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LIST OF ABBREVIATIONS

2-AF	2-amiofluorene
AFB_1	Aflatoxin B_1
B[a]P	Benzo[alpha]pyrene
t-BOOH	Tertiary butyl hydroperoxide
BR	Bilirubin
BRDT	Bilirubin- ditaurate
BVR	Biliverdin-reductase
BV	Biliverdin
CBR.	Conjugated bilirubin
CSC	Cigarette smoke condensate
CS	Cigarette smoke
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
FMN	Flavin mononucleotide
GSH	Glutathione
HMBP	6-hydroxymethylbeno[a]pyrene
HO (1,2,3)	Heme oxygenase (1,2,3)
1-NP	1-nitropyrene
2-NF	2-nitrofluorene
3-NFA	3-nitrofluoranthene
4NQO	4-nitropunoline-1-oxide
pH	Pondus Hydrogenii
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PROT	Protoporphyrin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S9	Metabolic activation system
SMBP	Sulfoxymethylbenzo[a]pyrene
SOD	Superoxidedismutase
TNFone	2-4-7 trinitrofluenone
UBR	Unconjugated bilirubin

1. INTRODUCTION

In case of jaundice or bruises the human skin changes in different colors and bile pigments become clearly visible. Bile pigments are natural endogenous colorful compounds, produced in the catabolism of heme and posses chemical structure belonging to the prophyrin family [BULMER, 2008].

Principal substances are bilirubin (yellow) and biliverdin (green). Further substances belonging to the group of bile pigments are bilirubin ditaurate (yellow/orange), a synthetic, water soluble conjugate and so called urobilinogens whereon science is focused to investigate possible positive modulatory effects.

In the past, it was thought, that bile pigments were use-less by products of the heme catabolism without any protective potential or function. From a medical point of view, the only role attributed to bile pigments has been considered as potentially toxic compounds, mainly associated with various causes of liver and neurological dysfunction, accumulation in the brain (causing kernicterus) or hemolytic diseases.

However, during the past 10 years, the interest in this field has been mounting. *In vivo* and *in vitro* studies, especially focussing on bilirubin, have demonstrated that these pigments act as anti-oxidants and possess preventative potential to protect the DNA against oxidative damage [www.univie.ac.at/antiox].

While the exact mode of action is hardly known yet, data show that bilirubin might reduce the risk of cardiovascular disease and colon cancer. As well, protective effects concerning lipids and proteins have been encountered.

Therefore the theory of a beneficial and physiological relevance of bile pigments is given.

This thesis is a sub-study of a FWF project: *"The physiological relevance of bile pigments: In vitro to in vivo evidence of antioxidant, antimutagenic, anticarcinogenic potential and their mechanisms of action".*

The diploma thesis at hand is especially focused on anti-mutagenic effects of specific bile pigments (bilirubin, biliverdin, bilirubin-ditaurate and protoporphyrin) and on the theory that the pigments protect against induced mutations, examined in the widely known and accepted *Salmonella reverse* mutation assay as a test model.

The aim of this research work was to enhance the understanding of the mechanisms and functionality of bile pigments in the human body and to communicate physiological effects.

Within this sub-study of the FWF project, so far two diploma theses were done in addition to this thesis, written by Andrea Steyrer (stercobilin, urobilin) and Gesa Ziesel (bilirubin dimethylester, biliverdin dimethylester).

2. LITERATURE SURVEY

In general, bile pigments come from the latin words “bilis” (bile) and “ruber” (red) and include following eight different key pigments: bilirubin (BR), biliverdin (BV), bilirubin ditaurate (BR-DT), portoporphyrin (PRO), stercobilin (S), urobilin (U), bilirubin dimethylester (BR-DME) and biliverdin dimethylester (BV-DME)

2.1. Historical review

Since thousands of years, bile pigments, especially BR plays an important role in the traditional Chinese and Indian medicine. Oral supplementation in different forms was considered as remedy with beneficial effects to prevent variety of disorders [BULMER, 2008].

Opposite to this, in the western medical practice, the physiological relevance of bile pigments were discussed and investigated only since the mid-20th century. It was in the 1950´s when fist studies occupied with the role of bile pigments, especially focused on the toxic effects of BR in neonates and how to reduce increased circulating BR levels to nontoxic levels.

In 2004 Stocker published a review where he summarized that bile pigments are able to stabilize vitamin A and beta-carotine during the intestinal uptake [BERNHARD et al., 1954]; further on, he reported on protective reactions of BR in conjunction with lipid peroxides [BRODERSEN et al., 1969]

During the past two decades, bile pigments have been intensely debated in terms of not only being useless toxic by-products of the heme catabolism. Although research work is lacking in this area, it is believed, that bile pigments possess physiological relevance in the human body.

Nowadays, it is actually presumed that people affected by Gilbert´s syndrome possessing a hereditary deficiency in hepatic glucuronosyl transferase activity –

which until today was assumed as negative - might have positive effects. It is believed that raised “safety factors” against cardiovascular diseases exist in the blood in these people, because of persistent enhanced levels of BR. This theory is widely believed, but published data is scarce.

Latest published studies confirmed ditto beneficial effects in various fields of cancer, cardio- vascular disease and congenital bilirubin- metabolism disorders, including several modulatory properties such as anti-complement, anti-inflammatory and anti-viral effects *in vitro* [BULMER et al., 2008].

2.2. Principles of physiology

Among the mammals, BR is the principal end product of the heme catabolism. The major source of BR are senescent erythrocytes, which are located mainly in the spleen and bone marrow. BR accrues in consequence of erythrocytes degradation and is referred to as *unconjugated* BR (UBR) [LÖFFLER; 2005].
BV, the biogenetic precursor of UBR is also formed during the catabolism of heme [SANJEEV and LIGHTNER, 2010]. UBR is released in the physiologic blood circulation- tightly bound to albumin – in order to be transported to the liver, from where it is removed mainly through uptake by hepatocytes. Less than 0.1% of the total BR is circulating in an unbound form (free BR); this free BR activates the diffusion of UBR into tissues - that´s why free BR is responsible for both - its beneficial and its toxic effects on cells [VITEK and OSTROW, 2009].

In the liver, conversion to *conjugated* BR happens by connecting with glucuronic acid and BR becomes more water soluble. Large quantities of CBR are produced daily in the human body which correspond roughly to the daily amounts of degraded hemoglobin- about 220mg /per day [LÖFFLER, 2005].

Within common blood tests, *total* BR (TBIL) is assessd which expresses parameter of all kinds of BR which appear in the blood.

2.2.1. Metabolism of bile pigments

The metabolism of bile pigments proceeds along a series of stations.
Degradation of heme, mainly located in the spleen, plays a key role in bile pigment metabolism. It is a very complex chemical reaction including many catalyzing enzymes like hemoglobin, myoglobin, cytochromes and other hemoprotein enzymes [BLANCKAERT, 1981].
Beside the pigments BR and BV, also PROT- hemin´s non-chelated precursor- is present in the metabolism and catabolism of heme. Research work is focused on PROT, because differences in anti-mutagenicity between PROT and heme are expected (based on their chemical structure) [BULMER et al., 2008]

In nature, important cyclic tetrapyrroles related to heme (beside hemoglobin and heme containing enzymes) are chlorophylls, and vitamin $B_{12.}$ Chlorophylls contain magnesium as the central atom and is derived from protoporphyrin-IX; Vitamin B_{12} is derived from uroporphyrinogen-III.
In birds and reptiles, the catabolism stops at the step of BV; no conversion to BR happens because BVR is lacking [BLANCKAERT, 1981]. Why the metabolism and catabolism of heme do not stop at the level of BV in humans? Due to the fact that the step of conversion to BR needs energy it is still on open question until today and is essential to be investigated in future [SEDLAK and SNYDER, 2004].

BV, the first oxidative product of heme decomposition is accomplished via the action of cytochrome P450 reductase and heme oxygenase. BV is immediately reduced by biliverdin-reductase to unconjugated bilirubin (UCB). UBR arrives- albumin bound into the liver, where UBR is transformed via the enzyme uridine glucuronosyl transferase to BR mono or diglucuronides. This chemical conversion is necessary to metabolize BR in a water-soluble form and to facilitate the secretion into the bile duct and the intestine [LÖFFLER, 2005, BULMER et al., 2008].

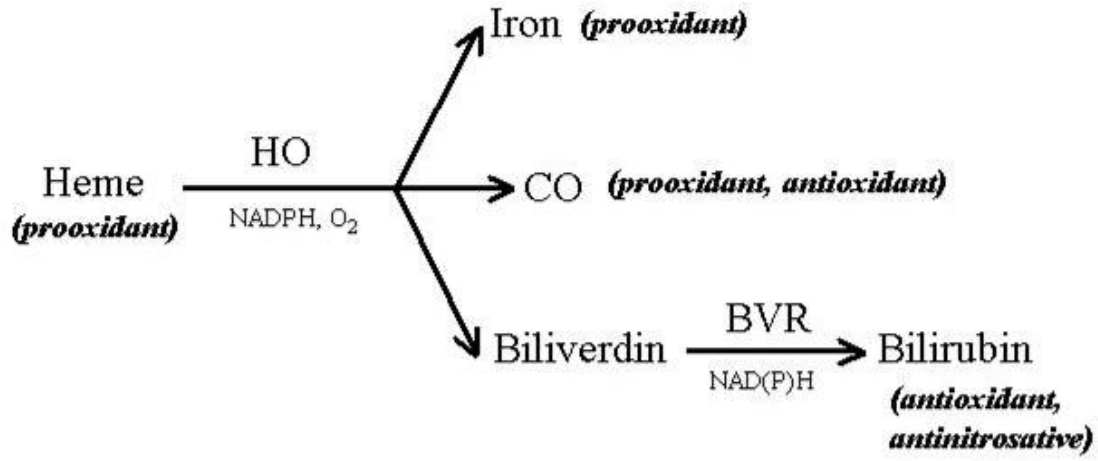

Figure 1 Reactions of heme degradation

Intestinal, bacteria decompose the glucuronic acid and transform BR to stercobilins and urobilins. Without this step, BR would accumulate to toxic levels.

As well, small proportions of the intermediate products are reabsorbed into the liver via enterohepatic circulation.

Residues reach the kidneys and get eliminated in the urine. The major part of stercobilin gets excreted with the faeces which is therefore responsible for the pigmentation of mammalian faeces [LÖFFLER, 2005].

In adults, the daily production of BR is about 250-350 mg; its excretion in the faeces is estimated to about 220 mg per day [BLANCKAERT, 1981].

