to 7 mg/dL in rats can reduce indicators of plasma oxidation, caused by hyperoxic exposure.

Later, in 1994, Schwertner *et al.* were successful with linking the total plasma concentration of bilirubin to the prevalence of atherosclerotic disease for the

very first time which illustrates a magnificent and encouraging advancement for further research.
Their repeatedly confirmed findings showed that individuals with higher plasma bilirubin concentrations were less likely to suffer from several variations of cardiovascular diseases including coronary artery plaque formation and peripheral arterial disease for instance (PERLSTEIN *et al*., 2008).
Concerning biliverdin it was shown that when acting together with unconjugated bilirubin in a redox cycle, this compound was a more potent antioxidant pool than on its own (BARANANO *et al*., 2002) and protects the mammalian feto-placental system from ROS overproduced in placental mitochondria (MYATT, 2006).

Multiple studies reinforced the antioxidant capacity of mildly elevated serum bilirubin levels, as well as activation of heme oxygenase, and pointed at their protection from pro-oxidative challenges, also at physiological concentrations.
Altogether there is abundant evidence supporting the thesis that circulating bilirubin concentrations show a negative relationship with the severity of atherosclerotic diseases. Now it is imperative to find ways of increasing the circulating bile pigment concentration, without causing toxicity resulting from accumulated unconjugated bilirubin.
The limiting factor of toxicity is the plasma's capacity to bind albumin (600 µM), according to this, a considerable scope between 10 µM up to 150 µM is supposed to be an adequate range for the protective effects of bile pigments and bilirubin in particular (BULMER, 2008).

#### *2.2.2.5 Mechanism of the antioxidative activity of bile pigments*

The antioxidant properties of bilirubin and biliverdin are predominantly due to their ability to scavenge free radical species such as superoxide anion and peroxide radicals (FARRERA *et al*., 1994).
More precisely it is hypothesised that the antioxidant effects of bilirubin and biliverdin are related to their capacity to donate electrons, as bilirubin can donate two electrons and biliverdin just one.

Stocker *et al*. demonstrated that bilirubin, during free radical reaction, formed a stable free radical with resonance stabilization extending over the entire bilirubin molecule (STOCKER *et al*., 1987a).
It can be adopted that similar free stable radicals are also able to be formed with other porphyrins which possess α-hydrogen atoms adjacent to the porphyrin ring (one of the possible products of a free radical reaction with protoporhyrin IX is given in Figure 2).
These radicals can be beneficial for the DNA since they are incapable of prolonging free-radical chains (BULMER *et al*., 2008).

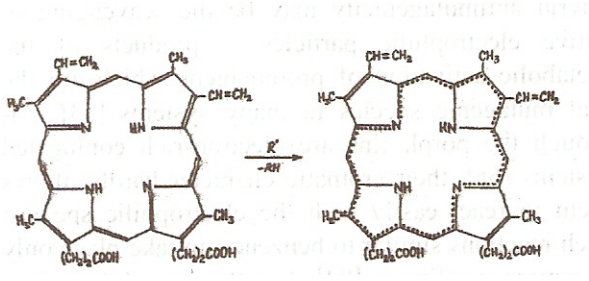


***Figure 2:*** *Proposed structure of a stable free porphyrin radical forming during interaction of a porphyne derivative with active reef radial R˙ (on the example of protoporphyrin IX). (ODIN AP, 1997)*

In 1987 Stocker *et al*. dealt intensely with the antioxidant activity of albumin-bound bilirubin. They pointed out that albumin-bound bilirubin, at concentrations found in plasma of healthy adults, was a very efficient peroxyl radical scavenger and protects fatty acids transported on albumin from oxidation by these radicals. The binding of bilirubin dianion to the primary binding site on albumin is thought to involve ion pairing, hydrogen bonding, interactions between amino acid side chains and the pigment, thereby fixing the two planar dipyrroles of the bilirubin molecule in an out-of-plane position (BRODERSEN, 1979).
Such asymmetric positioning of the bilirubin molecule on albumin is assumed to expose the reactive hydrogen atom at C-10 for initial hydrogen abstraction by peroxyl radicals while preventing the two dipyrroles moieties from oxidation. Thus, binding to albumin seems to afford both increased specificity and reactivity of bilirubin towards peroxyl radicals. Further, the results indicate that

each molecule of albumin-bound bilirubin can donate two hydrogens to scavenge two molecules of peroxyl-radicals, giving rise to albumin-bound biliverdin as the reaction product (STOCKER *et al*., 1987a).
Also unbound bilirubin, in solution or when incorporated in liposomes, efficiently scavenges peroxyl radicals (Stocker *et al*., 1987b).

### 2.2.3 Anti-mutagenic properties of bile pigments

#### *2.2.3.1 General aspects*

Because of an increased rate of mutations and the related possible risks of cancer in humans, discovery and exploration of chemical compounds with anti-mutagenic and anticarcinogenic potency are of great importance at the present time (ODIN, 1997).
Adjacent to the conclusion that bile pigments possess considerable antioxidative activity, the evidence for their additional anti-mutagenic potential has also been mounting during the last 20 years. These precisely not useless by-products of heme catabolism inhibit the mutagenic effects of numerous classes of mutagens (*e.g*. polycyclic aromatic hydrocarbons, heterocyclic amines, oxidants) but despite the encouraging *in vitro* anti-mutagenic effects of bile pigments, relatively little research probing their protective capacity in bacterial and cultured cell assays of mutation has been conducted to possibly link the available *in vitro* with the *in vivo* observations.
Spontaneous mutation is thought to occur as a result of imperfect DNA recombination and repair processes as well as from the production of intracellular free radicals that alter genomic structure.
As bile pigments show anti-mutagenic activity and are present at high concentrations in the bile and intestines of humans, their presence could possibly influence the genotoxicity of food derived mutagens in the human diet. Additional study of the bile and intestinal bile pigments could further elucidate the physiological and anti-mutagenic importance of these compounds in the gastrointestinal tract and the human body (BULMER *et al*., 2007).

Most of the anti-mutagenic investigations have utilized the *Salmonella* mutagenicity assay as a model. Table 3 gives a review of several studies that have been published concerning this matter, mainly with reference to this thesis (tested compounds, strains and mutagens).

### 2.2.3.2 *Anti-mutagenic effects of bile pigments in the Ames test*

Numerous experiments exploring the antigenotoxicity (equivalent with the term mutagenicity) of the porphyrins have enhanced bacterial cells.
In table 3 a selection of published data is summarised.
In most of the conducted tests besides bilirubin and biliverdin, tetrapyrolles including hemin, chlorophyllin or protoporphyrins have been tested,

Arimoto *et al*. explored the antigenotoxic affectivity of the porphyrins hemin, chlorophyllin, bilirubin and biliverdin against the mutagenicity of 6 amino acid pyrolysis products from soy bean, namely Trp-P-1, 3-amino-1-methyl-5H-pyridol[4,3-b]indole (Trp-P-2), Glu-P-1, 2-amino-dipyrido[1,2-α:3’2’d]imidazole (Glu-P-2), 2-amino-9H-pyrido[2,3-b]indole (amino-α-carboline), and 2-amino-3-methyl-9H pyrido[2,3-b]indole (aminomethyl-α-carboline). All of the experiments were conducted with metabolic activation. Hemin and chlorophyllin depicted strong anti-mutagenic activity in all classes. Protoporhyrin IX (hemin without central iron molecule) was active in three tests only (against Trp-P-1, Trp-P-2, and aminomethyl-α-carboline), biliverdin was less active while bilirubin had no effect. The addition of hemin to the preincubation mixture in the Ames test resulted in strong inhibitions for benzo[α]pyrene and Aflatoxin $B_1$. Generally, 50% inhibition could be reached by an amount of hemin 1-2 equivalent to the mutagen whereas the anti-mutagenic capacity of bilirubin, biliverdin and chlorophyllin in inhibiting benzo[α]pyrene-induced mutagenicity turned out to be significantly lower. These studies point out that the tetrapyrrole macrocycle is crucial for complexing. Therefore hemin, chlorophyllin, and protoporphyrin IX were significantly more effective than open tetrapyrroles such as biliverdin and bilirubin (ARIMOTO *et al.*, 1980b).

A further study by Arimoto *et al*. concentrated on the possible anti-mutagenic effects of hemin, bilirubin and biliverdin against benzo[α]pyrene, a polycyclic aromatic hydrocarbon derived from the combustion of fossils fuels. The findings indicated that bile pigments were potent anti-mutagens in the *TA98* strain with the 50% inhibition dose ($ID_{50}$) for bilirubin and biliverdin approximating 50 and 500 nmol/plate, respectively. Bilirubin was considered to be more effective than biliverdin in inhibiting mutations in the *TA98* strain caused by benzo[α]pyrene in this study (ARIMOTO *et al*., 1980a), but in more recently published data biliverdin exceeds the effectiveness of bilirubin and bilirubin ditaurate against benzo[α]pyrene. In the latter study the $ID_{50}$ for bilirubin, biliverdin and a water soluble bilirubin conjugate, bilirubin ditaurate equalled 658, 284 and 576 nmol/plate, respectively (BULMER *et al*., 2007).
Another investigation was made under similar conditions but with strain *TA100*. The effects of benzo[α]pyrene and its purified metabolites B[α]P-7,8-diol, B[α]P-4,5-epoxide and B[α]P-7,8-diol-9,10-epoxide on bilirubin and biliverdin were tested in the presence of metabolic activation, which depicts the main difference to the study mentioned before. In this experiment bilirubin and biliverdin inhibited benzo[α]pyrene induced mutation. The $ID_{50}$ for the two compounds constituted approximately 500 and 1000 µmol/plate, respectively, when incubated with 10 nmol/plate of the mutagen. Also a weak anti-mutagenic activity against the metabolites of benzo[α]pyrene, B[α]P-4,5-epoxide and B[α]P-7,8-diol-9,10-epoxide, was verified. In order to elucidate the mechanism of antimutagenicity, the porphyrins were incubated with benzo[α]pyrene and S9 while the synthesis of 3-hydroxyB[α]P was measured. Hemin and Fe-chlorin succeeded in inhibiting the above-mentioned synthesis, whereas bilirubin and biliverdin prevented the process to a lesser extent but were able to interfere with the activation of the precursor of benzo[α]pyrene. Another experiment followed in order to test whether the bile pigments accelerated the degradation of benzo[α]pyrene metabolites, but this assumption could not be verified (ARIMOTO *et al*., 1995).

However, by now the mechanisms of bile pigments on the binding and metabolism of benzo[α]pyrene are not clearly understood and thus further research is needed.

To pursue with the relevance of structural circumstances, Bulmer *et al*. intended to ascertain whether bile pigments could selectively inhibit the genotoxic effects of mutagens with specific three-dimensional conformations. For this purpose, previously untested combinations of bile pigments and mutagens such as 2,4,7 trinitrofluorenone (TNFone) (without metabolic activation) and 2-aminofluorene (2-AF) (with metabolic activation), which possess planar and non-planar stereochemistries (Figure 4), were applied.

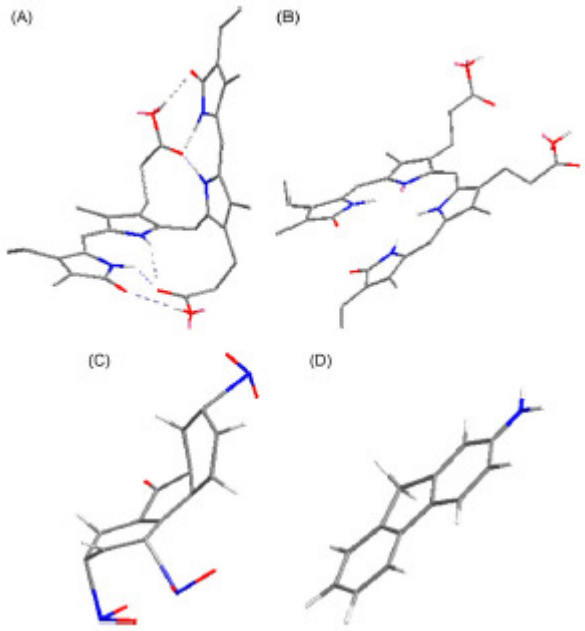



***Figure 3:*** *Three-dimensional, energy minimised (MM2) structures of unconjugated BR (A), unconjugated BV (B), 2,4,7 trinitrofluorenone (C) and 2-aminofluorene (D) (BULMER et al., 2007)*

Another intention of the experiments was to explore whether bile pigments would show anti-mutagenic effects when incubated with the oxidant tertiary-butyl hydroperoxide (*t*-BuOOH), suggesting a multi-modal mechanism of action. The tests were conducted in three strains of *Salmonella typhimurium* (*TA98*,

*TA100*, *TA102*) to establish whether the compounds were able to modulate spontaneous or induced genotoxicity.
Regarding the anti-mutagenic effects, the observation that bile pigments had inhibited benzo[α]pyrene induced mutation previously could be supported, presenting biliverdin as more potent than the other bile pigments tested. The $ID_{50}$ of bilirubin ditaurate amounted to 0.58 µmol, the one of BR to 0.66 µmol, suggesting the conjugation of bilirubin had no influence on its anti-mutagenic potential. For TNFone and 2-AF induced mutation, a common trend in all strains could be observed when the anti-mutagenic effects of the bile pigments were tested. The order of effectiveness in respect of the $ID_{50}$ turned out to be as follows:
BR≥BRT>BV for TNFone and BV≥BRT≥BR for 2-AF. As the results were similar in all strains when no metabolic activation was applied, this leads to the suggestion that bile pigments affected the ability of TNFone to disrupt DNA structure. Regarding 2-AF it should be noted that only in the presence of the microsomal S9 fraction, its metabolism to the mutagenic compound 2-acetylaminofluorene is possible. This might explain the anti-mutagenic effects of bile pigments against 2-AF induced mutation, since the cytosolic enzymes are not active in the S9 mix, in addition to binding/inactivation of the mutagens.
The affinity of porphyrins for planar aromatic compounds is known and suggests that bile pigments could protect from genotoxicity by interacting with these mutagens, possibly via π-stacking, hydrogen bonding or hydrophobic interactions (ARIMOTO *et al.*, 1995).

As already mentioned, the multi-modal mechanism of action of bile pigments was also tested, either with or without metabolic activation. For this purpose *TA102* was incorporated as a second strain. One characteristic of this strain is its susceptibility to oxidative stress induced mutation. The appropriate mutagen for this purpose was *t*-BuOOH, which causes DNA damage *in vitro* (EPE *et al.*, 1990) via the generation of reactive oxygen species (ROS), including alkoxyl radicals (MINOTTI *et al.*, 1986). The findings showed that bilirubin as well as biliverdin attenuated *t*-BuOOH induced mutation. Metabolic activation did not

influence the results. With an $ID_{50}$ of 0.556 µmol, amounting to approximately half of that of biliverdin, bilirubin exhibited its effectiveness to a much greater extent. According to observations (STOCKER, 2004), this could be explained by bilirubin's capacity to neutralise two radicals via initial hydrogen atom donation, resulting in a bilirubin radical, which binds to another radical forming a non-radical complex (STOCKER *et al*., 1987b). In contrary, biliverdin accepts only one electron with the formation of a resonance stabilised biliverdin radical complex (STOCKER *et al*., 1987b).

In summary, the results charge both, bilirubin and biliverdin, a more efficient protection against oxidative stress induced mutation than many other antioxidants. For instance caffeic acid and α-tocopherol had, compared to the bile pigments, only little (EDENHARDER and GRUNHAGE, 2003), if any (GREY and ADLERCREUTZ, 2003), anti-mutagenic potential in the presence of *t*-BuOOH.

The data presented cannot be translated to the *in vivo* condition but nevertheless they do fortify other anti-mutagenic and anti-carcinogenic evidence including bilirubin's apoptotic effects in colon cancer cell lines (KESHAVAN *et al*., 2004), and emphasize the negative correlation between circulating bilirubin concentrations and cancer mortality (TEMME *et al*., 2001).

Remarkably, the anti-mutagenic effects of bilirubin ranged between ~3 to 250 µM, encompassing the plasma bilirubin concentrations found in healthy persons (~ 12 µM) (HOPKINS *et al*., 1996), in those with Gilbert's Syndrome (~ 40 µM) (NAIMAN *et al.,* 1996) and in Crigler-Najjar syndrome (~ 80-280 µM) (YOUNOSSI and FOROOZAN, 1995).

To respond to the possible anti-mutagenic effects of porphyrin derivatives on cigarette smoke condensate and Swedish moist oral snuff, a study of Romert *et al.* will be presented in the following. The compounds hemin, biliverdin dichloride and chlorophyllin were tested in strains *TA98* and *TA100*.

Compared to hemin and chlorophyllin, biliverdin was not as effective, especially in strain *TA98* biliverdin did not decrease the number of mutant colonies beyond the 50% line.

To concretise the results, in *TA100* the $ID_{50}$ values for biliverdin amounted to approximately 0.4 µM, whereas for chlorophyllin it constituted about 0.1 µM (ROMERT *et al*., 1994).
However, in lower concentrations the results of the anti-mutagenic potential of chlorophyllin and hemin respectively were inconclusive.
The main difference in effectiveness between biliverdin and bilirubin compared to hemin and chlorophyllin become apparent in the compound's structures. Both, hemin and chlorophyllin, possess a chelated metal ion which seems to be crucial for the anti-mutagenic activity. According to this fact the automatic drawing of conclusions between the anti-mutagenic potential of the discussed compounds are limited.
Tang and Edenharder investigated the same compounds in regard of their potential to inhibit the induced mutagenicity of 2-nitrofluorene, 3-nitrofluoranthren and 1-nitropyrene in strain *TA98*. Strong anti-mutagenic activity was observed for hemin and bilirubin, while biliverdin and chlorophyllin were also active but to a lesser extent (TANG and EDENHARDER, 1997).

Another series of experiments focussed on the ability of bilirubin, and 89 other potential anti-mutagens, to inhibit cigarette smoke and 4-nitroquinoline-1-oxide (4NQO) induced mutation in strain *TA98* and *TA100*. Bilirubin showed considerable activity against both mutagens in strains *TA98* and *TA100*. Biliverdin was unable to affect the mutagenic effects of 4NQO (CAMOIRANO *et al.*, 1994) in the latter strain, in strain *TA98* no tests were conducted.

In summary all the studies mentioned support the theory that bile pigments possess anti-mutagenic skills. Nevertheless, further research is needed for a better understanding of the apparent *in vivo* importance and for exploring the therapeutic potential of these naturally occurring compounds.

### *2.2.3.3 Mechanism of the bile pigment's anti-mutagenic effects*

Bilirubin and biliverdin are a part of the porphyrin family of molecules. Porphyrin derivatives, such as chlorophylls and hemoglobins, which include metalloporphyrins as the active part are widespread in nature and display cyclic aromatic polyamines containing a system of conjugated double bonds along the whole contour of the porphyrin macrocycle. Thereby a planar molecule of strong aromatic nature is formed (BEREZIN and ENIKOLOPJAN, 1985).
As the chemical structure determines the unique biochemical and genetic properties of the porphyrins to a considerable extent, it will be discussed in the following.
Bilirubin and biliverdin are part of the physiological metabolism and catabolism of heme which is formed by adding iron to protoporphyrin. When the iron molecule is removed and the protoporphyrin ring is cleaved (-CO), biliverdin is formed and reduced to bilirubin in the next step.
When studying the anti-mutagenic potential of these compounds it becomes obvious that the specific addition and removal of functional groups could be of particular importance. Related to this, one would expect significant differences in anti-mutagenic effects between protoporphyrin and heme due to the incorporation of $Fe^{III}$ into the centre of the fused protoporphyrin ring and the resulting structural distinctions. In effect, the addition of a chelated iron molecule could confer the ability to bind additional molecules, possibly detoxifying them. Another indicator for this hypothesis is driven by the fact that adding metals, including copper, to chlorines increases the detoxification of polycyclic aromatic hydrocarbons. In addition, when the ring is opened and fully conjugated in consequence of the removal of CO, the structure of biliverdin is less potent than protoporphyrin, suggesting the facility that the planarity of porphyrins may affect their anti-mutagenic potential (ARIMOTO *et al.*, 1995).
In a study by Arimoto *et al.* bilirubin turned out not to be anti-mutagenic when being folded into a three dimensional ridge-tile conformation after the hydrogenation of biliverdin at the C10 methine bridge (see Figure 4).

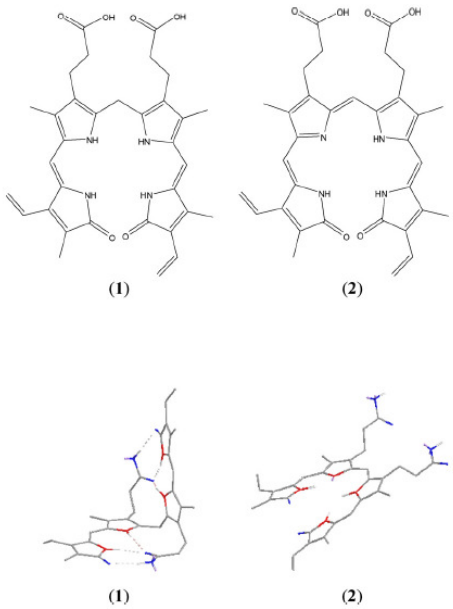



***Figure 4:*** *Two- and three-dimensional structures of bilirubin (1) and biliverdin (2) (Bulmer et al., 2008)*

Due to the hydrogen bonded and folded conformation of bilirubin, it is possible that a planar tetrapyrrolic structure (*e.g.* protoporphyrin, biliverdin) is also essential for anti-mutagenic binding of the amino acid pyrolysis products. Studying the structurally related porphyrins including protoporphyrin, heme, biliverdin, bilirubin and other hydrogenated and oxidised products, could support in establishing the structure-activity relationships of these compounds. In a study by Bulmer *et al*. (2008) the anti-mutagenic effects of the bile pigments were dependent on the mutagen that was tested and dependent of the strain used in the test, suggesting some interaction between the pigments and the mutagens

Currently, there is not a single known mechanism to fully explain the anti-mutagenic activity of porphyrins, only some common agenda concerning their antigenotoxic effects. Thorough discussion in order to clarify the possible role in mutagen detoxification and DNA repair for each compound of the porphyrin family is necessary (ODIN, 1997).

### *2.2.3.4 Mutagens used in the project*

- Aflatoxin $B_1$ ($AFB_1$)

Aflatoxins are a family of structure related mycotoxins and produced as secondary metabolites by the spoilage of fungi Aspergillus, particularly A. flavus and A. parasiticus (SWEENEY and DOBSON, 1998). Amongst others, $AFB_1$ is one of the most important members, depicting the most frequent metabolite in contaminated food samples with a clarification in the group I as carcinogens to humans (SIMON *et al.*, 1998). Aflatoxins are commonly found in peanuts (STROKA *et al.*, 2000), maize (SCUDAMORE and PATEL, 2000) and other nuts

***Picture 1**: Aflatoxin $B_1$*

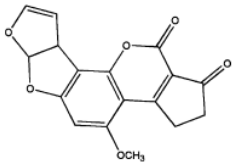



***Figure 5:** $AFB_1$*

- 2-Amino-1-Methyl-6-Phenylimidazo[4,5-*b*]Pyridine (PhIP)

PhIP belongs to the group of heterocyclic aromatic amines, a class of hazardous chemicals that are receiving heightened attention as a risk factor for human cancer. These heterocyclic aromatic amines arise during the cooking of meats, fish, and poultry, and several of these substances also occur in tobacco smoke condensate and diesel exhaust. DNA adducts of heterocyclic aromatic amines have been detected in human tissues, demonstrating that they induce genetic damage even though the concentrations of these compounds in cooked meats are generally in the low parts-per-billion (ppb) range (TURESKY, 2007).


***Picture 2:** PhIP*

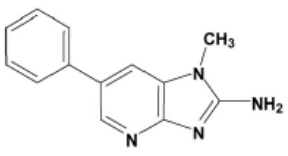



***Figure 6:** PhIP*

- *2,4,7 trinitrofluorenone (TNFone)*

TNFone is a non-planar polycyclic nitro-amine, not commonly found in the environment, but previously found in photocopy toner (KARI, 1992).

***Picture 3:*** *TNFone*

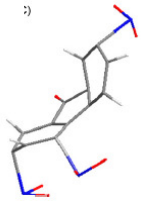


***Figure 7:*** *TNFone*

- *Tertiary butyl hydroperoxide (t-BuOOH)*

This mutagen is an organic hydroperoxide and oxidant.

Although rarely handled, oxidants have been used in several studies of anti-mutagenic investigation to figure out whether oxidative stress induced mutation and carcinogenicity can be inhibited by bile pigments (BULMER *et al.*, 2007).

***Picture 4:*** *t-BuOOH*

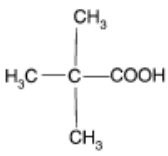



***Figure 8:*** *t-BuOOH*

| No. | Author | Salmonella Strain | Compounds | Mutagen | Metabolic Activation | Results/description |
|---|---|---|---|---|---|---|
| 1 | Arimoto *et al*. 1980b | *TA98* | BV (0-2 µmol), hemin, protoporphyrin, chlorophyllin | Amino acid pyrolysis products: Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, AαC, MeAαC | + S9 | Pyrrole pigments were effective against all six mutagens in the following order: hemin>chlorophyllin>BV<br>Protoporphyrin inhibited only the mutagenicity of Trp-P-1, Trp-P-2 and MeAαC<br>BR did not show any anti-mutagenic effect |
| 2 | Arimoto *et al*. 1980a | *TA98* | BR (0-1.6 µmol), BV (0-0.75 µmol), hemin, chlorophyllin | B[α]P | + S9 | According to the $ID_{50}$ for B[α]P the order of effectiveness was: Hemin>BR>chlorophyllin>BV<br>ID50 BR, BV: 0.05 vs. 0.5 µmol, hemin: 0.01 µmol |
| 3 | Bulmer *et al*., 2007 | *TA98, TA100, TA102* | BR, BV, BRT (0-2 µmol) | TNFone, 2-AF, $NaN_3$, *t*-BuOOH | B[α]P (*TA98*, + S9), TNFone (TA98, TA100, TA102, - S9), 2-AF (TA98, TA100, TA102, + S9), NaN3 (TA100, - S9), *t*-BuOOH (TA102, +/- S9) | The anti-mutagenic effects of the bile pigments depended on the tested mutagen. Endogenous bile pigments inhibit oxidative stress induced mutation.<br>TNFone: BR=BRT>BV<br>2-AF: BV>BRT>BR<br>B[α]P: BV>BRT>BR<br>*t*-BuOOH: BR>BV>BRT<br>ID50 BR, BV, BRT: 0.557;1;2.756 µmol (*t*-BuOOH – S9) |
| 4 | Arimoto *et al*., 1995 | *TA100* | BR, BV, Cu-chlorin, Fe-chlorin, hemin, protoporphyrin, chlorophyllin | B[α]P, B[α]P(7,8)D, B[α]P(4,5)E, B[α]P(7,8)D(9,10)E | +/- S9 | Order of efficiency without metabolic activation (-S9) was: Hemin=protoporphyrin> hematoporphyrin=Cu-chlorin> Fe-chlorin=chlorophyllin =BR>BV<br>Order for BP(4,5)E and BP (7,8)D(9,10)E: Hemin>chlorophyllin>proto-porphyrin>hematoporphyrin>BR=BV<br>Both, hemin and Cu-chlorin accelerated degradation of B[α]P(7,8)D(9,10)E,<br>BR and BV suppress B[α]P metabolism by S9, but do not accelerate B[α]P metabolite degradation |

| | | | | | | |
|---|---|---|---|---|---|---|
| 5 | Romert *et al.*, 1994 | *TA98*, *TA100* | BV (0-4 µmol), hemin, chlorophyllin | CSC, SNUS | +/- S9 | Explicit anti-mutagenic results only for hemin and chlorophyllin<br>BV was less effective, especially in TA98<br>Order of effectiveness in TA98:<br>Hemin=chlorophyllin>BV<br>TA100:<br>Chlorophyllin>BV?hemin<br>ID50 BV TA98: n/a, TA100:3.8 µmol |
| 6 | CAMOIRANO *et al.*, 1994 | *TA98*, *TA100* | BR, BV (0-10 µmol), hemin, chlorophylin (90 compounds in total) | Cigarette smoke, 4-nitroquinoline-1-oxide | Cigarette smoke (+ S9, *TA98*), 4NQO (-S9, TA100) | BR was the only compound capable of inhibiting mutagenesis of both mutagens. |
| 7 | TANG and EDENHARDER, 1996 | *TA98* | BV (1 µmol), BR (7 µmol), hemin, chlorophyllin | 2-nitrofluorene, 3-nitrofluoranthren, 1-nitropyrene | None | All porphyrins and related compounds exerted an anti-mutagenic effect against the three mutagens.<br>Order of effectiveness:<br>BR?hemin>BV?chlorophyllin |