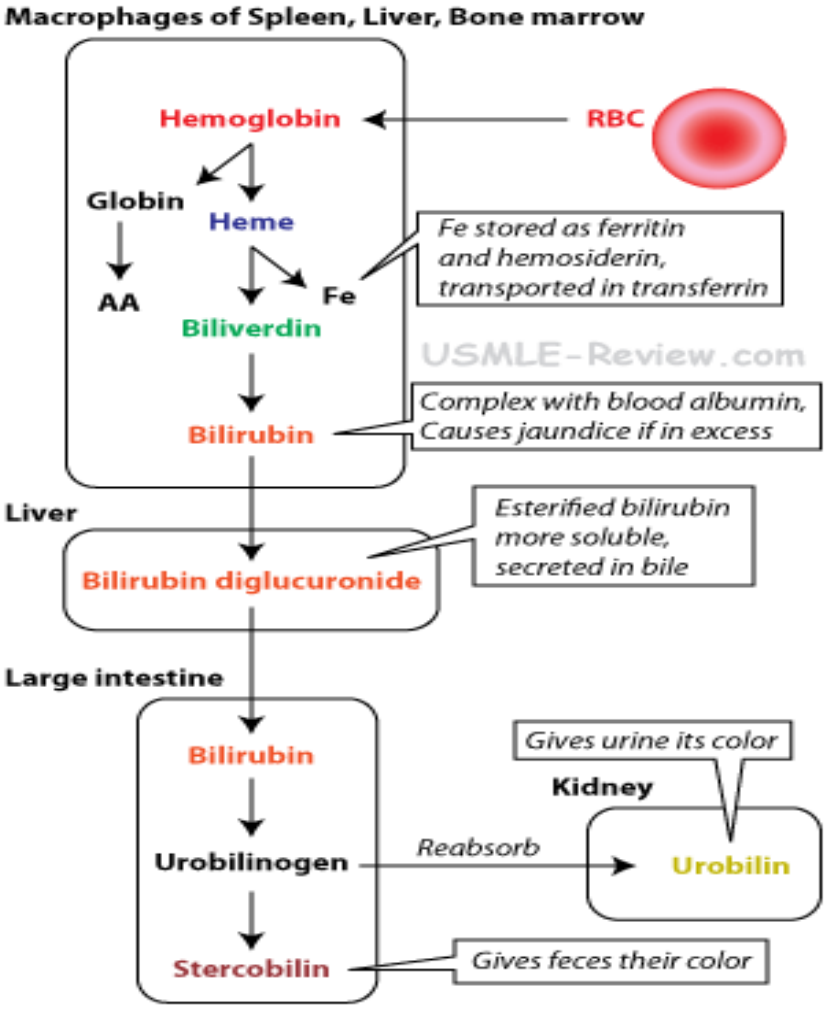

Figure 2 Steps of hemoglobin metabolism [http://usmle-review.com/]

To clarifying why BV is converted in the poorer soluble BR, science is focused. BR and its hydrophobic properties contain a range of disadvantages like neurotoxicity or propensity of gall stone but principally, a further metabolizing procedure is needed. Obviously, these disadvantages were compensated by the lipophilic antioxidative properties of BR [LÖFFLER, 2005].

2.2.2. Pathopysiolgogy

Non pathologic plasma concentrations of BR amount to 17 µmol/l. Hyperbilirubinemia is spoken of when plasma concentrations exceeded this

value. Concentrations at or above 30 µmol/l BR becomes visible in the skin [SILBERNAGEL, 2005].

Classifications of hyperbilirubinemia are very complex and may depend on various causes and conditions:

- Prehepatic icterus (enhanced BR production, for example appears in case of haemolysis, ineffective erythropoiese or blood loss)
- Intrahepatic icterus may become manifest in 3 different forms:
  - defected absorption of BR into hepatocytes (Gilbert –syndrome)
  - defected conjugation process in the liver (Crigler-Nijjar-syndrome, Gilbert-syndrome, Newborn icterus)
  - posthepatic icterus effects extrahepatic bile ducts, which can be caused by tumors, gall stones or pancreatitis [BESTE, 2003]

Different syndromes like the Gilbert -syndrome, Crigler-Najjar-syndrome, Dubin-Johnson syndrome, Rotor syndrome are part of the so called inherent hyperbilirubinemia, while the haemolytic, hepatocellular and newborn icterus belong to the acquired hyperbilirubinemia.
Further classification is possible according to the differentiation of BR concentrations of conjugated and unconjugated BR. Excessive levels of indirect BR is called unconjugated hyperbilirubinemia (for instance Gilbert -syndrome, haemolysis or in case of sepsis); dysfunction of secretion is called conjugated hyperbilirubinemia as it is the case with gall stones, occlusion or tumors for examples [BESTE, 2003].

General symptoms of all these diseases are the accumulation of BR which is reflected in jaundice or icterus. Therefore BR is generally regarded as potentially cytotoxic - that´s why BR needs to be excreted [BESTE 2003].

2.3. Chemistry of bile pigments

2.3.1. Structural aspects

Bile pigments share a few characteristics including porphyrin structure or linear tetrapyrrolic construction and possess two free or conjugated propionate groups. These groups are responsible for binding molecules which improve solubility excretion and transport around the body [BULMER, 2008].

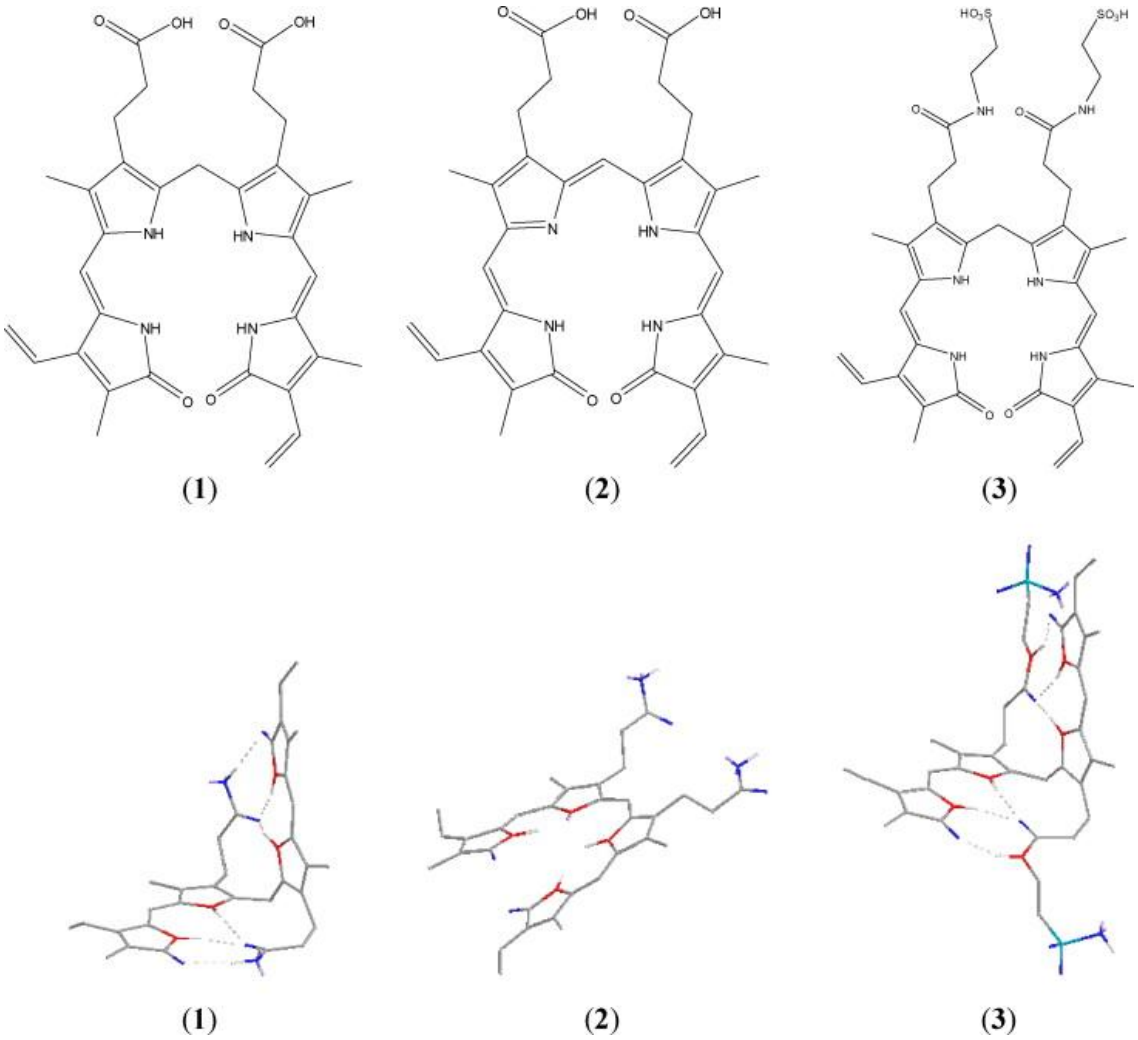

Figure 3 Two and three-dimensional structures of bilirubin (1), biliverdin (2) and bilirubin-ditaurate (3) [BULMER et al., 2008]

As depicted, BR and BV possess different molecular geometry. The most stable form of BV is the helical lockwasher shape; BR has a folded ridge tile conformation [GONCHAROVA and URBANVOVÁ, 2008].

Detailed information on the structural aspects of these compounds became available only with the advent of modern spectroscopic methods. Rich palettes of spectroscopic tools have been developed and have been used to solve constitution problems [FALK, 1989].

UBR as the principal degradation product of heme is the particular isomer in the human body. Generally, the chemical structure of bile pigments is very complex: naturally occurring pigments are formely known as BR IXalpha (Z,Z) and BV IX alpha (Z,Z). This isomer specification represents the different locations and forms of the side chains of the whole molecule [BLANCKAERT, 1981].

Nowadays these additional signs are omitted and chemical structures are simply called BR and BV. In older literature, many of such additional designations may still be used.

2.3.2. Solubility of bile pigments

For biological assays, it is truly essential to bring bile pigments into solution. Depending on pigments to be solved, complete solution is often a hard challenge and needs very special handling.

BR in all variations is a linear, lipophilic compound with water-insoluble properties at physiological pH (less than 1nM at pH=7 and about 0.1 µM at pH=8) [BLANCKAERT, 1981].

Due to the presence of two propionate groups one might expect that BR would dissolve in water- but in reality it is different! [BULMER, 2008].

Although the three-dimensional structures of BR and BV are very similar (see figure 3), their solution conformations differ greatly [GONCHAROVA and URBANOVÁ, 2007].

Methanol and other polar solvents are incapable of forming hydrogen bonds with BR, so there is no way to bring BR into solution with polar solvents. Nevertheless, UBR dissolves in chloroform, dimethyl formamide, alkali solutions, dimethly sulfoxide (DMSO) and in most lipid solvents [BRODERSEN, 1979].

In the *Salmonella* mutagenicity assay, the most effective and common way to solubilize bile pigments is the use of dimethly sulfoxide (DMSO) [BULMER et al., 2007] - a hydrogen bond-breaking solvent [OSTROW and CELIC, 2008].
The estimated solubility limit of bile pigments in DMSO amounts to 0.034 M, the maximum within the assays was about 3.42 µmol/plate (in 100 µL) [BULMER et al., 2008].

Pilot studies have been accomplished to investigate the maximum of a non toxic dose of DMSO without precipitation when PBS or S9 were added. The outcome of these solubility experiments was, that the dose of bile pigments could be maximized to 2 µmol/plate for BV, BRT and PROT and 0.75 µmol/plate for BR [BULMER et al., 2008].

In contrast to BR, BV is polar and more soluble. Both, BR and BV form non-covalent albumin complexes within the blood circulation (with optical activity) in order to increase the solubility of these compounds. This step in metabolism plays a crucial role in neutralizing the toxicity of BR [GONCHAROVA and URBANOVÁ, 2008].

In contrast to BR and BV, BRDT is a synthetic commercially available compound which has been used as a surrogate for bilirubin diglucoronide and in animal studies [GONCHAROVA and URBANOVÁ, 2007].

2.4. Antioxidative activities of bile pigments

In the past, bile pigments were generally regarded as harmful by-products of heme catabolism without any beneficial function. Damaging effects of BR and BV have been associated with jaundice, especially in newborns or hepatic insufficiency and were considered as toxic compounds [BULMER et al., 2008]. Plasma concentrations of >300 µM have been associated with accumulation as a consequence thereof with the risk of toxic effects in cell functions [STOCKER, 2004].