***Table 2:*** *Bile pigments and other pyrrolic compounds in the Ames test*

Abbreviations:

Compounds: BR=bilirubin, BV=biliverdin;

Mutagens:Trp-P-1=3-amino-1,4dimethyl-5H-pyrido[4,3-b]indole;Trp-P-2=3-amino-1-methyl-5H-pyrido[4,3-b]indole;Glu-P-1=2-amino-6-methyl-dipyrido[1,2-a:3',2'-d]imidazole;Glu-P-2=2-aminodipyrido-[1,2-a:3',2'-d]imidazole;AαC=2-amino-9H-pyrido[2,3-b]indole;MeAαC=2-amino-3methyl-9H-pyrido[2,3-b]indole;b(α)P=benzo(α)pyrene;TNFone=2,4,7 trinitrofluorenone;2-AF=2-aminofluorene;$NaN_3$=sodium azide; *t*-BuOOH=tertiary butyl hydroperoxide;S9=microsomal liver homogenate; B[α]P(7,8)D=benzo(α)pyrene-trans-7,8-dihydrodiol; B[α]P(4,5)E=4,5-dihydro-benzo[α]pyrene-4,5-epoxide; B[α]P(7,8)D(9,10)E=benzo[α]pyrene-trans-7,8-dihydrodiol-9,10-epoxide(anti); CSC=cigarette smoke condensate; CS=cigarette smoke;SNUS=extract and subfractions of Swedish moist oral snuff;4NQO= 4-nitroquinoline-1-oxide

### 2.2.4 The toxicity of bile pigments

As bilirubin is a weakly-polar, poorly-soluble compound, the lion's share of plasma bilirubin in circulation (5-15 µM) is tightly bound to serum albumin (BLOOMER *et al.*, 1971), with less than 0.01% of total bilirubin circulating in an unbound form (free bilirubin). This fraction allows the diffusion of UCB into tissues, and therefore free bilirubin is responsible for both its beneficial and toxic effects on cells. While normal to modestly elevated plasma bilirubin levels are cytoprotective, higher plasma and tissue bilirubin levels often show cytotoxic effects (VÍTEK and OSTROW, 2009).

Plasma concentrations which reach values greater than 300 µM are associated with the risk of developing neurological dysfunctions (MEUWISSEN and HEIRWEGH, 1982) impairment of cellular functions in the brain tissue (SCHENKER *et al.*, 1986) and neonatal hyperbilirubinemia since the liver's capacity to excrete bilirubin is exceeded. Disordered hepatic function, including mild (Gilbert's syndrome) and severe (Crigler-Najjar syndrome) deficiencies in UGT1A1 also result in increased circulating bilirubin concentrations. However, bilirubin toxicity is rarely observed in these persons and with the use of phototherapy or, if the worst comes to the worst, a liver transplantation, elevated bilirubin levels can be cured (STRAUSS *et al.*, 2006).

# 3. Materials and methods

## *3.1 Methods*

### 3.1.1 Experimental design

Based on other studies, six doses of BRDME and BVDME were used in the Ames test (0.01, 0.05, 0.1, 0.5, 1 and 2 µmol/plate). The assay was performed according to Mortelmans and Zeiger with 25 min of preincubation. The assay was performed in a dimly lit laboratory and the bile pigment solutions were protected from light using foil covered containers and amber cups. The pipetting sheme was as follows: 500 µL of PBS or S9 mix followed by 100 µL of overnight bacterial culture, 200 µL of bile pigment solution (DMSO) and finally 100 µL of mutagen solution (DMSO). The test tubes were incubated for 25 min at 37°C on a rotary shaker.

Afterwards 2 mL of molten agar was added to every tube, which was vortexed and poured onto minimum glucose agar plates. After 20 to 30 minutes drying time the plates were put in the incubator for 48 h at 37°C and the $His^+$ revertants were counted manually after that. Three plates were counted for every concentration of each condition and each test, except one ($AFB_1$ 120 µg/ml, *TA102*) was repeated on another day. In every test, three 'positive control' (mutagen only) and six 'negative control' (no mutagen or bile pigment) plates were included. The modulatory effects of bile pigments were investigated in two testing paradigms. Firstly, a series of experiments investigating the pro- and anti-mutagenic effects of the test compounds, in the presence of known mutagens, were conducted. In a second procedure the mutagenic effects of the bile pigments only was tested.

### 3.1.2 Chemicals

Bilirubin dimethyl ester [CAS # 19792-68-8] and biliverdin dimethyl ester [CAS # 10035-62-8] were acquired from Frontier Scientific (Logan, UT, USA). MP Biomedicals (Illkirch, France) delivered the S9 liver homogenate.

All mutagens and other reagents were of analytical grade or better and stored at – 80°C if necessary.

***Picture 5:*** *Biliverdin dimethyl ester*

### 3.1.3 Bacterial strains

The S. typhimurium strains *TA98* and *TA102* were obtained from Dr. Bruce N. Ames (University of California, Berkley, USA). Stock bacteria were stored at -80°C as frozen permanents prepared from bacterial overnight culture dissolved in DMSO (MORTELMANS and ZEIGER, 2000). For the experiments of this thesis the master plates from the foregoing working-team could be adopted.

### 3.1.4 Preparation of the samples

The bile pigment samples, tested for their anti-mutagenic and antioxidative potential respectively, were investigated at the University of Vienna, Department of Nutritional Sciences.

The compounds were mainly delivered in vials, containing 150 mg of the intensely coloured, powdered substances.

The preparation of the bile pigments followed the principle of a dilution series. Beforehand, 9 mg of the light sensitive samples were weighed in into brown coloured microtubes. For both compounds 6 different concentrations (2 µmol, 1 µmol, 0.5 µmol, 0.1 µmol, 0.05 µmol, 0.01 µmol), made of a stock solution, were tested.

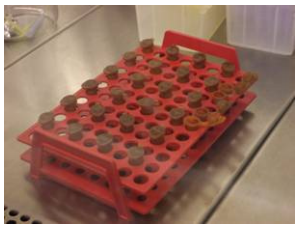
The stock solutions, prepared individually for BRDME and BVDME, consisted of the 9 mg weighted sample and 618 µL DMSO.

The solutions were then put into the ultrasonic bath for 3 min, to enhance the better solubility of the samples.

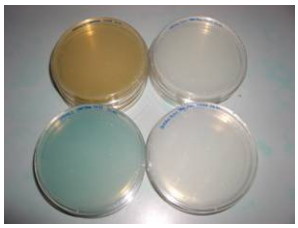
Afterwards the dilutions were conducted in the following order:

| concentrations | dilution |
|---|---|
| 2 µmol | 320 µl of stock solution + 480µL DMSO |
| 1 µmol | 160 µL of stock solution + 640 µL DMSO |
| 0.5 µmol | 80 µL of stock solution + 720 µL DMSO |
| 0.1 µmol | 100 µL (1 µmol) + 900 µL DMSO |
| 0.05 µmol | 100 µL (0.5 µmol) + 900 µL DMSO |
| 0.01 µmol | 100 µL (0.1 µmol) + 900 µL DMSO |

***Table 3:*** *Preparation of the samples*



***Picture 6:*** *Dilution of the samples*



***Picture 7:*** *Top left: BR at highest (2 µmol/plate), top left: BR lowest concentration (0.01 µmol/plate); below left: BV at highest concentration (2 µmol/plat), below right: BV at lowest concentration (0.01 µmol/plate)*

### 3.1.5 Evaluation of mutagenicity

The classification of the tested compounds as “mutagenic”, “pro-mutagenic” and “anti-mutagenic” was declared according to the guidelines published by Mortelmans and Zeiger (2000).

### 3.1.6 Mutagenic testing

The substances were identified as a “mutagen” if two criteria were applicable: Firstly a dose dependent increase in the number of revertants and secondly the number of revertants was equal to or exceeded 2fold negative control counts.

### 3.1.7 Pro/anti-mutagenic testing

The relative pro- and anti-mutagenic responses were calculated for each compound, in each condition, relative to the positive control, using the following equation.

$$\frac{\text{No. revertants formed [test compound] – No. revertants formed [negative control}*100}{\text{No. revertants formed [positive control] – No. revertants formed [negative control]}}$$

A compound was classified “anti-mutagenic” if the relative mutagenic response fell below 50%. A compound was classified “pro-mutagenic” if the relative mutagenic response increased beyond 200% (MORTELMANS and ZEIGER, 2000).
Additionally dose dependency was necessary according to Mortelmans and Zeiger. To facilitate the comparison between the different doses the results were specified per µmol/plate.
In order to visualise trends, mathematical algorithms were fitted to the data. The algorithm with the best fit for each compound in each condition was determined by observing the $R^2$ co-efficient and these algorithms were superimposed onto the results figures (MORTELMANS and ZEIGER, 2000). The results are presented in chapter 6, the 50% inhibition doses ($ID_{50}$) of the bile pigments and the inhibition percentage at 0.5 µmol/plate ($I_{0.5}$) and at the highest dose tested ($I_{max}$) are illustrated on page 37.

## *3.2 Main features of the Ames test*

### 3.2.1 Principle of the assay:

The *Salmonella typhimurium*/microsome assay (*Salmonella* test; Ames test) is a widely accepted short-term bacterial assay for identifying substances that can produce genetic damage that leads to gene mutations. The test uses a number of *Salmonella* strains with pre-existing mutations that leave the bacteria unable to synthesize the required amino acid histidine, and therefore unable to grow and form colonies in its absence (MORTELMANS and ZEIGER, 2000).

When the *Salmonella* tester strains are grown on minimal agar plates containing a trace of the amino acid histidine, only those bacteria are able to form colonies, that revert to histidine independence ($his^+$).
These days the bacterial reverse mutation assay has established itself as an initial screen to determine the mutagenic potential of new chemicals and drugs.

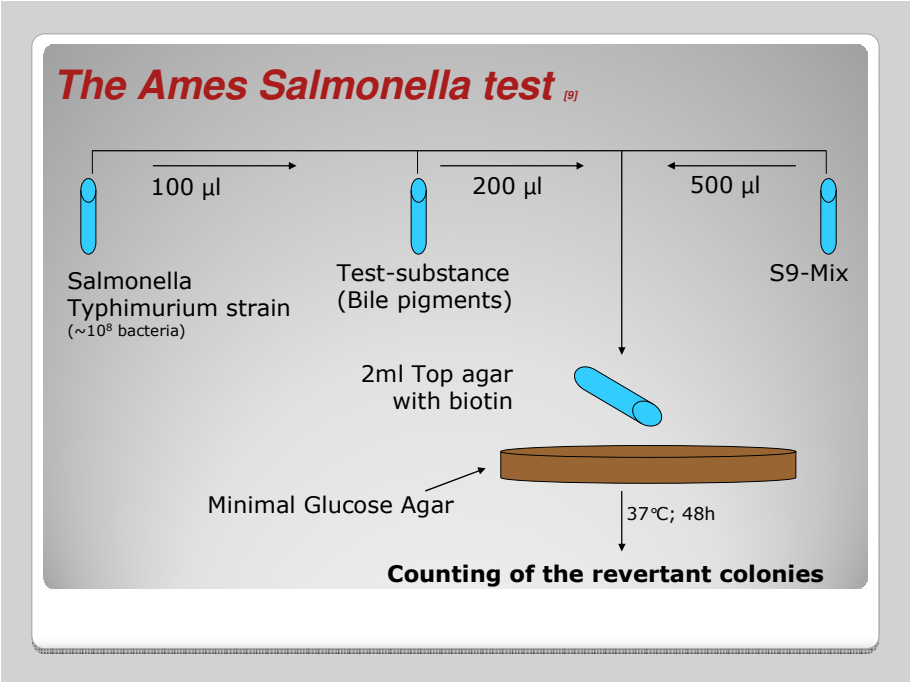



***Figure 9:*** *Steps depicting the preincubation assay*

## *3.3 Implementation of the Ames test*

### 3.3.1 Preparation of minimal glucose agar plates:

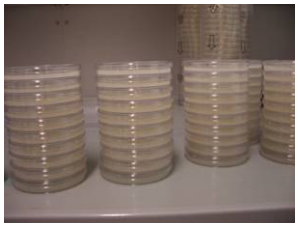
The outcome of every production of minimal glucose agar plates was approximately about 300 minimal glucose agar plates.
Therefore, 8 1.000 mL Erlenmayer flasks were filled up with 11.25 g of Oxoid agar, followed by 700 mL of distilled water. The flasks were closed with a dabber and wrapped with a piece of aluminium foil. Together with an adequate amount of glucose- and Vogel-Bonner solution, kept in 250 ml Pyrex flasks, they were put into the autoclave and sterilised. To control the sterilisation

operation, an indicator sticky tape was placed on each flask (For preparations of the solutions see below).

***Picture 8:*** *Autoclave with Erlenmayer flasks containing minimal glucose agar, Vogel-Bonner- and Glucose solution*

Before starting, sterile Petri-dishes needed to be prepared. Therefore they were unpacked and stacked – lid on top – in batches of 10. The casting of the plates was started at the bottom of each pile, by lifting the Petri-dishes above.
After the sterilisation process had been finished, the Erlenmayer flasks and the other solutions were immediately placed under the laminar flow.
Primarily 15 ml of Vogel-Bonner solution was added to each agar-containing Erlenmayer flask and, after shaking, 37.5 mL of glucose-solution followed. Afterwards the intermixture was stirred again and shortly after that the agar was filled into the arranged plates with an approximate thickness of 0.5 cm.

***Picture 9:*** *Erlenmeyer flasks and required solutions under the laminar flow*



***Picture 10:*** *Poured agar plates*

After a drying time of about 12 hours, the minimal glucose plates were repacked and closed firmly to avoid air inlet. The storage temperature amounted to 4°C.

### 3.3.2 Vogel-Bonner solution

In 670 mL of distilled water at a temperature of 45 °C, 10 g of magnesium sulphate heptahydrate, 100 g of citric acid monohydrate, 500 g of dibasic potassium phosphate and 175 g of sodium ammonium phosphate tetrahydrate were added and dissolved. The solution was then divided in Pyrex flasks and sterilised after preparation and each time before use.

### 3.3.3 Glucose solution (40%)

The 40% solution was produced with 400 g of glucose, dissolved in 1.000 mL of boiling deionised water. Before each time of use the glucose solution was autoclaved.

### 3.3.4 Preparation of the nutrient broth (NB) and the overnight culture (OVNC)

5 g of Nutrient Broth were weighed in a 250 mL Pyrex flask, filled up with 200 mL of deionised water and put in the autoclave. Before use, the solution needed to be cooled-down. Then, according to the *Salmonella* strain used, the NB was inoculated with the specific antibiotics just before starting the OVNC:

| *Salmonella strain* | *Antibiotics* |
|---|---|
| *TA98* | 625 µL of Ampicillin |
| *TA102* | 625 µL of Ampicillin<br>156 µL of Tetracyclin |

***Table 4:*** *Strain specific antibiotics pipetted into the nutrient broth*

Thereof, 12 mL each were transferred into two sterile 100 mL Erlenmeyer flasks. To each of the flasks the particular bacterial colony (either strain *TA98* or *TA102*) was taken from a master plate, added, and whirled.
In succession, the flasks were recapped with a dabber and aluminium film and put into the incubator at 37°C, for 12 hours.
During the incubation, the OVNC was continuously shaken at 55 rpm.

***Picture 11:*** *Prepared OVNC in the incubator*

### 3.3.5 Ampicillin solution (0.8%)

0.2 g of ampicillin were weighed into a capable plastic tube and mixed with 2 5 mL of 0.2N sodiumhydroxide. Afterwards the solution was sterilised through an aseptic filter, and could be stored at 4°C.

### 3.3.6 Tetracycline solution

To 80 mg of tetracycline - weighed in into an adequate plastic tube – 10 mL of 0,2N hydrochloric acid werte added. After sterilising the solution through an aseptic filter, it could be stored at 4°C for two weeks.

### 3.3.7 Histidine solution

0.25 g of histidine was mixed with 50 mL of deionised water and filter-sterilized. The solution could be stored at 4°C.

### 3.3.8 Biotin solution

Into a capable plastic tube, 6.1 mg of D-biotin were weighed in and mixed with 50 mL of deionised water. After aseptic filter sterilisation, the solution was kept at 4°C.

### 3.3.9 Preparation of the master plates

For the preparation of two master plates (two per strain), 3.2 g of Oxoid agar was weighed into a 250 mL Pyrex flask and filled up with 200 mL of deionised water. The flask was put in the autoclave, together with the required amount of glucose- and Vogel-Bonner solution. After the sterilisation process, 4.3 mL Vogel-Bonner- and 10.8 mL glucose solution were added, followed by 2.17 mL of histidine- and 1.3 mL of biotin solution, under sterile conditions.

After each compound the flask was shaken to obtain a homogenous dispersion. The (strain specific) antibiotics are sensitive to heat, therefore the agar had to be cooled down before the addition.
For strain *TA98* 682 µL of ampicillin were used, whereas *TA102* additionally needed 54 µL of the tetracycline solution.
After this step the content of the flask was mixed again.
The agar was then ready to be poured into two sterile Petri dishes, whereupon the thickness of the agar in the dishes amounted to approximately 1 cm.
The plates were left in the powered laminar flow to dehumidify for 24 hours.
After drying, the intended bacterial strains, taken from a prepared bacterial overnight culture, were applied on the plates lattice-like. After another drying time of about 15 - 20 minutes and under sterile conditions, the master plates were put in the incubator at 37°C for 48 hours.
After incubation the plates were stored at 4°C. Once inoculated, the master plates could be kept for either up to 2 months (*TA98*) or 2 weeks (*TA102*) respectively.

### 3.3.10 Preparation of the top agar

For the top agar 1.2 g of Oxoid agar and 1 g of sodium chloride were weighed in into a 250 mL Pyrex flask and filled up with 200 mL deionised water.
Subsequently the agar was put in the autoclave to be ready shortly before the test started. Before use, 20 mL of the also autoclaved histidine-biotin solution were added under sterile conditions and then put into the water bath at 55°C.

***Picture 12:*** *Top agar and histidine-biotin solution in the autoclave*

### 3.3.11 Preparation of the histidine-biotin solution

The histidine- biotin solution consisted of 30.9 mg D-biotin and 24 mg L-histidine which were weighed in with the laboratory scale into a weighing boat. Afterwards the amino acids were carefully transferred into a 250 mL Pyrex flask and filled up with 250 mL of deionised water. The solution was sterilised each time before use, and was kept at 4°C.

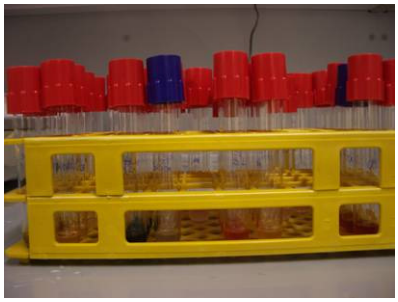
| *Denotation* | *Abbreviation/ Empirical formula* | *Source* | *Order number* | *Utilisation* |
|---|---|---|---|---|
| Agar No 1 | | Lab M/Bertoni | LP011P | Plates, top Agar |
| Ampicillin Trihydrate | | Sigma | A6140 | Master plates, OVNC |
| D-Biotin | $C_{10}H_7NO_2$ | Sigma | B4639 | Master Plates |
| Dimethylsulfoxide | DMSO | Sigma | D5879 | Frozen Permanents, solvent |
| D-Glucose | $C_6H_{12}O_6$ | Sigma | G8270 | MG-Agar-Plates, Master Plates |
| Glucose-6-Phosphat | Glu-6-P | Sigma | G7250 | S9-Mix |
| Potassiumchloride | KCl | Sigma | P5405 | S9-Mix |
| Potassiumphosphate, dibasic | $K_2HPO_4$ | Sigma | P3786 | Vogel-Bonner solution |
| L-Histidine | $C_6H_9N_3O_2$ | Sigma | H8125 | Top Agar, Master Plates |
| Magnesiumchloride | $MgCl_2 \cdot 6H_2O$ | Sigma | M9272 | S9-Mix |
| Magnesiumsulphate | $MgSO_4 \cdot H_2O$ | Sigma | M9397 | Vogel-Bonner solution |
| Nutrient Broth No 2 CM 67 | | Lab M/Bertoni | CM067B | OVNC, NB |
| Sodiumammonium-phosphate | $Na_2NH_2PO_4 \cdot 4H_2O$ | Sigma | S9506 | Vogel-Bonner solution |
| Sodiumchloride | NaCl | Sigma | S5886 | Top Agar |
| Sodiumhydroxide | NaOH | Sigma | O6203 | Ampicillin solution |

| | | | | |
|---|---|---|---|---|
| Nicotineamid-Adeninedinucleotide-phosphate | NADP | Sigma | N0505 | S9-Mix |
| Phosphatebuffer | PBS | PAA Labor | H15002 | Pre-incubation, S-9 Mix |
| Rat liver homogenate | S9 | MP Biomedicals | 50412 | Trials with metabolic activation |
| Tertiary-Butyl-hydroperoxide | *t*-BOOH | Sigma | T3383 | Oxidative mutagen |
| Tetracycline Hydrochloride | | Sigma | T3383 | Master Plates, OVNC |
| 2,4,7-Trinitro-9-Fluorenone | TNFone | - | - | Standard mutagen in the Ames test |
| Citric acid monohydrate | $C_6H_8O_7 \cdot H_2O$ | Sigma | C1909 | Vogel-Bonner solution |
| 2-Amino-1-Methyl-6-Phenylimidazo[4,5-*b*]Pyridine | PhIP | Toronto Scientific | A617000 | Mutagen |
| Aflatoxin $B_1$ | $AFB_1$ | Sigma | A6636 | Mutagen |

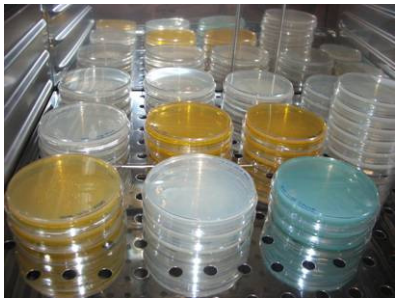
***Table 5:*** *Index of chemicals used within the test procedures*

### 3.3.12 Characterisation of the pre-incubation test

The workflow was performed in the following manner:
Under sterile conditions, chronologically 500 µL of PBS buffer, 100 µL of the OVNC as well as 100 µL of the intended samples, here the bile pigments, were pipetted into sterile test tubes. If metabolic activation was necessary, the buffer was replaced by 500 µL of the S9-Mix.
As soon as the first sample or the positive control respectively was added, a timer, set to 25 min, was started.
As soon as the entire range of compounds had been added, the test tubes in the rag were stirred and then put into the incubator until the time elapsed.
During incubation the tubes were shaken at 55 rpm at a temperature of 37°C.



***Picture 13:*** *Filled test tubes ready for preincubation*



***Picture 14:*** *Poured plates in the incubator*

In the following, the rag was taken out and placed under the laminar flow to add 2 mL of top agar to each of the tubes. After vortexing, the content of each test tube was poured and instantly dispersed on compatibly labelled minimal glucose agar plates.

The plates were then dried for 25 to 30 min and subsequently stored (lid down) in the incubator for 48h at 37°C.

All the trials implemented during the project were performed in terms of pre-incubation practices.

### 3.3.13 Positive and negative controls in use

Acquainted mutagenic substances were used as ***positive controls***, specifically for each tester strain, to reveal reversion that appears when bacteria are influenced by mutagens.
Following mutagenic substances were used in the tests:

| *Strain* | *Without metabolic activation* | *Including metabolic activation* |
|---|---|---|
| *TA98* | 2,4,7-Trinitro-9-Fluoroenone (TNFone) [0.0001 mg/mL] | 2-Amino-1-Methyl-6-Phenylimidazo[4,5-*b*]Pyridine (PhIP) [0.1 µmol/mL] |
| | | Aflatoxin $B_1$ ($AFB_1$) [5 µg/mL] |
| *TA102* | 2,4,7-Trinitro-9-Fluoroenone (TNFone) [0.07 mg/mL] | |
| | Tertiary-Butyl Hydroperoxide (*t*-BuOOH) [0.75*10^-6 mol/plate] | Tertiary-Butyl Hydroperoxide (*t*-BuOOH) [0.75*10^-6 mol/plate] |
| | | Aflatoxin $B_1$ ($AFB_1$) [120 µg/mL] |

***Table 6:*** *Diagnostic mutagens/positive control*

In order to prove the number of spontaneous revertants, ***negative controls*** were applied. Since the samples within the trial were dissolved in DMSO, this solvent was also used as the negative control.

### 3.3.14 Preparation of the S9-Mix

Since bacteria do not have a cytochrome-based P450 metabolic oxidation system, like humans and lower animals, an exogenous mammalian organ activation system needed to be added. Therefore the S9-Mix, representing an enzymatic mixture made from rat liver homogenate, was applied in the *in vitro* test.

The metabolic activation system for a maximum of 100 plates consisted of: 19.75 mL sterile deionised water, 25 mL PBS buffer (without Ca and Mg), 1 mL $MgCl_2$/KCl solution, 2 mL NADP solution as well as 250 µL glucose-6-phosphate solution. All compounds were pipetted into a 50 mL centrifuge tube, at last the rat liver homogenate, which had been stored at -80°C, was added.

It is necessary to prepare the S9-Mix freshly just before the beginning of the test, and to cool it constantly on ice during the experiment, since the solution contains highly susceptible enzymes; the mix is applicable for a maximum of 60 min.

### 3.3.15 Preparation of the glucose-6-phosphate (304 mg/mL)

1 g of glucose-6-phosphate was solved in 3.289 mL of deionised water. Then, the solution was aseptically filtered and pipetted in portions of 260 µL each, into cryo tubes. The prepared quantity represents the volume needed for the mixture of one S9-Mix.
The tubes were stored at – 20°C.

### 3.3.16 Preparation of the $MgCl_2$/KCl solution

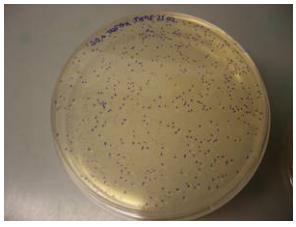
61.5 g of KCl as well as 40.7 g of $MgCl_2$ were dissolved in 50 mL of deionised water. The solution was sterilised in the autoclave.

### 3.3.17 Preparation of the NADP solution (0.135 g/2mL)

1g of NADP was dissolved in 14.814 mL of deionised water. The solution was then sterilised through an aseptic filter. Cryo tubes each containing 2 mL of the NADP solution were frozen at -20°C. The portion size was consistent with the amount needed for each S9-mix.

### 3.3.18 Colony counting

The plates were incubated for 48 hours, and with a with a colony-meter, the colonies were counted. To enhance the detection of the white spots, it is beneficial to place the plates onto a flat lamp. The results were expressed as revertant colonies per plate.
Mutagenic tendencies were existent, if the number of revertants on a sample-plate exceeded the double negative control.



***Picture 15:*** *Counted plate*

## 3.4 Statistical analysis

The data were entered into Microsoft Excel and that followed transferred to Sigmastat (Systat Software, Inc. ver.17.0). First the Kruskal-Wallis one-way analysis on ranks was applied to ensure that the data are normally distributed. Further arithmetic means and standard deviations were determined.
To figure out whether a significance can be found, the bile pigment's different concentrations were tested in comparison to the positive control (anti-mutagenic testing) and the negative control (mutagenic testing) respectively. For this purpose the one-way analysis of variance (ANOVA) was applied.
In order to identify the post-hoc differences between the revertants formed at different concentrations and the positive and the negative control data, the Bonferroni and the Tukey B tests were performed.
A *p*-value $<0.05$ was considered significant.