Scientific research for the antioxidant activity of BR and BV was started in the mid 19 th century. It was already in the early 1950 when for the first time BR was reported to prevent oxidation of lipids (e.g. linoleic acid), vitamin A and ß-carotene during intestinal uptake [SEDLAK and SNYDER, 2004; STOCKER, 2004].
Although these data were published already in the 1950, it was only in the late 1980 when Ames and co-workers established further research findings, that BR´s antioxidative capacity toward lipid peroxidation overtopped that of vitamin E [BURTON and INGOLD, 1986].

In fact, since the last two decades there is a continuing interest by scientists in the research field of bile pigments to investigate the beneficial effects as physiological antioxidants which have been reported by various groups [VITEK, 2005; HATFIELD and BARCLAY, 2004]. As well, it is suggested that BR possesses multiple biological activities [LIU et al., 2008]
As mentioned above, BR possesses beneficial and damaging effects in biological systems [HATFIELD and BARCLAY, 2004]. The concentration, localization as well as binding and blood circulation of BR are crucial to achieve

the balance between deleterious cytotoxic and beneficial anti-oxidative effects [TOMARO et al., 2002].
This chapter is focused on the positive and preventative properties of these compounds.

2.4.1. General aspects of antioxidants

Human health is protected and controlled by an antioxidant defense system. Therefore endogenous enzymes like GSH, SOD, catalase, glutathione peroxidase, are essential for maintaining health in the human body. Under non pathological conditions, this protection system is at least in balance between pro-oxidants (*e.g.* light, radiation, metalloions, chemical substances) and antioxidants, favouring for the latter. This “network” protects living organisms from tissue injury and the deleterious process of radicals [THEILER, 2009].

An imbalance in favor of oxidants leads to oxidative stress and consequently to high levels of reactive oxygen species (ROS) and nitrogen species (RNS) [VERTUANI et al., 2004]. Both species, ROS and RNS, evolutionary developed as natural waste products in the metabolism, possess deleterious function in the body and work as cell signaling and mediator molecules (damaging cell structures, lipids, membranes, proteins and DNA) in the living organism [VALKO et al., 2007].

To achieve the balance between the deleterious and beneficial face of these radicals, the human body requires a number of proteinous (endogenous enzymes) and nonproteinous antioxidants (micronutrients absorbed via food).

Endogenous antioxidants like specific enzymes, bilirubin, uric acid, glutathione or ubiquinon and exogenous (non enzymatic) antioxidants such as tocopherols, caritinoids or ascorbic acid possess a central role and generally act together in cellular defense [THEILER, 2009].

Together, an overproduction of ROS results in oxidative damage which is an important factor in numerous pathological conditions and occupies a central role in a variety of diseases like cancer, atherosclerosis, rheumatoid arthritis, viral infections [NEUZIL et al., 1993] cardiovascular diseases or diabetes, as well as in infections, inflammations [SEDLAK and SNYDER, 2004] and in the ageing process [VALKO et al., 2007].

2.4.2. Antioxidative capacities of bile pigments in literature

To interact as antioxidants, the chemical structure of bile pigments is a decisive factor. BR in its different forms (free, albumin-bound and conjugated) has conjugated double bonds and a pair of reactive hydrogen atoms which are responsible for the H-donation, the main pathway for its antioxidative activity.
In contrast, BV´s chemical structure only exhibits conjugated double bonds, so that the antioxidative capacity of BV is due to the formation of a resonance-stabilized, carbon centered radical [STOCKER, 2004].

2.4.2.1. Modulatory effects towards oxidation of lipids and proteins

As mentioned above, Stocker and Co-workers (1987) were the first to publish a series of *in vitro* studies on the behavior and physiological reactions of bile pigments as a protective agent with possible antioxidant potential in living organisms.

It was in 1987 when the idea of a ´beneficial´ role of BR arose with the suggestion that BR suppressed lipid peroxidation to a greater extent than alpha tocopherol [STOCKER et al., 1987].

An antioxidant-protective function of BR was also confirmed by Neuzil and Stocker (1993). In general, when BR interacts as an antioxidant, it is itself oxidized to BV and then rapidly reduced back to BR via BVR, an abundant

enzyme with a high turnover rate [SEDLAK et al., 2008; SEDLAK and SYNDER, 2004; BARANANO et al., 2002].

The published review form Stocker (2004) described a chemically defined and controlled thermolabile test model system to examine bile pigments, focussing on BR, UBR, CBR and BV. Published data came to the result, that all forms of BR were effective against peroxyl radicals in homogeneous solutions or multilamellar liposomes.
Under these conditions, the antioxidative potential of BR in liposomes actually exceeded that of alpha tocopherol, the most active form of vitamin E, which is generally regarded as the best known protective agent concerning lipids and proteins [NEUZIL et al., 1993]. Under these conditions, BV, compared with all forms of BR, was still more effective in scavenging radicals [STOCKER, 2004].

A study of Ritter (2002) also confirms that nanomolar amounts of BR were sufficiently working against peroxyl radicals.

Besides the above mentioned effects, BR and BV were also described as synergists with membrane-bound alpha tocopherol, demonstrated within the liposome membranes in the presence and absence of membrane bound alpha tocopherol.
Summarizing, it can be concluded that in absence of alpha tocopherol, the lipid peroxidation proceeded linearly; micormolar amounts of CBR or BV inhibited this oxidation and the induction period increased substantially in the presence of alpha tocopherol and one of the two bile pigments. The same results the prevention of lipid peroxidation were shown with CBR and UBR [STOCKER, 2004].

In another experiment, Hatfield and Barclay (2004) focused on the antioxidative potential and activity of BR reactions in 3 different media. The antioxidative capacity of BR was mainly depend on the used medium. A weak antioxidant

activity was shown in nonpolar solution (styrene or cumene in chlororbenzene); strong effects were demonstrated in aqueous lipid bilayers or in micelles of SDS (in polar solutions).

It has also been reported that BR and BV were able to scavenge various other RONS. It is highly probable, that BR and BV were effective against both, 1e-and 2e- oxidants, similar to ascorbate. This behavior is a special feature of BR and BV, because other antioxidants are exclusively effective against one class of oxidants [STOCKER, 2004].
As mentioned above, BR and BV are effective reducing agents and possess antioxidant activity in various *in vitro* systems. In *in vivo* studies, the antioxidative role of these pigments is less clear and less proven. Up to date, there exists only indirect evidence in such *in vivo* situations, like the role of HO-1 and its impact on the cellular heme and iron status.

Although, most findings are not entirely evident, the antioxidative activity of BR and BV is believed in situations of raised oxidative stress or low protection system.

To improve further research work, it might be useful to investigate the effect of altered pigment concentrations on established *in vivo* markers *e.g.* oxidized lipids [STOCKER, 2004]. For instance, Dennery *et al.* (1995) documented a decrease of oxidative damage makers (thiobarbitureic acid and lipid hydroperoxides) after exposure to oxygen, in hyperbilirubinemic gun rats compared with nonjaundiced littermates. The study yielded showed an *in vivo* antioxidant activity of BR, but for future studies more direct testing would be necessary to confirm these first results.

2.4.2.2. Antioxidative effects of albumin-bound bilirubin

Stocker *et al.* (1987) published data whereupon BR protects fatty acids transported on albumin from oxidative damage. Further on, albumin-bound BR

was a 30 times more effective radical scavenger than unbound BR was [STOCKER 2004].

But albumin-bound BR (up 15 µM) is not the major radical scavenger in the extracellular space; main competitors are ascorbate (less or equal 50 µM) and urate (less or equal 300 µM). However, more important than the quantitative character are qualitative considerations, for instance which antioxidant is first depleted and limited in case of increased oxidant production [STOCKER, 2004].

In the nineties, studies of Frei *et al.* (1988) investigated the time dependent consumption of antioxidants in conjunction with the accumulation of lipid hydroperoxides and came to the result that albumin-bound BR represents a second line antioxidant defense against peroxyl radical-induced lipid peroxidation in extracellular matrix and further, that albumin-bound BR inhibits lipoprotein lipid peroxidation in absence of ascorbate and urate.

Besides, a range of studies display that albumin-bound BR protects proteins against oxidative damage by different types of RONS [KAPITULNIK, 2004].

Neuzil *et al.* (1993) confirms also that BR act as protector of albumin-bound fatty acids against peroxyl-radical-mediated oxidation *in vitro;* further on, when BR was absorbed in liposomes, they suggested that BR acted as an agent with possible chain-breaking antioxidant activity as effective as alpha tocopherol [STOCKER et al., 1987].

In summary binding of BR to albumin increased specificity and reactivity against various RONS and increased also protection of lipids and protein [STOCKER, 2004].

2.4.2.3. The theory of a possible redox-cycle of bilirubin and biliverdin

As mentioned above, for several years, BR and BV have been considered as strong antioxidants. While the basic mechanism how BR can protect cells

against oxidative damage is currently unknown, it is believed that the antioxidative function of BR arises from a redox cycle in which BR is oxidized to BV and then recycled back by the ubiquitous BVR [MAGHZAL et al., 2009].

The theory of such mechanism are based on studies, which assessed the heme oxygenase system [SEDLAK and SYNDER, 2004].

Test procedures of cell membrane-bound BR (carried out with chloroform and hydrogen peroxide) found that both substances did not generally oxidize albumin-bound BR to BV and BVR is not an important cellular protector. Nonetheless, it is possible that BVR possess an antiapoptotic activity, but further studies in this field are necessary in future [MAGHZAL et al., 2009]. Whereas Baranano *et al.* (2002) published a study, whereupon data proposed that this redox cycle may represent the cytoprotective role of BVR cycle as the principal physiological function of BR.

These findings are not clear in assuming the theory that the BVR mediated redox cycle plays an important role in cellular defense and demonstrate, that scientific research in this field is still at the very beginning [MAGHZAL et al., 2009].

2.4.2.4. The role of heme and heme oxigenase

In vertebrates, the most abundant source of heme is haemoglobin. The potential toxicity of heme is a critical factor concerning heme degradation in the cellular metabolism. Through enzymatic conversion of the HO system, (HO exists in three isoforms) bile pigments are formed in the blood.

HO-1 (regarded as one of the heat shock proteins) is described as “remarkable inducible enzyme” and occurs ubiquitary in many tissues; the most substantial source is the spleen, where it is activated by heme from senescent red blood cells [SEDLAK et al., 2008; SEDLAK and SYNDER, 2004].

HO-2 and HO-3 are considered as “constitutive enzymes” [STOCKER, 2004] - synthesis is arranged by neuronal activity- and are mainly located in the brain and testes. In the peripheral nervous system, the heme ring of HO is cleaved and CO is produced (which seem to possess a neutrotransmitter function). Due to this fact, BV is formed, which is immediately reduced back to BR by BVR [SEDLAK et al., 2008; SEDLAK and SYNDER, 2004]. The reason, why the more water soluble and therefore more readily excreted BV is metabolized back to BR remains unclear until today [SEDLAK et al., 2008], because it is an energetically expensive, potentially toxic and apparently unnecessary step in mammal metabolism. One possible approach would be, that BR is placenta crossing and therefore a convey from fetal to maternal circulation can happen more easily [BARANANO et al., 2002].

Generally, catabolic heme pathways including HO activity produce both, pro- and antioxidant compounds which might have effects on the cellular redox potential [TOMARO et al., 2002].

Induction of HO-1 is a defense reaction in cells in case of oxidative stress. For instance, it is implied that, HO-1 induction works against lipid-peroxidation and vasospasm [MATZ et al., 1996].
One main result of gained HO-1 activity is increased bile pigment concentrations in the body. BR and BV possess antioxidative properties and represent the adaptive response of cells to oxidative stress [TOMARO et al., 2002].