For a further interpretation of the test results, the data were divided in classification groups according to Mortelmans *et al*. (2000):

- *Mutagenic:*

If a compound produces a reproducible, dose-related increase in the number of revertant colonies in one ore more strains or when their numbers of His+ revertant colonies exceed doubled negative control counts, it is considered a mutagen.

- *Weak mutagen:*

If the tested substances produce a reproducible, dose-related increase in the number of His+ revertants in one ore more strains but the number of the revertants is not double the background number of colonies.

- *Inconclusive:*

If substances cannot be clearly assigned as mutagenic or a non-mutagenic, the results are classified as inconclusive.

- *Non-mutagenic:*

The compounds are classified as non-mutagenic if no concentration-dependent increase in the number of revertant colonies is observed in at least two different experiments.

# 4. Results of the trials and discussion

Bilirubin dimethyl ester and biliverdin dimethyl ester were tested in two strains of *Salmonella typhimurium* (*TA98*, *TA102*) in order to detect whether they could modulate spontaneous or induced genotoxicity. When the bile pigments were tested alone, without a positive control, they were neither mutagenic nor did they cause toxicity in the two *Salmonella* strains used ($p<0.001$, see chapter 6.4). In the following, only that data of the anti-mutagenic experiments that show a significant difference between the distinct tested concentrations of the bile pigments and the positive control will be presented in diagrams or histograms. The mentioned $ID_{50}$ displays the amount of bile pigments in µmol that is necessary to inhibit 50% of the added mutagen.

## *4.1 TA98 – anti-mutagenic testing*

### 4.1.1 2,4,7 trinitrofluoreneone induced mutagenicity

2,4,7 trinitrofluorenone (TNFone) induced genotoxicity was used to test the possible anti-mutagenic effects of bile pigments bilirubin dimethyl ester and biliverdin dimethyl ester in the absence of metabolic activation. The first 3 concentrations (2 to 0.5 µmol) exhibited significant difference to the positive control ($p$<0.05). All compounds inhibited the TNFone induced genotoxic effects in a dose dependent manner (Figure 10). The $ID_{50}$ for BRDME amounted to 1.32 µmol, the one for BVDME 1.70 µmol.

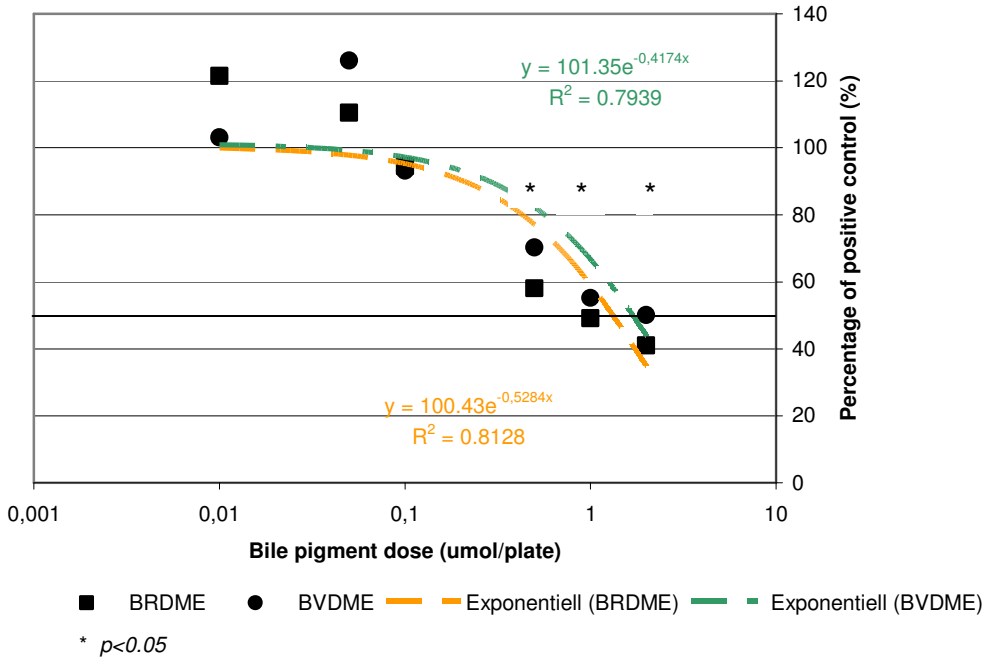



***Figure 10:*** *The modulatory effects of bilirubin dimethyl ester (BRDME) and biliverdin dimethyl ester (BVDME) on TNFone induced genotoxicity in TA98 Salmonella typhimurium (n=6).*

### 4.1.2 2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]Pyridine induced mutagenicity

For testing the capacity of BRDME and BVDME to inhibit mutagenicity induced by PhIP, metabolic activation was applied. Figure 11 illustrates that the curves of both compounds proceeded below the 50 % inhibition line. The whole concentration range from 0.01 to 2 µmol significantly reduced the number of $His^+$ revertants ($p$<0.001).

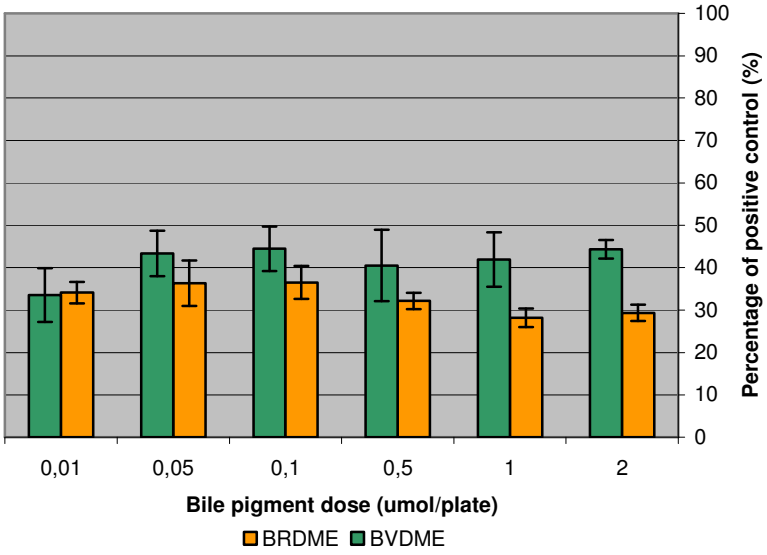



***Figure 11:*** *The modulatory effects of BRDME and BVDME on PhIP induced genotoxicity in TA98 Salmonella typhimurium (n=6). All concentrations are significant different to the positive control (p<0.001).*

### 4.1.3 Aflatoxin B1 induced mutagenicity

$AFB_1$ induced genotoxicity was used to investigate the possible anti-mutagenic effects of the bile pigments including metabolic activation. The entire concentration range as well for BRDME as for BVDME exhibited a significant difference to the positive control ($p$<0.001). The $ID_{50}$ of BVDME with 0.73 µmol was clearly lower than the one of BRDME, requiring 2.20 µmol to inhibit 50% of the tested mutagen (Figure 12).

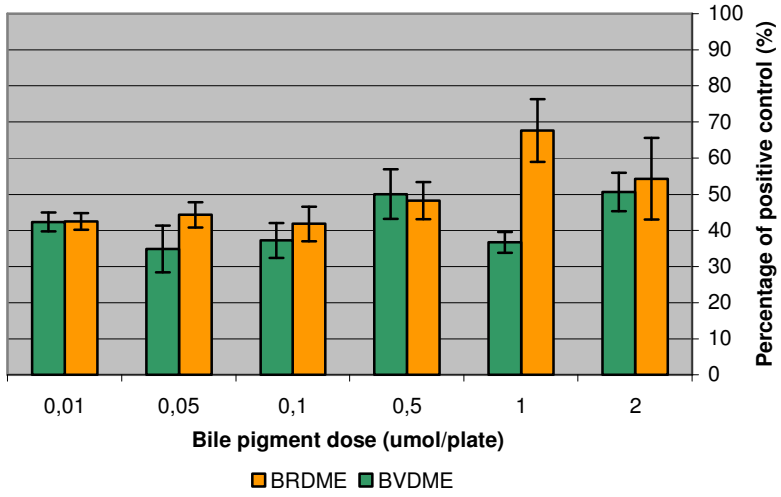



***Figure 12:*** *The modulatory effects of BRDME and BVDME on $AFB_1$ induced genotoxicity in TA98 Salmonella typhimurium (n=6). All concentrations are significant different to the positive control (p< 0.001).*

## 4.2 TA102 – anti-mutagenic testing

### 4.2.1 2,4,7 trinitrofluorenone induced mutagenicity

When TNFone caused mutation in the *TA102* strain, without the application of the S9-fraction, BRDME and BVDME inhibited this response in a dose dependent manner. Both bile pigments significantly reduced the number of $His^+$ revertants in each tested concentration range and, moreover, in Figure 13 it is apparent that the curves of the two compounds ran along or even below the 50% line. The $ID_{50}$ of BRDME amounted to 0.19 µmol, the one of BVDME 0.03 µmol.

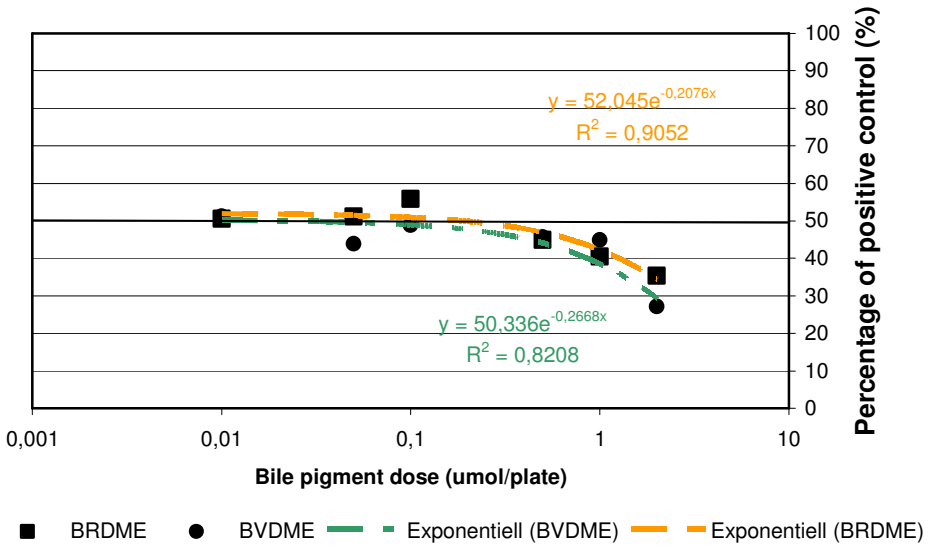



***Figure 13.:*** *The modulatory effects of bile pigments on TNFone induced genotoxicity in TA102 Salmonella typhimurium (n=6). All concentrations are significant different to the positive control (p<0.001).*

### 4.2.2 2- Aflatoxin $B_1$ induced mutagenicity

$AFB_1$ induced genotoxicity was used to test the possible anti-mutagenic effects of the bile pigments BRDME and BVDME in the presence of metabolic activation. In this condition, both compounds significantly reduced the number of $His^+$ revertants ($p<0.001$). In any tested concentration an anti-mutagenic impact of the bile pigments could be demonstrated (Figure 14).

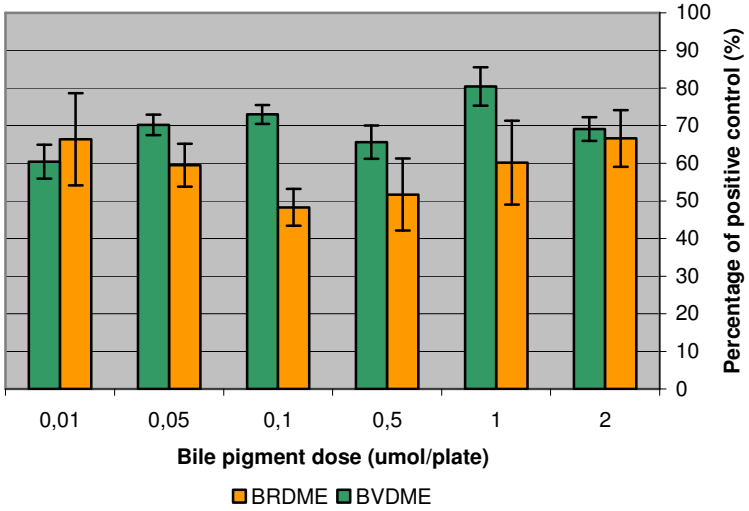



***Figure 14:*** *The modulatory effects of bile pigments on $AFB_1$ induced genotoxicity in TA102 Salmonella typhimurium (n=3). All concentrations are significant different to the positive control (p<0.001).*

## *4.2.1 TA102 – antioxidant testing*

### 4.2.1.1 *t*-BuOOH induced pro-oxidative effects without S9

With *t*-BuOOH possible antioxidant effects of bile pigments were tested. When metabolic activation was absent, BRDME and BVDME inhibited *t*-BuOOH induced mutagenic response only to a partly significant extent. BRDME reduced the number of $His^+$ revertants significantly at 1 and 0.5 µmol and BVDME at 1 and 0.1 µmol (see table 7).

| *Concentration* | *% of Positive Control BRDME* | *% of Positive Control BVDME* |
|---|---|---|
| *2* | 91.9 ± 6.6 | 89.05 ± 3.6 |
| *1* | 79.8*± 8.8 | 79.25 ± 6.4 |
| *0.5* | 71.3* ± 3.35 | 85.5 ± 7.75 |
| *0.1* | 80.15 ± 7.15 | 71.3* ± 5.3 |
| *0.05* | 90.15 ± 5.9 | 96.15 ± 7.1 |
| *0.01* | 83.85 ± 7.1 | 82.45* ± 10.95 |

***Table 7**: Averaged percentages of the positive control values of BRDME and BVDME tested with t-BuOOH without metabolic activation; (* p<0.05) ± the averaged percentages of standard deviations of BRDME and BVDME tested with t-BuOOH without metabolic activation.*

### 4.2.1.2 *t*-BuOOH induced pro-oxidative effects with S9

In the presence of metabolic activation, the effects of the bile pigments concerning their ability to inhibit *t*-BuOOH induced mutation, improved considerably. Both compounds exhibited a significant anti-mutagenic effect on revertant formation ($p<0.05$), (Figure 15).