Tomaro *et al.* (2002) investigated the field of HO and evidenced that BR was an effective marker against oxidative damage in the liver of rats. Treated with various chemicals, it became apparent that ROS rapidly decreased reduced glutathione levels and after that, they induced HO activity.
This HO induction triggered an increase of ROS and a decrease of the anti-oxidant defense system. By having used BR before the treatment with an

oxidative stress inducer was started, it was clearly evident that BR prevented HO induction, GSH decrease and ROS increase in all assays.
In conclusion, collected data of the study imply that UBR is a chain breaking anti-oxidant and scavenger of various peroxide radicals.

Together, BR plays a key role in cellular protection and works efficiently against oxidative damage [TOMARO et al., 2002].

2.4.2.5. Complementary antioxidative and cytoprotective role of bilirubin

2.4.2.5.1. Cytoprotective effects of bilirubin and gluthatione

It was long believed that glutathione (GSH), a tri-peptide and well established cellular antioxidant, formed by glutamic acid, cysteine and glycine, was the most important cellular antioxidant to protect cells from oxidative damage, because of its high concentration in tissues (millimolar) [SEDLAK et al., 2004].

Although glutathione was considered as the most important and prominent cellular antioxidant during the last 80 years [RITTER, 2002] today, it is arguable, that BR provides increased cytoprotective effects *in vitro*, although BR levels in tissues were 10.000 times lower than GSH. 10 nm BR protected cells against 10.000 fold higher concentrations of hydrogen peroxide [SEDLAK et al., 2004; MAGHZAL et al., 2009].

Besides the above mentioned differences in tissue concentrations, also the enzymatic regulation differentiates in these two systems. GSH recycling requires peroxidase and reductase activity for synthesizing the antioxidant; BV is directly oxidized to BR without the need of other enzymes [BARANANO et al., 2002].

Although remarkable differences can be found in tissue concentration and enzymatic regulation, several parallels can also be demonstrated. For instance,

both substances imply an oxidized and a reduced form (the latter particularly arises under healthy conditions) [BARANANO et al., 2002].

Why do cells evolve two different antioxidant defend systems? Due to the fact, that BR (highly lipophilic) was found in the blood at high concentration, it is conceivable, that BR is the major protector for cell membranes against lipid peroxidation, while GSH (highly hydrophilic) interacts protectively with water soluble proteins inside cells. A possible overlapping function of both antioxidant defense systems is subject of debate [SEDLAK et al., 2004; SEDLAK et al., 2008].

Besides the above mentioned effects of BR and GSH, it appears that cells use multiple “antioxidant network” to defend human health against oxidative stress and ROS, *e.g.* free heavy metals are regulated by chelators (such as ferritin and transferrin), together SOD and catalase convert superoxide to water or ascorbate and vitamin E act as direct antioxidants by catching free radicals [SEDLAK and SYNDER, 2004; BARANANO et al., 2002].

2.4.2.5.2. Further modulatory effects of bile pigments

The beneficial and physiological actions of bile pigments as an endogenous tissue protector was also examined by Nakagami *et al.* in 1993. Published data show that bile pigments possess anti-complement properties and therefore could be involved in tissue protection. Examinations showed inhibitory effects of BV in complement cascade reactions *in vitro* and also in associated complement reactions in guinea pigs; UBR showed complement-dependent reactions *in vitro*. So this study represents another example for a protective role of bile pigments in tissue protection [NAKAGAMI et al., 1993].

Immunological importance of bile pigments was confirmed and published by Liu *et al.* (2008). The results clearly showed significantly powerful suppressing effects on both, polyclonal and Ag-sepcific T cell responses, while other known antioxidants did not exhibit these effects. Liu *et al*. identified BR as a potent

immunomodulator that might protect mammals against immune disorders (multiple sclerosis and other autoimmune diseases).

Furthermore, it is believed that, bile pigments may have an important biological function in other fields to maintain human health. For instance, it is discussed that BR plays an evolutionary role in the REM sleep and also has a large share on the light´s anti-depressant effects [OREN et al., 1997].
Besides the above mentioned capacities of bile pigments, several other modulatory effects of bile pigments were discussed in literature, *e.g.* anti-viral, anti-apoptotic and anti-mutagenic effects (see next chapter) [BLUMER et al., 2008].

2.5. Antimutagenic properties of bile pigments in the *Salmonella* Ames Test

Within this sub-study, the widely accepted and popular *Salmonella* mutagenicity assay was used as test model to detect potential anti-mutagenic effects of BR, BV, BRDT and PROT.

2.5.1. The *Salmonella* Ames Test

The Ames Test is a short-term bacterial reverse mutation assay designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations [MORTELMANS et al., 2000].

To describe and report substances, capable of inducing mutations or cancer, the Ames Test is used world-wide as an initial screening method to elicit mutagenic potential of chemicals and drugs [MORTELMANS et al., 2000].

Over the years, the Ames Test has been securely established and developed, so that today uniformity of testing procedures can be ensured Therefore, within scientific communities and corporations, the Ames Test is a commonly used method and pays a key role in safety assessment [MORTELMANS et al., 2000].

2.5.1.1. Historical background

The Ames test has been developed in 1964 when Bruce Ames found different mutants of *Salmonella typhimurium bacteria*, unable to form the amino acid histidine and therefore, unable to form colonies. Due to the fact, that bacteria reverted to their wild form after adding mutagenic substances, this test is often referred to as “reversion assay”.

Over the years, mutants were selected which were sensitive to reversion by a variety of chemicals mutagens. Within test procedures, various tester strains were used to differentiate types of mutations in the histidine operon like base-pair mutants and frameshift mutants [MORTELMANS et al., 2000].
In 1973, Ames *et al.* improved the spot test procedure of 1966 (mutagen screening with an *E.coli* strain) and created the so called incorporation assay procedure. Over the years different groups developed this incorporation assay to improve the sensitivity of the test so that nowadays it is possible to test a wide range of chemicals, gases and volatile substances [MORTELMANS et al., 2000].

2.5.1.2. Metabolic activation systems

In mammalian, several carcinogenic chemicals are biologically active until they are metabolised to inactive forms or vice versa via the P450 metabolic oxidations system, which is mainly located in the liver. Due to the fact, that bacteria do not have a similar metabolic oxidation system like the cytochrome-based P450 system, an exogenous metabolic activation system needs to be added within the test procedure.

In fact, it was the key to success that Ames *et al.* (1973) included this S9 fraction in the test procedure. Consequently, the test procedure became highly sensitive, more quantitative and meaningful for science. This activation system is called S9 microsomal fraction and is a mix of oxidase enzymes obtained from animals (liver homogenate), treated with Acrolor 1254. For chemicals, which

include azo and diazo bonds, the metabolic activation system also contains reductive enzymes.

In mammalian, metabolism and reduction of chemical substances a part takes in the intestional wall place via the anaerobic intestinal microflora or in the liver. In *in vitro* studies, this reductive metabolism is generated based on liver homogenate supplemented with FMN (Flavin mononucleotide) and as well based on rat intestinal microflora preparations (see chapter “Materials and Methods”) [MORTELMANS et al., 2000].

2.5.1.3. *Salmonella* tester strains

A set of standard tester stains were used within mutagenicity testing of chemicals to detect different types of mutations in the histidine operon. Additional other mutations/genetic alterations have been inserted in the standard tester strains to make these strains more sensible to detect mutagenic substances in the test procedure (*e.g*. uvrB-mutation, rfa mutation or pKM101).

Within this sub-study, tester strains *TA 98* and *TA 102* were used:

Mutation	Bio chid uvrB gal	LSP defect	Plasmid
TA 98 his D3052	Deletion mutation	rfa	pKM101 (ampicilin restistence)
TA 102 his G428	Wild type	rfa	pKM101, PAQI (tetracycline resistance)

Table 1 Genotype of the exerted *Salmonella* tester strains [MÖLZER, 2007]

TA 98 and *TA 102* contain a *rfa* mutation that leads to a damaged lipopolysaccharide (LPS) layer (*rfa* mutation affects the permeability of the cell wall) and increase permeability in the bacteria to large chemicals which do not permeate under normal conditions.

TA 98 has a *his D3052* frameshift mutation which affects the DNA sequence –C-G-C-G-C-G-C-G-.

In contrast, *TA 102* has an AT base pair at the *his G428* mutant site and belongs to the set which are susceptible to oxidative compounds [MÖLZER, 2007].

2.5.1.4. Commonly used mutagens within the Ames Test

Commonly used mutagens within the Ames Test can be divided in three classes [BULMER et al., 2008]:

- Mutagens, derived from processes involving combustion: *e.g*. benzo[alpha]pyrene- which is polycyclic aromatic hydrocarbon derived from fossils fuels and commonly used in the *Salmonella* mutagenicity assay (B[a]P); nitroarenes derived from combustion of diesel fuel; 4-nitropunoline-1-oxide (4NQO) which accrues form the combustion of tabacco; these mutagens are mainly present in the atmosphere and therefore contribute to the development of cancer, particularly of the respiratory tract.

- Mutagens, found in food products, formed as a consequence of processing or cooking. It is thought that its metabolism can result in genotoxicity; *e.g.* amino acid pyrolysate products of tryptophan, glutamic acid or soy beans, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), aflatoxin B_1 (AFB_1)

- Miscellaneous mutagens: *e.g.* 2-4-7 trinitrofluenone (TNFone), a nonplanar, polycyclic nitro-amine which is found in photocopy toner, 2-aminofluorene which is a planar heterocyclic amine and synthetically produced; tertiary butyl hydroperoxide (*t*-BOOH) which is an organic hydroperoxide and oxidant.

Within this sub-study aflatoxin B_1 (AFB_1) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) were used as representatives for mutagens, since they are particularly found in food products [BULMER et al., 2008].

2-4-7 trinitrofluenon (TNFone) and tertiary butyl hydroperoxide (*t*-BuOOH) were included in test procedure representing pro-oxidants for TA 102 and are commonly not found in environment.

2.5.2. Antimutagenic effects of bile pigments in the *Salmonella* Ames Test

Bulmer *et al.* published the first review on anti-mutagenic effects of bile pigments in 2008. They summarised 12 studies describing antimutagenic properties of bile pigments, within cultured bacterial studies.
Most of these studies dealing with the antimutagenic effects of porphyrins were realized in the period from 1980 to 2000.

2.5.2.1. Literature survey of studies of mutagens, derived from processes involving combustion

Arimoto *et al.* (1980) investigated different kinds of porphyrins (hemin, BR, BV) and confirmed that these compounds inhibited induced mutagenicity by B[a]P in tester strain *TA 98*. The results showed a potent antimutagenic effect of all tested substances. Within the bile pigments, BR showed a greater effect than BV.

Hemin, a chelated porphyrin, seemed to be more effective than bile pigments and could inhibit the mutagenic response of B[a]P - it is believed, that hemin can chemically bind B[a]P [ARIMOTO et al., 1980]. However, in a recently published study by Blumer *et al.* (2007) BR and BV showed complete conflictive results in effectiveness.

In a later study of Arimoto *et al.* (1995) once again an anti-mutagenic effect of BR and BV was confirmed in tester strain *TA 100*, this time in presence of metabolic activation. Further weak antimutagenic effects were shown against B[a]P metabolites B[a]P-4,5-epoxide and B[a]P-7,8-diol-9,10-epoxide.

Further metabolites of B[a]P (Sulfoxymethylbenzo[a]pyrene (SMBP) and 6-hydroxymethylbeno[a]pyrene (HMBP)) were tested by Cho *et al*. (2000) in the bacterial strain *TA 98*. Mutagenesis by the above mentioned mutagens was tested with selected porphyrins: BV, PROT, hemin, chlorophyllin and phtalocyanines. The results showed that the porphyrins were more effective against SMBP compared to HMBP. More precisely, BV and hemin were absolutely ineffective against HMBP.
Studies concerning antimutagenicity testing of mutagens and metabolites of tobacco compounds were done by Romert *et al.* in 1994 and in 1992. In 1992 no antimutagenic effects were displayed referring to tobacco specific nitrosamine and BV. This result was critically discussed, since it can be distorted as a consequence of too low doses of BV and mutagen.

In a further study by Romert *et al*. in 1994 porphyrins, including BV were investigated in the bacterial strains *TA 98* and *TA 100* in the presence of metabolic activation. The applied mutagen was cigarette smoke condensate (CSC). Due to the fact, that BV showed greater antimutagenic effects in *TA 100* than in *TA 98* it is believed, that BV is able to prevent base-pair mutations. In 1994 Camoirano *et al.* also confirmed antimutagenic effects of BR in *TA 98* and *TA 100* with the same mutagenic condensate of CS.

Concerning 4NQO, studies were done by De Flora *et al*. (1994) and Camoirano *et al*. (1994). Camoriano tested BR´s response with 4NQO in *TA 98* and *TA 100* and found an antimutagenic effect. Besides BR, BV was also tested in this study in *TA 100* but no protective effect was shown in this set of experiments.

De Flora *et al.* (1994) confirmed the above mentioned results of Camoirano *et al.* Antimutagenic effects of BR against genotoxicity of 4NQO in *TA 100* were shown. In 1994 De Flora *et al*. investigated the possible antimutgenic potential of BR more precisely and tested the spontaneous mutations in *TA 102* and *TA 104*, as a marker for deficient DNA recombination, repair processes and production of free radicals that modify genomic structure. Many compounds

were tested within this study; BR was only tested in *TA 104* and interestingly, no effect in spontaneous mutation was shown in this experiment.

2.5.2.2. Literature survey of studies of mutagens, found in food products

Humans are regularly exposed to food toxins, formed during the cooking process. Typical food mutagens are particularly arcylamide, hetereocylic amines, nitrosamines or polyaromatic hydrocarbons. Exposure can increase the risk of human cancer [JAGERSTAD and SKOG, 2005].

Arimoto *et al.* (1980) used pyrolysate products of tryptophan, glutamic acid and soy bean to investigate potential antimutagenic effects of bile pigments. In presence of S9 and *TA 98*, BV showed the most pronounced antimutagenic effect, but concurrently BV was less effective than hemin, chlorophyllin and PROT (under certain conditions).

The reason behind BR´s non antimutagenic effects in this study is presumed to be due to a different chemical structure of BR (hydrogen bonded and folded conformation) compared to the planar tetrapyrrolic structure of PRO and BV. This is a possible theory and may be essential for the antimutagenic binding of the amino acid pyrolysis products.

In 1989, Arimoto and Hayatsu continued their investigations of amino acid pyrolysis products. The same porphyrins were tested in *TA 98* including metabolic activation. Again, antimutagenic effects were confirmed, whereby the planar molecules of PROT, BV and chlorophyllin were more effective than BR.

Tang and Edenharder (1997) investigated possible antimutagenic effects of hemin, chlorophyllin, chlorophyll, BV and BR in *TA 98* without metabolic activation in the presence of nitroarenes: 2-nitrofluorene (2-NF), 3-nitrofluoranthene (3-NFA) and 1-nitropyrene (1-NP). The results showed

stronger antimutagenic effects of BR and BV than hemin and chlorophyllin. The results showed that BR was more effective than BV.

Due to the fact that bile pigments possess antimutagenic activities (see above mentioned studies) and the fact that they are present in the bile and intestine of humans in high concentrations, it is possible that they can influence the genotoxicity of food toxins in human diet [BULMER et al., 2008].

2.5.2.3. Literature survey of studies of miscellaneous mutagens

Examples for such mutagens are 2,4,7 trinitrofluenone (TNFone), 2-aminofluorene (2-AF) or tertiary butyl hydroperoxide (*t*-BOOH).

Bulmer *et al.* (2007) published data on the antimutgenic effects of BR, BV and BRDT in the bacterial strains *TA 98, TA 100* and *TA 102* tested with TNFone, 2-AF and *t*-BOOH. It became apparent that antimutagenic effects of the bile pigments were dependent on the tested mutagen but independent from the bacterial strains which were used.

In this study BR and BV were the most effective compounds against TNFone and 2-AF. Related to *t*-BOOH, results showed that BR was more effective than BV. However, BRDT showed no effect referred to *t*-BOOH.

Due to this study-outcome, the theory has been discussed that antimutagenic effects of BR and BV stem from the ability that they donate electrons (BR donates 2 electrons, BV donates one electron). Why BRDT showed no antioxidative effect remains unclear. It is suggested, that this behaviour of BRDT is attributed to structural differences between BR and BRDT or to the inability of BRDT to cross lipid membranes.

Summing the data up, the study of Bulmer *et al*. (2007) was the first that confirmed a preventative role of endogenous bile pigments related to oxidative

stress induced mutation in *TA 102*. Therefore it is suggested that bile pigments possess possible physiological importance in biological systems.

3. MATERIALS and METHODS

3.1. Basic principles of the Ames Test

The so called *Salmonella* microsome mutagenicity assay is a popular and widely accepted test method to detect chemical substances that possess mutagenic properties. Over the years, the Ames Test has been steadily developed by different research groups to enhance particularly the sensitivity of test procedure [MORTELMANS, 2000].
Within the test, various *Salmonella* test strains are used with typical known foregoing mutations in different genes in the histidine operon. Due to these certain types of mutations, bacteria cannot produce the amino acid histidine, which is a growth factor to form colonies. A second mutation can reconstruct the gene´s function and allow the cells to synthesize histidine and to form colonies. These colonies can grow in absence of histidine and will be counted after incubation.

To identify substances, capable of inducing mutations, the Ames test is used world- wide as an initial screen. Developed international guidelines guarantee the uniformity of test procedures; for this reason the Ames Test became a key role in safety assessment [MORTELMANS, 2000].

Within our study, the plate incorporation assay with and without metabolic activation was realized in order to analyze the antimutagenic/mutagenic and antioxidative/oxidative manner of BR, BV, BRDT and PRO with *Salmonella* tester strains *TA 98* and *TA 102.*

3.2. Preparatory work of the Ames Test

3.2.1. Preparatory work of used agar

3.2.1.1. Minimum glucose agar plates

In each 1000 ml Erlenmeyer flask, 11,25g of Oxid agar were dissolved in 700ml water. Before autoclaving, the Erlenmeyer flasks were closed with a piece of cotton, capped with aluminium foil and indicator tape to ensure, that a temperature of 121° Celsius was reached during autoclaving. Along with the agar flasks, Pyrex flasks of glucose solution and Vogel-Bonner solution were also autoclaved.

After the sterilization process, the Erlenmeyer flasks were opened under the laminar flow and inoculated first with 15 ml Vogel-Bonner solution. After shaking 37.5 ml glucose solution were added.

Now, the agar was ready-made to pour it in prepared sterile Petri-dishes (thickness about 5 mm).

After approximately 12 hours the plates were well dried and were repacked in plastic bags and stored at 4° Celsius (dated and packed in sealed plastic bags to prevent dehydration).

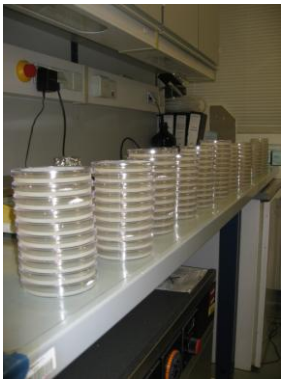

Figure 4 Batches of poured Petri-dishes

3.2.1.2. Top Agar (TA)

1,2 g of Oxiod agar and 1g NaCl was weighed in a Pyrex-flask. The flask was then filled to 200 ml with *aqua dest*. After sterilization, 20 ml of histidine-biotin solution was added. After shaking, the top agar was kept warm in a water bath under the laminar flow at 48 °Celsius.

Top agar is one of the most critical medium components in the Ames test because it contains the trace amount of histidine for limited growth.

3.2.1.3. Master plates Agar (MP)

Master plates were prepared for availability of bacteria. For the preparation of two plates, 3.2 g Oxiod agar was dissolved in 200 ml of *aqua dest.* After autoclaving, 4.3 ml Vogel-Bonner- and 10.8 ml Glucose solution were added, subsequently, the agar was completed with 2.17 ml of histidine- and 1.3 ml of biotin solution. Then, specific antibiotics were pipetted in the cooled-down agar. For strain *TA 98,* the agar was inoculated with 625 µl of ampicillin solution. Strain *TA 102* furthermore needed 54.3 µl of tetracycline solution.

The mixed agar was filled in Petri-dishes; thickness about 1 cm. After the drying process (about 24 hours; laminar switched on) the bacteria solution was spread in fine lines onto the agar. When incubation was completed (48 hours at 37 ° Celsius) the plates were stored in the refrigerator.

Due to the used antibiotics (Tetracyclin- stable for two weeks; Ampicillin- stable for two month) master plates of *TA 98* could be used for two months; plates of *TA 102* could only be stored for two weeks due to the number of spontaneous revertant colonies that increases over time.

3.2.2. Preparation of used solutions

3.2.2.1. Preparation of the S9 mix (metabolic activation system)

Bacteria do not have an equivalent metabolic system, like the cytochrome-based P-450 metablic oxidation system in humans and lower animals. Thus, an exogenous mammalian metabolic activation system was included in the test procedure in order to imitate the mammalian hepatic metabolisms.
The used S-9 fraction was prepared from rat liver chemically treated with Acrolor 1254.

The S-9 Mix is an enzymatic mixture and contained 19.75 ml of sterile *aqua dest.*, 25 ml of PBS (without Ca and Mg), 1 ml of MgCl2/KCl solution, 2 ml of NADP solution and 250 µl of glucose-6-posphate solution. The rat homogenate was added at last step. The solution was vortexed carefully from time to time.

Since the added enzymes are very sensitive, the mixture needed to be cooled on crashed ice and prepared freshly each time before starting the test. It could be used for a maximum of 60 minutes after opening the flask.

3.2.2.2. Preparation of the Nutrient Broth (NB) and Overnight Culture (OVNC)

For cultivating bacteria, 5 g of the Nutrient Broth (weighed into a Pyrex-flask) were dissolved in 200 ml of *aqua. dest*. Subsequently, the flask were capped with aluminium foil and indicator tape and transferred into the autoclave.

Before starting the OVNC, specific antibiotics were pipetted into the cooled NB. Depending on the used tester strains, different amounts of ampicillin and tetracycline were inoculated: for *TA 98,* 625 µl ampicillin, for *TA 102*, 625 µl ampicillin plus 156 µl tetracycline were added.

Figure 5 OVNC

Thereof, 12 ml were transferred into a sterile Erlenmeyer-flask and inoculated with the adequate bacteria strain (swirled through a loop), which were scraped off the master plates. The Erlenmeyer-flask was closed with a piece of cotton and coated with aluminium foil. The OVNC was incubated at 37° Celsius for 12 hours and shaken continuously at about 100 rpm.

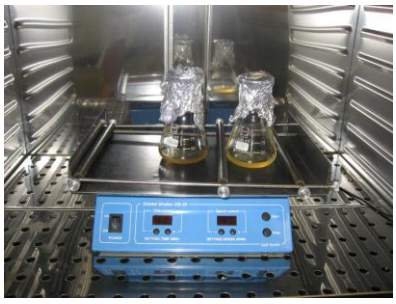

Figure 6 OVNC- were put in incubator

To be on the safe side, OVNC was carried out in duplicate.

3.2.2.3. Preparation of positive and negative controls

Specific positive controls are needed to indicate the reversion of the applied bacteria strains. This reversion of bacteria emerges from the reaction of bacteria and the added mutagen.