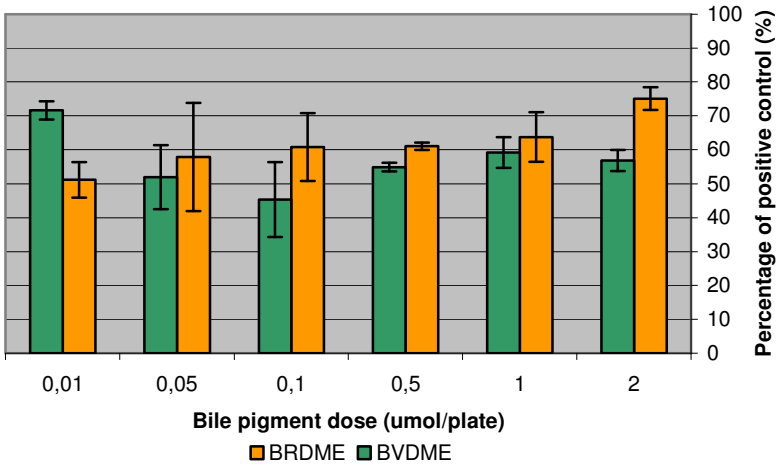



***Figure 15:*** *The modulatory effects of bile pigments on t-BuOOH induced genotoxicity in TA102 Salmonella typhimurium with metabolic activation (n=6). All concentrations are significant different to the positive control (p<0.05).*

## *4.3 Summary of mutagenic modulation*

In Table 9 a summary of the bile pigments capacity to modulate genotoxicity in the *TA98* and *TA102* bacteria is presented. The concentrations that inhibit mutagenicity to 50% of the positive control ($ID_{50}$), the percentage anti-mutagenic response at the highest common dose (0.5 µmol/plate; $I_{0.5}$) and the highest respective dose tested ($I_{max}$) are presented to facilitate the identification of the relative anti-mutagenic effects of the bile pigments.

| Strain | Mutagen (mol/plate) | S9 | Compound | $ID_{50}$ (µmol) | $ID_{75}$ (µmol) | $I_{0.5}$ (%) | $I_{max}$ (%) | $His^+$ |
|---|---|---|---|---|---|---|---|---|
| *TA98* | 2,4,7 TNFone ($3.17*10^{-11}$) | - | BRDME | 1.32 | 2.63 | 58.05 | 41 | 422 |
| | | | BVDME | 1.70 | 3.38 | 70.25 | 50.1 | |
| *TA98* | PhIP ($0.1*10^{-7}$) | + | BRDME | - | 2.93 | 32.15 | 29.35 | 442 |
| | | | BVDME | - | | 40.5 | 44.3 | |
| *TA98* | $AFB_1$ ($1.22*10^{-8}$) | + | BRDME | 2.20 | - | 48.25 | 54.3 | 295.5 |
| | | | BVDME | 0.73 | - | 50.05 | 50.56 | |
| *TA102* | 2,4,7 TNFone ($2.22*10^{-8}$) | - | BRDME | 0.19 | 2.62 | 44.9 | 35.35 | 1217.5 |
| | | | BVDME | 0.03 | 3.35 | 45.75 | 27.2 | |
| *TA102* | $AFB_1$ ($2.9*10^{-7}$) | + | BRDME | - | - | 51.7 | 66.6 | 1041 |
| | | | BVDME | - | - | 65.6 | 69.1 | |
| *TA102* | *t*-BuOOH ($0.75*10^{-6}$) | - | BRDME | - | - | - | 91.9 | 1259 |
| | | | BVDME | - | - | - | 89.05 | |
| *TA102* | *t*-BuOOH ($0.75*10^{-6}$) | + | BRDME | - | - | - | 75.05 | 1229 |
| | | | BVDME | - | - | - | 56.85 | |

***Table 8:*** *The modulatory effects of bile pigments on genotoxicity in the TA98 and TA102 Salmonella strains*

$ID_{50,}$ dose of compound required for 50% inhibition; $ID_{75}$, dose of compound required for 75% inhibition; $I0_{.5}$, percent inhibition at 0.5 µmol/plate; $I_{max}$, percent inhibition at the maximum dose tested; $His^+$, mean number of histidine positive revertant colonies in the positive control plates of two experiments; BRDME, BVDME.

## *4.4 Mutagenic testing*

To assure that the bile pigments do not provoke mutations on their own, mutagenic experiments were conducted. In these, some of the mutagens could be taken together, as the positive control was not added to the test tubes which contained the bile pigments.

For the statistical analysis the results of the $his^+$ revertants were compared to the $2^{nd}$ fold negative control. Since, the complete achieved results appeared with a significant difference to the $2^{nd}$ fold negative control values ($p$<0.001),

this implied a significant difference to the positive control as well and was therefore not tested separately.

### 4.4.1 Mutagenic testing - strain *TA98*

#### *4.4.1.1 Mutagenic testing with TNFone*

The data shown in Figure 16 illustrates, that the curves of BRDME and BVDME did not even reach the negative control values and were therefore definitely not mutagenic.

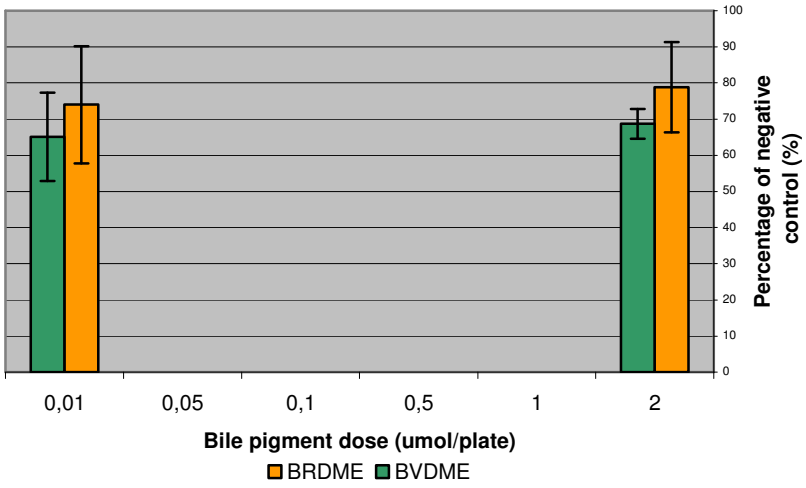



***Figure 16*:** *Mutagenic testing of BRDME and BVDME with TNFone in strain TA98 (n=3).*

### 4.4.1.2 Mutagenic testing with $AFB_1$ and PhIP

When testing the bile pigments bilirubin dimethyl ester and biliverdin dimethyl ester with $AFB_1$ and PhIP, interestingly the diagram's balks of bilirubin dimethyl ester increased with the concentration range, amounting to about 120 % of the negative control at 2 µmol/plate. BVDME, in contrast, proceeded continuously below the negative control (Figure 17).

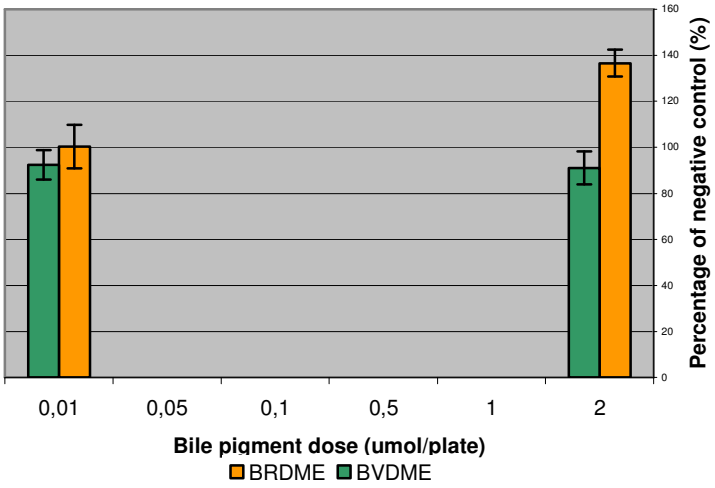



***Figure 17:*** *Mutagenic testing of BRDME and BVDME with $AFB_1$ and PhIP in strain TA98 (n=3).*

### 4.4.2 Mutagenic testing – strain *TA102*

#### *4.4.2.1 Mutagenic testing with TNFone and t-BuOOH (without metabolic activation)*

The curves of both compounds were below the negative control (Figure 18), thus suggesting that the bile pigments do not induce mutagenicity.

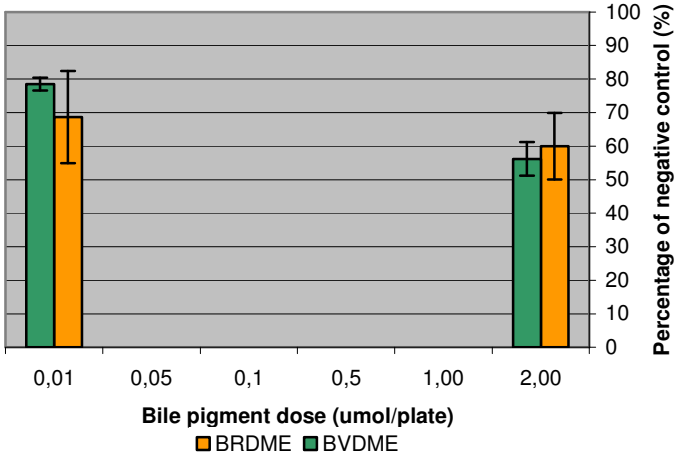



***Figure 18:*** *Mutagenic testing of BRDME and BVDME with TNFone and t-BuOOH (without metabolic activation) in strain TA98 (n=3).*

### 4.4.2.2 Mutagenic testing with $AFB_1$ and t-BuOOH

Also for $AFB_1$ and *t*-BuOOH, the $his^+$ revertants did not exceed the negative control and therefore it can be said that bilirubin dimethyl ester or biliverdin dimethyl ester alone did not provoke any mutation (Figure 19).

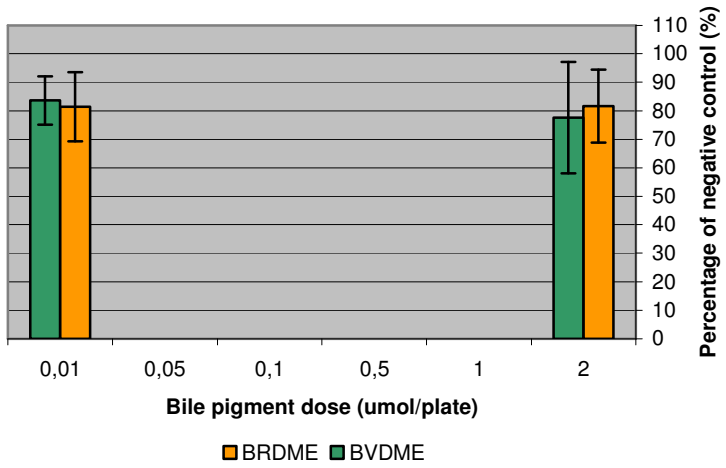



***Figure 19:*** *Mutagenic testing of BRDME and BVDME with $AFB_1$ and t-BuOOH in strain TA102, including metabolic activation (n=3).*

## 4.5 Discussion

As already mentioned in the literature survey (see chapter 4.2.2 and 4.2.3) the antioxidant capacity of the bile pigments is well established, but explicit data on their anti-mutagenic potential is lacking.
In this thesis, the Ames *Salmonella* test was chosen, in order to consider the impact of bile pigments' in the presence of genotoxic compounds which possess different three-dimensional conformations (Figures 5-8). In addition, the bile pigment's antioxidant effects were tested.

The four different tested mutagens in this thesis were *t*-BuOOH, TNFone, PhIP and $AFB_{1,}$ whereas actually and to the best of our knowledge, assumptions for the anti-mutagenic potential of the bile pigments is only existent for *t*-BuOOH and TNFone.

In order to evaluate the findings in sequence the experiments with *t*-BuOOH will be examined first.
*t*-BuOOH, an organic hydroperoxide, was selected in order to test the multi-modal mechanism of action of bile the pigments in strain *TA102*, which is susceptible to oxidative stress induced mutation (LEVIN *et al*., 1984). *t*-BuOOH, which causes DNA damage *in vitro* (EPE *et al*., 1990) via the generation of reactive oxygen species, including alkoxyl radicals (MINOTTI *et al*., 1986), was used to promote mutation. Bile pigments possess the ability to inhibit peroxyl radical chain reactions in lipids via a hydrogen donation mechanism (STOCKER *et al*., 1987a). As alkoxyl and peroxyl radicals share similar structures it is thinkable that bile pigments are also efficient alkoxyl radical scavengers, providing a mechanism whereby bile pigments could interfere with *t*-BuOOH induced DNA damage (MAHLER *et al*., 2001).
With the results of this thesis it could be shown that BRDME and BVDME attenuated *t*-BuOOH induced mutation, even though the effects appeared not too strong.

Without metabolic activation, both bile pigments inhibited the positive control to an extent of about 10 to 20% (Table 7 and 8), while in the presence of enzymatic catalysis the results improved and amounted to a 25% - 45% inhibition (Figure 15).
An explanation for this observation could be provided by Stocker in 2004, who detected that bilirubin is able to neutralise two radicals via initial hydrogen atom donation, forming a bilirubin radical, which binds to another radical forming a non-radical complex. In contrary, biliverdin can only accept one electron with the formation of a resonance stabilised biliverdin radical complex (STOCKER *et al.*, 1987b).

The question about what is accountable for the anti-mutagenic effects of the bile pigments leaded to their structural conformation.

With reference to Bulmer *et al.* (2007), it is known that unconjugated bilirubin and biliverdin inhibit the mutagenic effects of TNFone. This is founded on their acquirement to affect the ability of TNFone to disrupt DNA structure. Our results support this observation, though the difference between the anti-mutagenic potential within the two pigments was not clearly attachable. In strain *TA98*, BRDME showed slightly stronger effects than BVDME. For the former an $ID_{50}$ of 1.32 µmol was calculated, while the $ID_{50}$ of biliverdin dimethyl ester amounted to 1.70 µmol.
In strain *TA102* the results appeared converse, producing an $ID_{50}$ of 0.03 µmol for BVDME and 0.19 µmol for BRDME.