Following chemicals were utilized within test procedure:

Bacteria strains:

	TA 98	***TA102***
-S9	TNFone	TNFone
		BOOH
+S9	AFB_1 +S9	BOOH +S9
	PhIP +S9	AFB_1 +S9

Table 2 Overview of utilized chemicals and bacteria strains

Negative controls are used in order to display the numbers of spontaneous revertants. As a negative control, we used the identical solvent utilized for preparing the test samples, but without adding the test substance.

Vogel-Bonner solution	The following salts were dissolved one by one in 670 ml of aqua dest. (45° Celsius): - 10 g of magnesium sulphate heptahydrate - 100 g of citric acid monohydrate - 500 g of dibasic potassium phosphate - 175 g of sodium ammonium phosphate tetrahydrate The solution was autoclaved each time before use.
Glucose solution (40%)	400 g Glucose was solubilised in 1000 ml of heated aqua dest. The solution was autoclaved each at time before use.
Biotin-Histidine solution	30,9 mg D-biotin and 24 mg L-histidine were weighed into a weighing boat, transferred into a Pyrex-flask and made up to 250 ml. The solution was stored at 4° Celsius and autoclaved each time before use.
Ampicillin solution	0,2 g of ampicillin is vortexed with 25 ml of 2N NaOH; solution was sterilized through an aseptic filter and stored at 4° Celsius
Tetracycline solution	80 mg of tetracycline was vortexed with 10 ml of 0,2N HCl; solution was sterilized through an aseptic filter and stored at 4° Celsius
Histidine solution	0,25 g of histidine was mixed with 50 ml of aqua dest.; solution was sterilized through an aseptic filter and stored at 4° Celsius
Biotin solution	6.1 mg of D-biotion was mixed with 50 ml of aqua dest.; solution was sterilized through an aseptic filter and stored at 4° Celsius

MgCl2/KCl solution	61.5 g of KCl and 40,7 g of MgCl2 was solubilised in 500 ml of aqua dest. and sterilized in the autoclave
NADP solution (0,135 g/2ml)	1 g of NADP was solved in 14.814 ml of aqua dest.; solution was sterilized through an aseptic filter and kept in portions of 1 ml at -20° Celsius
Glucose-6-phosphate solution (304 mg/ml)	1 g of glucose-6-phosphate was dissolved in 3.289 ml of aqua dest.; filter sterilized solution kept in portions of 1 ml at -20° Celsius
Nutrient Broth	5 g nutrient broth were weighed in into a Pyrex-flask and filled up with 200 ml aqua dest.; solution was autoclaved and inoculated with strain specific antibiotics

Table 3 Index of solutions used within the test procedure

Denotation	Abbreviation	Use
Aflatoxin B_1	AFB_1	Standard mutagen (+S9)
Agar No 1 (Oxoid Agar)		Plates, Top Agar
Ampicillin Trihydrate		Master Plates, OVNC
Citric Acid Monohydrate	$C_6H_8O_7$	Vogel-Bonner solution
D-Biotin	$C_{10}H_{16}N_2O_3S$	Master Plates
D-Glucose	$C_6H_{12}O_6$	MG-Agar Plates, Master Plates
Dimethylsulfoxide	DMSO	Organic solvent
Glucose-6-phosphate	Glu-6-P	S9-Mix
Hydroclorid acid	0,2N HCl	Tetracyline solution
L-Histidine	$C_6H_9N_3O_2$	Top Agar, Master Plates
Magnesiumchloride	MgCl2	S9-Mix
Magnesiumsulphate heptahydrate	$MgSO_4$. $7H_2O$	Vogel Bonner solution
Nicotineamid-adeninedinucleotide-phosphate	NADPH	S9- Mix
Phosphatebuffer	PBS	Pre-incubation, S9-Mix
Potassiumchloride	KCl	S9-Mix
Potassiumphosphate, dibasic	K_2HPO_4	Vogel-Bonner solution
Rat liver homogenate	S9	Tests with metabolic activation
Sodiumammonium-phosphate-tetrahydrate	$Na_2NH_2PO_4$. $4H_20$	Vogel-Bonner solution
Sodiumhydroxide	2N NaOH	Ampicillin solution
Tertiary-Butyl-Hydroperoxide	*t*-BOOH	Standard mutagen (+/-S9)
Tetracycline Hydrochloride		Master Plates, OVNC (+/-S9)
2-4-7-Trinitro-9-Fluorenone	TNFone	Standard mutagen
2-Amino-1-Methyl-6-Phenylimidazol [4,5-B]Pyridine	PhIP	Standard mutagen (+/-S9)

Table 4 Index of chemicals used within the test procedure

3.2.3. Bacterial strains used within the test procedure

Within the test procedure, various *Salmonella typhimurium* tester strains can be used. All these bacterial strains have different mutations in the histidine operon, which is why they are histidine dependent.

Both bacteria strains (*TA 98* and *TA 102*) used in the study show different mutations and alterations in various sections and plasmids in the genes.
TA 98 carries the *hisD3052* mutation which is named frame shift mutation, leading to a completely different sequence of bases, resulting in a changed sequence of amino acids.
TA 102 contains the *hisG28* mutation which is a transitions/transversions mutation. These two types of mutations are categories of so called gene point mutations which affect the succession of bases.

3.2.4. Preparation of the test samples

Solubility experiments of BR, BV, BRDT, PRO
Preliminary, solubility tests were performed in order to gather information on solubility - behaviour of these specific bile pigments. The different stock solutions were diluted with PBS and Dimethylsulfoxid (DMSO) as organic solvent and analyzed photometically at three various time points.
These pre-tests showed, that the pigments were hardly dissolved, especially BR. Therefore, to ensure adequate solubility of the bile pigments, sonication was used alongside with gently warming up the samples in a water bath (37°).

3.2.4.1. Preparation operations of uses samples

The samples (BR, BV, BRDT, PRO) were prepared in amber cubs according to the scheme of dilution (see table 5) and stored in the freezer in a light-proof box. On the day of testing the samples were defrosted and diluted with DMSO through a well conceived scheme of dilution. BR was tested in 5 concentrations (0.01µmol/plate, 0.05 µmol/plate, 0.1 µmol/plate, 0.5 µmol/plate, 0,75 µmol/plate), BV, BRDT and PRO were analyzed in 6 concentrations (0.01 µmol/plate, 0.05 µmol/plate, 0.1 µmol/plate, 0.5 µmol/plate, 1.0 µmol/plate, 2.0

µmol/plate). These concentrations were applied to make our study comparable with previous research work and particularly to have a concentration area which is recommended for the test.

Dilution-scheme			
Sample	**Stock solution** (Sample material+ DMSO)	**investigated concentrations** (µmol/plate)	
BR	4 mg + 0.274 µl DMSO	0.75 µM	120 µl Stock + 680 µl DMSO
		0.5 µM	80 µl Stock + 720 µl DMSO
		0.1 µM	16 µl Stock + 784 µl DMSO
		0.05 µM	100 µl [0.5 µM] + 900 µl DMSO
		0.01 µM	100 µl [0.1 µM] + 900 µl DMSO
BRDT	13mg+ 617 µl DMSO	2.0 µM	320 µl Stock + 480 µl DMSO
		1.0 µM	160 µl Stock + 640 µl DMSO
		0.5 µM	80 µl Stock + 720 µl DMSO
		0.1 µM	100 µl [1.0 µM] + 900 µl DMSO
		0.05 µM	100 µl [0.5 µM] + 900 µl DMSO
		0.01 µM	100 µl [0.1 µM] + 900 µl DMSO
BV	9mg + 618 µl DMSO	2.0 µM	320 µl Stock + 480 µl DMSO
		1.0 µM	160 µl Stock + 640 µl DMSO
		0.5 µM	80 µl Stock + 720 µl DMSO
		0.1 µM	100 µl [1.0 µM] + 900 µl DMSO
		0.05 µM	100 µl [0.5 µM] + 900 µl DMSO
		0.01 µM	100 µl [0.1 µM] + 900 µl DMSO
PRO	9mg + 618 µl DMSO	2.0 µM	320 µl Stock + 480 µl DMSO
		1.0 µM	160 µl Stock + 640 µl DMSO
		0.5 µM	80 µl Stock + 720 µl DMSO
		0.1 µM	100 µl [1.0 µM] + 900 µl DMSO
		0.05 µM	100 µl [0.5 µM] + 900 µl DMSO
		0.01 µM	100 µl [0.1 µM] + 900 µl DMSO

Table 5 Dilution scheme

Due to the chemical structure, bile pigments are highly photosensitive.
In order to avoid any chemical reactions of the substances (e.g. oxidation processes), the samples dilution had to be prepared freshly each time before starting the test.

3.3. Experimental design

We used the plate incorporation assay procedure according to Ames *et al*. in 1973. As compared with the previously used spot test, this procedure provides higher sensitivity and is more quantitative

The standard practice is structured in many steps which must be carried out in sequential order. It is important to work in a timely manner and to stick to the time limits. Therefore a high level of time management is needed in order to maintain quality of investigation and to prevent distortion of results.

Before starting the tests, the following preparations had to be performed daily, approximately one hour before starting the test procedure:

labeling the minimum glucose agar plates (date, sample, concentration, bacterial strain)
labeling test tubes (sample, concentration): - three tubes for each concentration to be examined - 6 tubes for negative control - 3 tubes for positive control
preparing dilutions of the samples in the right concentrations according to the pipetting scheme
preparing the positive control
thawing of the stock solutions
inoculating the sterilized TA with the biotin-histidine solution
get the laminar flow ready for the test

Table 6 Overview of pre- test steps

First, TA was ready-made for the autoclave; the next steps were done during the period of TA sterilization.

Ready prepared samples as well as sterile equipment (*e.g.* test tubes, Erlenmeyer-flasks, petri dishes) must be available in the laboratory.
This checkup should already be done some days before a test run is scheduled.

The following scheme illustrates all steps of the Ames Test:

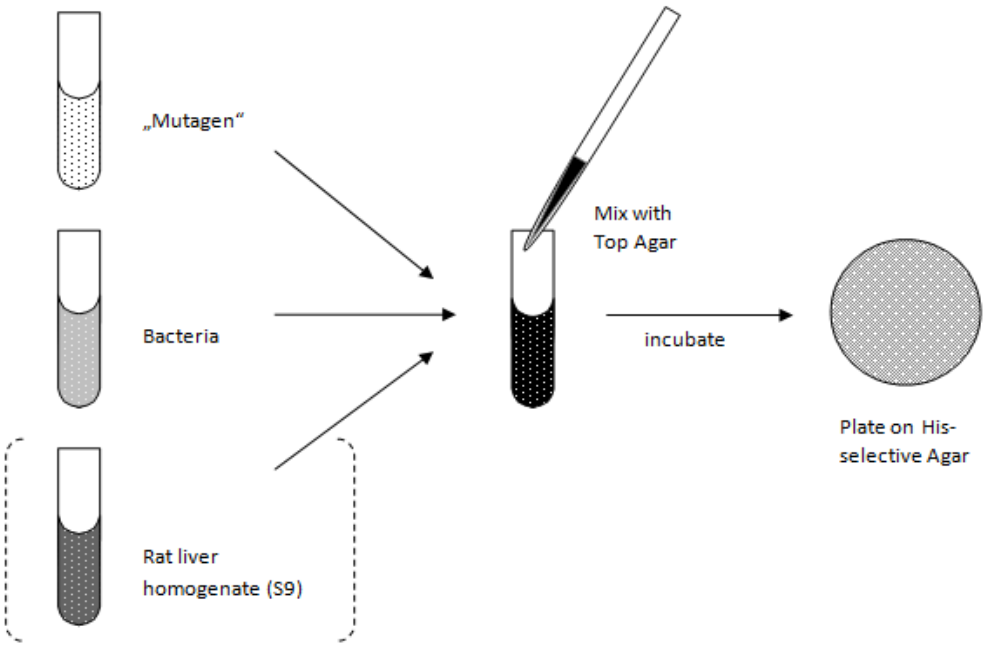

Figure 7 Scheme of the Ames Test

In our study, each test was carried out in duplicates (rerun on another day); for each concentration three plates were prepared. (total n=6/concentration)

3.3.1. Test procedure in pairs

First of all 500 µl of buffer and 100 µl of bacteria-suspension were alternately pipetted, followed by 100 µl of positive control (except negative control tubes) and 200 µl of sample. Along with pipetting the first sample, the stopwatch (period of twenty-five minutes) was pushed for starting the pre-incubtion period.