Regarding PhIP and Aflatoxin $B_{1,}$ particularly hazardous mutagens commonly occurring in food products, it can only be speculated about the reasons for the bile pigment's anti-mutagenic effects since data is lacking.
Most probably also these prosperities may be due to similarities within the structures of the bile pigments and the mutagens, or maybe the explanation is once more provided by the hydrogen donation system mentioned before.

Interestingly, in our trials bilirubin dimethyl ester's 3D ridge tile conformation (Figure 4) most effectively inhibited PhIP (strain *TA98*, Figure 5) whereas biliverdin dimethyl ester (Figure 4) most effectively inhibited TNFone (strain *TA102*, Figure 7).

Although the data presented cannot be transferred to the *in vivo* condition, the presumption of the bile pigment's anti-mutagenic and antioxidative potential in strains *TA98* and *TA102* could be strenghtened again.
It is well known that the information value of the Ames *Salmonella* assay is limited due to inaccuracies in counting the revertant colonies, especially when exceeding 1500, and various other circumstances that can hardly be influenced. *E.g.* the season, affecting the room temperature and hence resulting in a reduced cooling capacity of the fridge. But also chemicals, for instance different S9 charges, may exert considerable influence on the results.

The anti-mutagenic effects of the bile pigments bilirubin dimethyl ester and biliverdin dimethyl ester tested in the course of this thesis were definitely crowned with success as in the complete testing series, the compounds collectively achieved anti-mutagenic and antioxidant effects respectively. Moreover, this could be confirmed with the mutagenic tests in which the bile pigments had never exceeded the 2fold negative control values.

# 5. Conclusion

The aim of this study was a further investigation of the possible anti-mutagenic and antioxidant properties of bile pigments. For this purpose it was explored whether bilirubin dimethyl ester or biliverdin dimethyl ester could prevent DNA mutation in the *Salmonella* typhimurium strains *TA98* and *TA102*.
Strain *TA102* was included to probe the potential antioxidant capability of the pigments, as repeated evidence for this hypothesis is existent, *e.g.* by Stocker (2004).

Besides, the carcinogen PhIP, belonging to the group of heterocyclic aromatic amines, and $AFB_1$, a well known and often occurring consequence of spoiled food, were implicated. As far as known, there do not exist any other findings with these two substances in the Ames test, investigating the bile pigments' anti-mutagenic properties.
On the contrary, the positive impact of the compounds in inhibiting $his^+$ revertants induced by TNFone and *t*-BuOOH, already published by Bulmer *et al.* (2007), could be confirmed.

For all of the four mutagens tested in the course of this thesis, the two bile pigments exerted a considerable anti-mutagenic effect in strain *TA98*, as well as in *TA102*. Moreover, it was verified that none of the compounds was mutagenic itself, supporting their anti-mutagenic and antioxidant potential and therefore their postulated physiological relevance.

In strain *TA98* the lines of both bile pigments proceeded continuously below the 50% line at PhIP induced mutation (Figure 11). A similar outcome could be gained when $AFB_1$ was applied to induce mutagenic response: the curves of BRDME and BVDME predominantly lay below the 50% line (Figure 12). Interestingly, at higher concentrations, at 1 and 2 µmol respectively, the number of mutants was slightly or markedly above this level.
Using TNFone as mutagen, a clear dose dependent decrease of the $his^+$ revertants could be observed (Figure 10), though all in all the anti-mutagenic

effects achieved by the bile pigments were rather weak until a concentration range of approximately 0.5 µmol or higher. At this amount 30% - 40% of the positive control could be inhibited by BVDME and BRDME respectively. At the highest concentration of 2 µmol, both compounds passed the 50% line, BRDME even gained a 60% inhibition of the positive control.

In strain *TA102* the most auspicious anti-mutagenic results could be reached for TNFone, presenting a continuous decrease in revertants. From 0.5 µmol BRDME and BVDME inhibited the TNFone induced mutation to an extent of more than 50%, reaching the maximum at 73% provoked by BVDME at the highest concentration of 2 µmol (Figure 13).
For $AFB_1$ induced genotoxic response, the bile pigments' effectiveness in inhibiting mutation ranged between 35% and 45% for BRDME and between 25% and 35% for BVDME respectively.

Regarding the antioxidative capacity of the two compounds tested with *t*-BuOOH in strain *TA102*, especially the outcomes of the experiments conducted without metabolic activation showed that the impact of the bile pigments was weak. As for both pigments, the inhibitory effects were only between 10% - 20% (Table 7).
In the presence of enzymatic catalysis, a 45% inhibition of BRDME and BVDME could be revealed at the lowest concentration range (0.01 µmol). Interestingly, at least for BRDME the higher concentrations of the pigment were linked with an augmented number of revertants, achieving only about 25% of inhibition of the positive control value at the highest concentration of 2 µmol (Figure 14), whereas BVDME seemed to appear rather independent of the bile pigment dose.

It was hypothesised that the bile pigments would inhibit mutation caused by mutagens that shared similarities in their respective three dimensional structures. And, moreover, the bile pigments were considered to be able to

inhibit mutation caused by tertiary butyl hydroperoxide, due to their well known antioxidant potential.
Regarding the relevance of the structural similarity, the existent results could only partially be confirmed.
Referring to a study of Bulmer *et al*. (2007) it was evidenced that unconjugated bilirubin, exhibiting a non planar structure, is more effective in inhibiting mutation caused by 2,4,7 trinitrofluorenone, which is also a non planar compound and that biliverdin stronger inhibited 2-aminofluorene, a planar compound. Indeed an anti-mutagenic potential could be observed in the tests conducted for this thesis, but this applied as well for the non planar BRDME as for the planar BVDME, since no mentionable differences in the effectiveness between the two compounds in inhibiting TNFone induced mutation could be observed.

Also for tertiary butyl hydroperoxide, independent whether experiments were conducted with or without metabolic activation, the effectiveness of bilirubin dimethyl ester and biliverdin dimethyl ester appeared without remarkable distinctions (Table 7, Figure 15). The results were related to their antioxidant potential and suggest that the bile pigments act as ‘intercepting’ molecules with the capacity to bind mutagens and prevent them from rearranging DNA structure, additionally to preventing mutation, via oxidative modification.
Moreover, it can be assumed that bilirubin might exert anti-mutagenic effects at physiological concentrations, seen in the circulation. Further, bilirubin compounds could incept mutagens at physiological concentrations seen in the intestine.
Therefore, the possibility of supplying bile pigments via oral administration could emerge a pioneering success.

However, at present this cannot be verified due to the fact that bilirubin becomes potentially toxic in higher ranges of concentrations.
To conclude, the findings obtained in the course of this thesis provided consistent evidence for the anti-mutagenic and antioxidant potential of the bile

pigments. As *in vitro* data is rising, the next step will be to gain more insight in the compounds' *in vivo* behaviour to reinforce the existent and auspicious evidence of the bile pigment's beneficial properties.

# 6. Summary

In order to reveal the anti-mutagenic and antioxidant properties of the two synthetic bile pigments bilirubin dimethyl ester and biliverdin dimethyl ester, the Ames *Salmonella* assay, applying the preincubation test, was chosen.
The Ames *Salmonella*/microsome mutagenicity assay is a short-term bacterial reverse mutation assay which was specifically designed to detect the mutagenic potential of various chemical substances but which otherwise also exposes possible beneficial effects of the investigated compounds against genotoxic DNA alterations.

In this thesis bilirubin dimethyl ester and biliverdin dimethyl ester were tested for their potential to inhibit mutagenicity induced by 2,4,7-TNFone, 2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]Pyridine (PhIP), Aflatoxin $B_1$ and the oxidizing agent tertiary-butyl hydroperoxide in *Salmonella typhimurium* strains *TA98* and *TA102*. To investigate a possible dose dependency, the following concentration ranges were tested: 0.01; 0.05; 0.1; 0.5; 1; 2 µmol/plate.

The achieved results give reason to the evident anti-mutagenic potential of BRDME and BVDME, since in every conducted test an inhibition of induced mutagenicity could be accomplished. A dose dependency became particularly apparent when TNFone was applied as a mutagen, but especially in strain *TA98* the anti-mutagenic capacity of the two bile pigments against PhIP and $AFB_1$ were prominent.
The potential to defend *t*-BuOOH mediated oxidative mutations was existent with and without the presence of metabolic activation, but turned out to be considerably stronger with the application of the S9-fraction.
To conclude, this study confirmed the anti-mutagenic and antioxidant potential of the bile pigments. However, the mechanisms that account for their ability to inhibit induced mutagenicity is still not fully understood. More knowledge about these processes is essential to explore the therapeutic potential of these promising compounds.

# 7. Zusammenfassung

Ziel dieser Studie war es sowohl das antimutagene als auch das antioxidative Potential der zwei synthetisch hergestellten Gallenfarbstoffe Bilirubin Dimethyl Ester und Biliverdin Dimethyl Ester aufzuzeigen. Zu diesem Zweck wurde der Ames *Salmonella* Mutagenitätstest herangezogen, der eine rasche und verhältnismäßig kostengünstige Möglichkeit darstellt um das erbgutverändernde Potential von chemischen Substanzen (z.B. Medikamente und Pestizide) aufzudecken bzw. liefert dieser Hinweise auf Substanzen die möglicherweise DNA-schützende Effekte aufweisen.

Im Zuge dieser Arbeit wurden das antimutagene Potential von Bilirubin Dimethyl Ester und Biliverdin Dimethyl Ester gegenüber 2,4,7 TNFone, 2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]Pyridin (PhIP), Aflatoxin $B_1$ und dem oxididativ wirksamen Tertiär Butyl Hydroperoxid in den *Salmonella typhimurium* Stämmen *TA98* und *TA102* untersucht. Um zusätzlich auf eine mögliche dosisabhängige Wirkungsweise der Gallenfarbstoffe schließen zu können wurden folgende 6 Konzentrationsstufen (in µmol/Platte) bei den Tests inkludiert: 0,01; 0,05; 0,1; 0,5; 1 und 2.
In jedem der durchgeführten Experimente konnte die induzierte Mutation, ohne wesentliche Unterschiede zwischen den beiden getesteten Substanzen, inhibiert werden, was einen weiteren Hinweis sowohl hinsichtlich des antimutagenen als auch des antioxidativen Potentials der Gallenfarbstoffe darstellt. Eine Dosisabhängigkeit wurde besonders bei den Tests mit TNFone deutlich, wobei die stärksten antimutagenen Effekte der Gallenfarbstoffe im Bakterienstamm *TA98* bei PhIP und Aflatoxin $B_1$ erreicht werden konnten. Die Fähigkeit der Ester *t*-BuOOH induzierte Mutationen zu verhindern konnte sowohl mit als auch ohne metabolischer Aktivierung belegt werden, wobei Erfolge mit dem S9-Mix wesentlich deutlicher ausfielen.

Wesentliches Ziel für die Zukunft ist es die Mechanismen der antimutagenen Wirkungsweise der Gallenfarbstoffe soweit zu verstehen um das therapeutische Potential dieser Substanzen ausschöpfen zu können.

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# 9. APPENDIX

## *9.1 TA98*

### 9.1.1 Single revertant numbers for *TA98*

| ***Mutagen*** | ***Concentration (µmol/plate)*** | | | | | |
|---|---|---|---|---|---|---|
| ***TNFone [0.001 mg/mL) TEST 1*** | **2** | **1** | **0.5** | **0.1** | **0.05** | **0.01** |
| BRDME | 214 | 286 | 287 | 450 | 476 | 484 |
| | 158 | 220 | 275 | 473 | 465 | 527 |
| | 220 | 265 | 334 | 455 | 455 | 476 |
| *Mean* | 197 | 257 | 299 | 459 | 465 | 496 |
| *Standard deviation (sd)* | 34.2 | 33.7 | 31.2 | 12.1 | 10.5 | 27.4 |
| BVDME | 264 | 246 | 375 | 418 | 546 | 473 |
| | 297 | 281 | 382 | 451 | 521 | 428 |
| | 242 | 274 | 322 | 407 | 558 | 532 |
| *Mean* | 268 | 267 | 360 | 425 | 542 | 478 |
| *Standard deviation (sd)* | 27.7 | 18.5 | 32.8 | 22.9 | 18.9 | 52.2 |
| ***TNFone [0.001 mg/mL] TEST 2*** | | | | | | |
| BRDME | 130 | 155 | 169 | 368 | 451 | 543 |
| | 210 | 168 | 206 | 315 | 450 | 505 |
| | 105 | 164 | 213 | 335 | 451 | 476 |
| *Mean* | 148 | 162 | 196 | 339 | 451 | 508 |
| *Standard deviation (sd)* | 54.8 | 6.7 | 23.6 | 26.8 | 0.6 | 33.6 |
| BVDME | 171 | 195 | 231 | 426 | 530 | 488 |
| | 163 | 197 | 223 | 346 | 541 | 381 |
| | 151 | 207 | 261 | 293 | 447 | 293 |
| *Mean* | 162 | 200 | 238 | 355 | 506 | 387 |
| *Standard deviation (sd)* | 10.1 | 6.4 | 20.0 | 67.0 | 51.4 | 97.7 |
| ***PhIP [0.1 µmol/mL]+S9 TEST 1*** | | | | | | |
| BRDME | 124 | 120 | 152 | 120 | 231 | 205 |
| | 121 | 133 | 171 | 146 | 142 | 170 |
| | 118 | 145 | 159 | 157 | 221 | 197 |
| *Mean* | 121 | 133 | 161 | 141 | 198 | 191 |
| *Standard deviation (sd)* | 3.0 | 12.5 | 6.9 | 19.0 | 48.8 | 18.3 |
| BVDME | 218 | 241 | 187 | 222 | 263 | 159 |
| | 237 | 188 | 254 | 201 | 250 | 199 |
| | 232 | 207 | 214 | 223 | 216 | 118 |
| *Mean* | 229 | 212 | 218 | 215 | 243 | 159 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Standard deviation (sd)* | 9.8 | 26.9 | 33.7 | 12.4 | 24.3 | 40.5 |
| ***PhIP [0.1 µmol/mL]+ S9 TEST 2*** | | | | | | |
| BRDME | 148 | 124 | 116 | 174 | 128 | 119 |
| | 131 | 110 | 129 | - | 130 | 112 |
| | 123 | 112 | 127 | - | 124 | - |
| *Mean* | 134 | 115 | 124 | 174 | 127 | 116 |
| *Standard deviation* | 12,8 | 7,6 | 7 | 0 | 3,1 | 4,9 |
| BVDME | 154 | 183 | 116 | 196 | 130 | 150 |
| | 172 | 128 | - | 195 | 136 | 143 |
| | 169 | 169 | 171 | 140 | 172 | 117 |
| *Mean* | 165 | 160 | 144 | 177 | 146 | 137 |
| *Standard deviation* | 9.6 | 28.6 | 38.9 | 32.0 | 22.7 | 17.4 |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***$AFB_1$ [5 µmol/plate]+ S9 TEST 1*** | | | | | | |
| BRDME | 202 | 243 | 129 | 142 | 137 | 156 |
| | 135 | 191 | 153 | 122 | 128 | 142 |
| | 142 | 175 | 139 | 137 | 146 | 136 |
| *Mean* | 160 | 203 | 140 | 134 | 137 | 145 |
| *Standard deviation (sd)* | 36.8 | 35.6 | 12.1 | 10.4 | 9.0 | 10.3 |
| BVDME | 162 | 101 | 159 | 104 | 121 | 128 |
| | 121 | 86 | 112 | 127 | 69 | 118 |
| | 157 | 64 | 107 | 90 | 77 | 127 |
| *Mean* | 147 | 94 | 126 | 107 | 89 | 124 |
| *Standard deviation (sd)* | 22.4 | 10.6 | 28.7 | 18.7 | 28.0 | 5.5 |
| ***$AFB_1$ [5 µmol/plate]+ S9 TEST 2*** | | | | | | |
| BRDME | 148 | 179 | 163 | 109 | 138 | 108 |
| | 195 | 207 | 145 | 132 | 116 | 104 |
| | 140 | 202 | 126 | 96 | 118 | 102 |
| *Mean* | 161 | 196 | 145 | 112 | 124 | 105 |
| *Standard deviation (sd)* | 29,7 | 14,9 | 18,5 | 18,2 | 12,2 | 3,1 |
| BVDME | 161 | 115 | 182 | 103 | 119 | 133 |
| | 152 | 125 | 171 | 122 | 107 | 129 |
| | 144 | 113 | 159 | 113 | 126 | 114 |
| *Mean* | 152 | 118 | 171 | 113 | 117 | 125 |
| *Standard deviation (sd)* | 8.5 | 6.4 | 11.5 | 9.5 | 9,6 | 10 |