For the remaining time (after having finished the pipetting), the tubes were covered and the whole tube rack was placed in the incubator at 37° Celsius (rotary shaker about 100 rpm). Afterwards 2 ml of top agar (kept in the water bath at 48° Celsius) was added to each test tube. Then, test tubes were vortexed once again and poured onto minimum glucose agar plates and stacked in the laminar; after about 20 minutes the plates were well dried and placed in the incubator for 48 hours (lid downwards).

Colony counting was started right after the incubation period.

For the test with metabolic activation, the S9-mix was pipetted instead of the buffer.

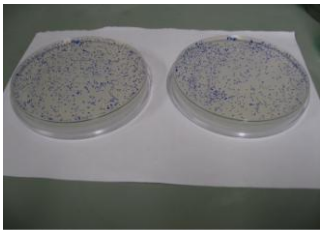

Figure 8 Counted MG-Plates

3.4. Statistics

Test data was analyzed using SPSS (SPSS Software, Inc., Vers. 16.0) and Microsoft Excel (Windows Vista 2009).
For graphic representation and to calculate arithmetic means and standard deviation, raw data were entered into Microsoft Excel.
Data were tested for normal distribution with the Kolmogorov-Smirnov test. To detect possible effects of the bile pigments in revertant growth within different concentrations, One-way Analysis of Variance (ANOVA) was selected. For post hoc evaluation the Bonferroni and Scheffé test was realized. For testing homogenous subsets, the Tukey test was used.
Significant level for statistical analysis was considered at $p< 0.05$.

Following published criteria of Mortelmans *et al.* (2000) were further used to interpret our results:

- Mutagenic:

A compound was classified as mutagenic, if test procedure showed a concentration dependency and further, when the numbers of his+ revertant colonies were higher than double negative values.

- Weak mutagenic:

Substances were identified as weak mutagenic when the numbers of his+ revertant colonies were not exceeded double negative values, but a concentration dependency was demonstrated.

- Non-mutagenic:

If no concentration dependency could be proven within test procedure, samples were rated as not mutagenic.

- Anti-mutagenic

If the relative mutagenic response fell below 50 percent, substances were classified as anti-mutagenic.

- Inconclusive:

If compounds cannot be clearly interpreted as mutagen or nonmutagen, the results were discussed as inconclusive.

4. RESULTS AND DISSCUSSION

To detect the possible antimutagenic and antioxidative effects of BR, BV; BRDT and PRO, tester strains were used in order to achieve selective information about frameshift mutations (*TA 98*) and data about oxidative stress (*TA 102*).
The following table shows the mutagens which were used within the test procedure:

Anti-mutagenic/ anti-oxidative test procedure in the *Salmonella* Ames assay:

TA 98	***TA 102***
AFB_1 $0.8*10^{-7}$ µmol/plate + S9	AFB_1 $0.24*10^{-6}$ µmol/plate + S9
TNFone $0.3*10^{-6}$ µmol/plate	TNFone $0.2*10^{-7}$ µmol/plate
PhIP $0.1*10^{-7}$ µmol/plate + S9	*t*-BOOH $0.75*10^{-6}$ µmol/plate
	t-BOOH $0.75*10^{-6}$ µmol/plate +S9

Table 7 Overview of used mutagens and bacterial strains

After the practical procedure in the lab and manual colony counting, statistical analysis was performed. The following chapter gives an overview of obtained results.

A detailed overview of all counted single revertant numbers is shown in chapter nine (Appendix).

4.1. Antimutagenic Testing

4.1.1. TA 98

4.1.1.1. Mutagenicity induced by PhIP 0.1 µmol/ml + S9

In this condition, BR, BV, BRDT and PRO were tested in the presence of metabolic activation.

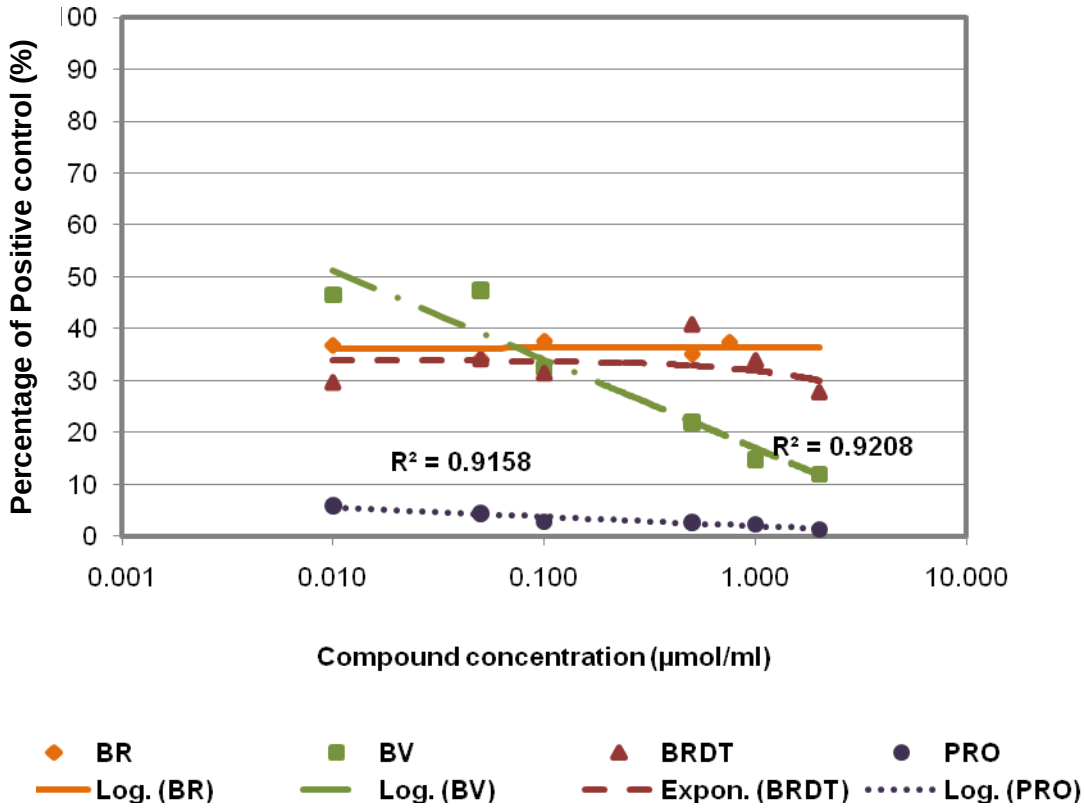

Figure 9 The modulatory effects of bilirubin (BR), biliverdin (BV), bilirubin-ditaurate (BRDT) and protoporphyrin (PRO) on PhIP induced mutagenicity in *TA 98*

Figure nine shows that all tested substances were able to reduce PhIP induced mutagenicity. All compounds passed the 50 percent line.

PRO was the compound yielding the most pronounced antimutagenic effect. Less than 10% of the induced colonies were still countable, which means that more than 90% were inhibited. Highly significant reductions of His+revertants

became obvious when all tested concentrations of BR, BV, BRDT and PRO were compared to the positive control ($p<0.01$).

BV and PRO inhibited PhIP induced mutagenicity in a dose-dependent manner as depicted in figure 10:

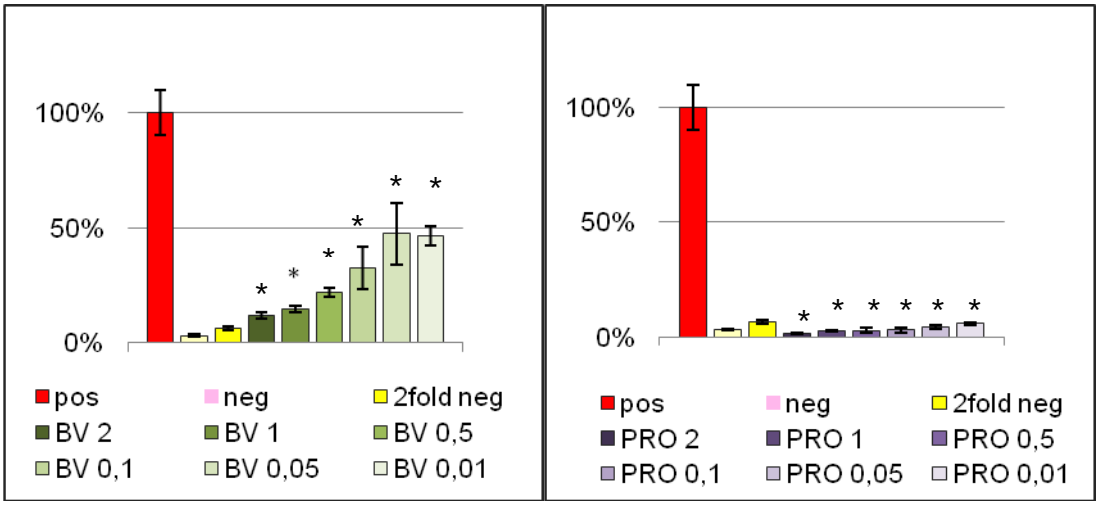

Figure 10 Colony counts after incubation with BV (left) and PRO (right), tested in *TA 98* with PhIP +S9; (positive control= 100%); * significantly different from positive values

All six tested concentrations (2.0; 1.0; 0.5; 0.1; 0.05; 0.01) of BV and of PRO, showed highly significant reductions of colony counts compared to the positive control ($p<0.01$). More precisely, BV yielded a difference in statistical significance ($p<0.05$) between all tested concentrations, except concentration BV 0.05 to BV 0.01. A highly significant difference became obvious between the highest (2.0) and the lowest (0.01) concentration of BV ($p<0.01$); so, the 2.0 concentration was more efficient in inhibition of colony formations than the 0.01 dose. The same was true for PRO ($p< 0.01$). Significant differences were also observed between PRO 0.1 to 0.05 and PRO 0.05 to 0.01 ($p<0.05$).

The ID_{50} for BV of 0.011 µmol/ml was respectably low, which means that 0.011 µmol/ml were required for a 50 percent inhibition of the mutagenic response of PhIP.

Homogeneity of variance was tested within the Tukey test. All tested concentrations of BR, BRDT and PRO showed heterogenic tendences compared to the positive control.

4.1.1.2. Mutagenicity induced by TNFone 0.0001 mg/ml

In the absence of metabolic activation BR, BV, BRDT and PRO were tested with the standard mutagen TNFone.