### 9.1.2 Positive control values *TA98*

| *Mutagen* | *Positive 1* | *Positive 2* | *Positive 3* | *Mean* | *sd* |
|---|---|---|---|---|---|
| TNFone Test 1 | 470 | 452 | 520 | 481 | 35,2 |
| TNFone Test 2 | 353 | 381 | 355 | 363 | 15,2 |
| PhIP Test 1 | 527 | 463 | 481 | 490 | 33,0 |
| PhIP Test 2 | 323 | 425 | 433 | 394 | 61,3 |
| $AFB_1$ Test 1 | 162 | 291 | 284 | 288 | 4,9 |
| $AFB_1$ Test 2 | 260 | 328 | 321 | 303 | 37,4 |

### 9.1.3 Negative control values *TA98*

| *Mutagen* | *Neg 1* | *Neg 2* | *Neg 3* | *Neg 4* | *Neg 5* | *Neg 6* | *Mean* | *sd* |
|---|---|---|---|---|---|---|---|---|
| TNFone Test 1 | 37 | 37 | 45 | 50 | 43 | 34 | 41 | 6,0 |
| TNFone Test 2 | 88 | 81 | 61 | 84 | 105 | 86 | 84 | 14.1 |
| PhIP Test 1 | 62 | 52 | 51 | 57 | 59 | 64 | 58 | 5.2 |
| PhIP Test 2 | 73 | 80 | 70 | 70 | 70 | 67 | 72 | 4.5 |
| $AFB_1$ Test 1 | 47 | 63 | 61 | 44 | 47 | 41 | 51 | 9.2 |
| $AFB_1$ Test 2 | 49 | 51 | 52 | 55 | 48 | 42 | 50 | 4.4 |

### 9.1.4 No treatment values *TA98*

| *Mutagen* | *No. of revertants* | *No. of revertants* | *No. of revertants* | *Mean* | *sd* |
|---|---|---|---|---|---|
| TNFone | 417 | 425 | 434 | 425 | 8.5 |
| PhIP + S9 | 349 | 385 | 422 | 385 | 36.5 |
| $AFB_1$ + S9 | 123 | 151 | 162 | 145 | 20.1 |

## *9.2 TA102*

### 9.2.1 Single revertant numbers for *TA102*

| ***Mutagen*** | ***Concentration (µmol/plate)*** | | | | | |
|---|---|---|---|---|---|---|
| ***TNFone [0.07 mg/mL) TEST 1*** | **2** | **1** | **0.5** | **0.1** | **0.05** | **0.01** |
| BRDME | 488 | 505 | 620 | 571 | 704 | 700 |
| | 505 | 542 | 624 | 759 | 637 | 673 |
| | 514 | 567 | 633 | 742 | - | 551 |
| *Mean* | 502 | 538 | 626 | 691 | 671 | 641 |
| *Standard deviation (sd)* | 13.2 | 31.2 | 6.7 | 104.0 | 47.7 | 79.4 |
| BVDME | 221 | - | 711 | 672 | 547 | 583 |
| | 410 | 644 | 637 | 567 | 567 | 581 |
| | - | 643 | 597 | - | 552 | 587 |
| *Mean* | 316 | 644 | 648 | 620 | 555 | 584 |
| *Standard deviation (sd)* | 133.6 | 0.7 | 57.8 | 74.2 | 10.4 | 3.1 |
| ***TNFone [0.07 mg/mL] TEST 2*** | | | | | | |
| BRDME | 440 | 540 | 469 | 632 | 543 | 612 |
| | 328 | 375 | 484 | 653 | 595 | 545 |
| | 321 | 422 | 460 | 682 | 573 | 602 |
| *Mean* | 363 | 446 | 471 | 656 | 570 | 586 |
| *Standard deviation (sd)* | 66.8 | 85.0 | 12.1 | 25.1 | 26.1 | 36.1 |
| BVDME | 333 | 481 | 473 | 521 | 444 | 600 |
| | 341 | 430 | 492 | 602 | 591 | 712 |
| | 330 | - | 449 | 555 | 476 | 612 |
| *Mean* | 335 | 456 | 471 | 559 | 504 | 641 |
| *Standard deviation (sd)* | 5.7 | 36.1 | 21.5 | 40.7 | 77.3 | 61.5 |
| ***$AFB_1$ [120 µg/mL]+ S9 TEST 1*** | | | | | | |
| BRDME | 613 | 656 | 474 | 501 | 581 | 546 |
| | 700 | 725 | 488 | 555 | 688 | 783 |
| | 769 | 500 | 653 | 454 | 591 | 744 |
| *Mean* | 694 | 627 | 538 | 503 | 620 | 691 |
| *Standard deviation (sd)* | 78.2 | 115.3 | 99.6 | 50.5 | 59.1 | 127.1 |
| BVDME | 683 | 779 | 635 | 739 | 703 | 672 |
| | 735 | 851 | 689 | 789 | 760 | 579 |
| | 742 | 882 | 726 | 752 | 729 | 637 |
| *Mean* | 720 | 837 | 683 | 760 | 731 | 629 |
| *Standard deviation (sd)* | 32.2 | 52.8 | 45.8 | 25.9 | 28.5 | 47.0 |

| ***t-BuOOH [0,75*10^-6 mol/plate] + S9 TEST 1*** | | | | | | |
|---|---|---|---|---|---|---|
| BRDME | 988 | 862 | - | 572 | 538 | 658 |
| | 981 | 714 | 742 | 581 | 996 | 635 |
| | 861 | 537 | 724 | 788 | 768 | 969 |
| *Mean* | 943 | 788 | 733 | 647 | 767 | 663 |
| *Standard deviation (sd)* | 71.4 | 104.7 | 12.7 | 122.2 | 229.0 | 30.8 |
| BVDME | 635 | 620 | 525 | 572 | - | 922 |
| | 621 | 609 | 522 | 420 | 768 | - |
| | 731 | 603 | 525 | - | 624 | - |
| *Mean* | 662 | 611 | 524 | 496 | 696 | 922 |
| *Standard deviation (sd)* | 59.9 | 8.6 | 1.7 | 107.5 | 101.8 | 0 |
| ***t-BuOOH [0,75*10^-6 mol/plate] + S9 TEST 2*** | | | | | | |
| BRDME | 880 | 835 | 743 | 935 | 782 | 610 |
| | 874 | 745 | 772 | 912 | 617 | 652 |
| | - | 700 | 751 | 714 | 481 | 455 |
| *Mean* | 877 | 760 | 755 | 854 | 627 | 572 |
| *Standard deviation (sd)* | 4.2 | 68.7 | 15.0 | 121.5 | 150.7 | 103.8 |
| BVDME | 736 | 863 | 818 | 666 | 562 | 783 |
| | 721 | 961 | 880 | 760 | 419 | 835 |
| | - | 739 | 834 | 435 | 682 | 688 |
| *Mean* | 729 | 854 | 844 | 620 | 554 | 809 |
| *Standard deviation (sd)* | 10.6 | 111.3 | 32.2 | 167.2 | 131.7 | 36.8 |
| ***t-BuOOH [0.75*10^-6 mol/plate] - S9 TEST 1*** | | | | | | |
| BRDME | 1091 | 1081 | 982 | 928 | 992 | 1045 |
| | 1060 | 993 | 972 | 1044 | 983 | 1073 |
| | - | 883 | 1033 | 822 | 966 | 912 |
| *Mean* | 1076 | 986 | 996 | 931 | 980 | 1010 |
| *Standard deviation (sd)* | 21.9 | 99.2 | 32.7 | 111.0 | 13.2 | 86.0 |
| BVDME | 1293 | 1025 | 1125 | 998 | 1002 | 1022 |
| | 1193 | 1121 | 1248 | 936 | 1103 | 1053 |
| | 1152 | 953 | 1002 | 893 | - | 898 |
| *Mean* | 1213 | 1033 | 1125 | 942 | 1053 | 991 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Standard deviation (sd)* | 72.5 | 84.3 | 123.0 | 52.8 | 71.4 | 82.0 |
| ***t-BuOOH [0,75*10^-6 mol/plate] - S9 TEST 2*** | | | | | | |
| BRDME | 1320 | 976 | 740 | 1017 | 1468 | 1133 |
| | 1326 | 921 | - | 1141 | 1219 | 1167 |
| | 1073 | 1157 | 815 | 1110 | 1218 | - |
| *Mean* | 1240 | 1018 | 778 | 1089 | 1302 | 1098 |
| *Standard deviation (sd)* | 144.4 | 123.5 | 53.0 | 64.5 | 144.0 | 92.2 |
| BVDME | 1005 | 993 | 1055 | 883 | 1457 | 959 |
| | 992 | 988 | 1044 | 889 | 1303 | 1313 |
| | 1018 | 861 | 934 | 743 | - | 973 |
| *Mean* | 1005 | 947 | 1011 | 838 | 1380 | 1082 |
| *Standard deviation (sd)* | 13.0 | 74.8 | 66.9 | 82.6 | 108.9 | 200.5 |

### 9.1.2 Positive control values *TA102*

| ***Mutagen*** | ***Positive 1*** | ***Positive 2*** | ***Positive 3*** | ***Mean*** | ***sd*** |
|---|---|---|---|---|---|
| TNFone Test 1 | 1306 | 1402 | 1375 | 1361 | 49.5 |
| TNFone Test 2 | 1051 | 1091 | 1081 | 1074 | 20.8 |
| $AFB_1$ + S9 | 1115 | 1006 | 1003 | 1041 | 63.8 |
| *t*-BuOOH + S9 TEST 1 | 1095 | 1112 | 1115 | 1107 | 10.8 |
| *t*-BuOOH + S9 TEST 2 | 1251 | - | 1450 | 1351 | 140.7 |
| *t*-BuOOH – S9 TEST 1 | 1238 | 1050 | 1235 | 1174 | 107.7 |
| *t*-BuOOH – S9 TEST 2 | 1487 | 1311 | 1235 | 1344 | 129.3 |

### 9.1. 3 Negative control values *TA102*

| ***Mutagen*** | ***Neg 1*** | ***Neg 2*** | ***Neg 3*** | ***Neg 4*** | ***Neg 5*** | ***Neg 6*** | ***Mean*** | ***sd*** |
|---|---|---|---|---|---|---|---|---|
| TNFone Test 1 | 371 | 318 | 235 | 369 | 320 | 399 | 335 | 58.4 |
| TNFone | 276 | 234 | 268 | 268 | 255 | 291 | 265 | 19.4 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Test 2 | | | | | | | | |
| $AFB_1$ Test | 421 | 495 | 415 | 433 | 461 | 445 | 445 | 29.6 |
| *t*-BuOOH + S9 TEST 1 | 444 | 308 | 431 | 412 | 411 | 391 | 400 | 48.4 |
| *t*-BuOOH +S9 TEST 2 | 418 | 509 | 517 | 511 | 450 | 515 | 487 | 42.1 |
| *t*-BuOOH -S9 TEST 1 | 388 | 333 | 348 | 346 | 331 | 355 | 350 | 20.7 |
| *t*-BuOOH - S9 TEST 2 | 434 | 439 | 383 | 486 | 505 | 396 | 441 | 48.1 |

### 9.2.4 No treatment values *TA102*

| ***Mutagen*** | ***No. of revertants*** | ***No. of revertants*** | ***No. of revertants*** | ***Mean*** | ***sd*** |
|---|---|---|---|---|---|
| TNFone | 1106 | 1121 | 1109 | 1112 | 7.9 |
| *t*-BuOOH + S9 | 1902 | 1914 | 2003 | 1940 | 55.2 |
| *t*-BuOOH – S9 | 1641 | 1653 | - | 1647 | 8.5 |
| $AFB_1$ + S9 | 725 | 701 | 814 | 747 | 59.5 |

# 10. Curriculum Vitae

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