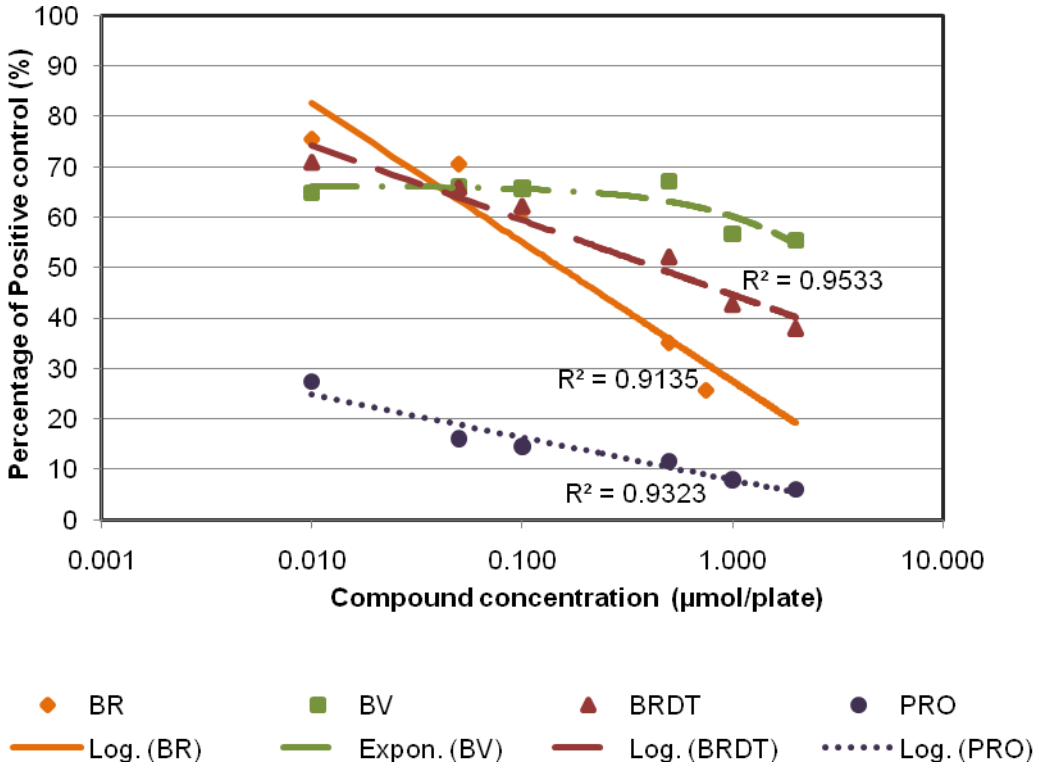

Figure 11 The modulatory effects of bilirubin (BR), biliverdin (BV), bilirubin-ditaurate (BRDT) and protoporphyrin (PRO) on TNFone induced mutagenicity in *TA 98*

Test results indicated antimutagenic trends for all tested substances. BR (0.75; 0.5) and BRDT (2.0; 1.0) passed the 50 percent line, as well as all tested concentrations of PRO. Highest tested doses of PRO (2.0; 1.0) inhibited more than 90 percent of TNFone´s induced mutagenicity and showed a clearly antimutagenic effect. BV showed the smallest potential tendencies.

Compared to the positive control, BR 0.75 and 0.5 were significantly lower ($p<0.01$; $p<0.05$); BRDT 2.0 differed also significantly from the positive control ($p<0.05$), as well as BRDT 1.0 ($p<0.01$). PRO depicted highly significant reductions of His+revertants in each tested concentration compared to the positive control ($p<0.01$). For BV, there was no significant outcome.

Test results of BR, BRDT and PRO exerted a dose dependent impact on TNFone:

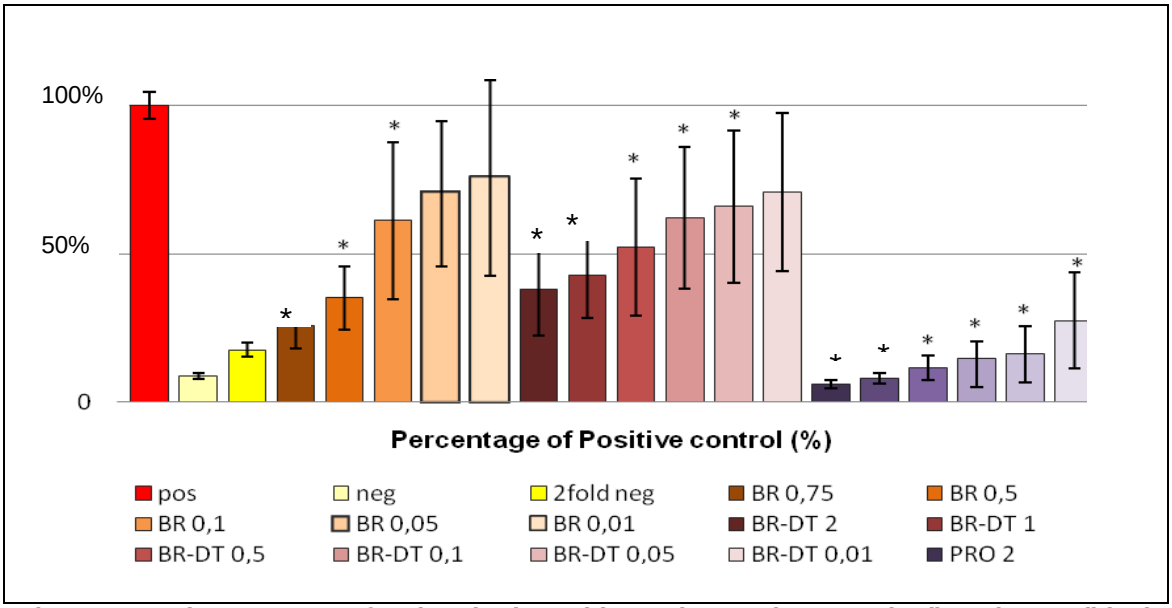

Figure 12 Colony counts after incubation with BR (orange), BRDT (red) and PRO (blue) tested in *TA 98* with TNF one; (positive control= 100%) * significantly different from positive values

Comparing the highest (0.75) with the lowest (0.01) tested concentration of BR, a highly significant difference was observed ($p<0.01$); this means that the highest concentration was more efficient in inhibition His+revertants after incubation than the lowest tested dose. The same was true for BRDT (2.0 vs 0.01; $p< 0.05$) and PRO (2.0 vs. 0.01; $p<0.05$).

Especially for BR the ID_{50} with 0.153 µmol per plate was remarkably low; for BRDT, the ID_{50} was about three times higher with 0.434 µmol per plate. That implies that two third more BRDT is required to reach the 50 percent inhibition of the mutagenic response of TNFone.

Within testing homogeneity of variance, BR depicted heterogeneity in the doses 0.75 and 0.5 compared to the positive control. BRDT showed a difference compared to the positive control in the concentrations 2.0 and 1.0. For PRO heterogeneity of variances can be considered for all tested concentrations compared to the positive control.

4.1.1.3. Mutagenicity induced by AFB_1 35µg/ml + S9

In the presence of metabolic activation and AFB_1, all compounds (except PRO) showed a non expected increase of mutagenicity.

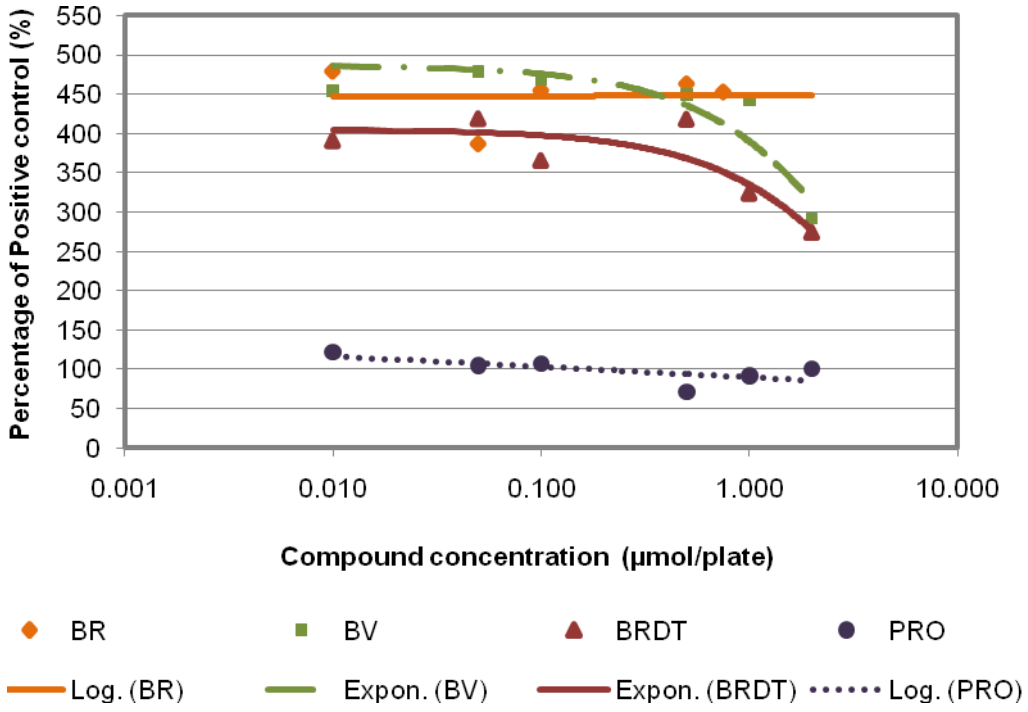

Figure 13 The modulatory effects of bilirubin (BR), biliverdin (BV), bilirubin-ditaurate (BRDT) and protoporphyrin (PRO) on AFB_1 induced mutagenicity in *TA 98*

Each of the Ames Test trials was repeated two times on different lab days. In total, the test results of *TA 98* induced mutagenicity by AFB_1 are uncommonly high and indefinably pronounced increase of mutagenicity in the presence of S9. Similar results cannot be found in literature.

Nevertheless, repetitions of the approach confirm these surprisingly high values.

Previously Bulmer *et al.* (2007) tested BR, BRDT and BV in the absence of metabolic activation and confirmed that all tested compounds inhibited the TNFone induced mutagenicity in a dose dependent manner.

Similar results were shown in our study. We confirmed antimutagenic tendencies in all 4 tested substances (BR, BRDT, BV, PRO) while BV showed the lowest and PRO the highest effect. Compared to the study of Bulmer *et al*., BR and BR-DT equally exerted a dose dependent impact on TNFone. However, a dose dependent manner of BV was not confirmed in our study.
Taken these two studies together, data support the hypothesis of an antimutagenic potential of BR, BRDT; for BV, a great antimutagenic trend was confirmed. Due to the fact, that PRO has been not tested before; more studies will be needed in the future to reaffirm these results, however, PRO showed the most promising results.

Bulmer *et al.* (2007) also tested the mutagens 2-aminofluorene (2- AF, which is found in the cooking process) and benzo [a]pyrene (B[a]P belongs to the group of mutagens induced from combustion) in the presence of S9 and found for BR, BRDT and BV antimutagenic effects. Especially, against B[a]P, all substances showed a dose dependent reduction of colony formation. Romert *et al.* (1994) and Camoirano *et al.* (1994) also tested bile pigments in the test in the presence of S9 and found antimutagenic effects against cigarette smoke (CS), a cigarette smoke condensate (CSC) and 4-nitropunoline-1-oxide (4NQO, a mutagen derived from the processes of combustion).

The results of Arimoto *et al.* (1980) also confirmed antimutagenic effects of bile pigments in the Ames test, tested against B[a]P and concluded that BR was more effective than BV. A further study of Arimoto *et al.* in 1995 showed weak antimutagenic effects of BR and BV against B[a]P metabolites.

However, the recently published study of Bulmer *et al.* (2007) presented oppositional data: BV was the most effective pigment in the same condition as

mentioned above. The theory that among this condition, pigments are capable of directly binding to B[a]P is speculative. Since data are limited on B[a]P and bile pigments clear mechanistic conclusions cannot be drawn [BULMER et al., 2008].

Data on the anti-mutagenic behaviour of bile pigments against AFB_1 and PhIP, two mutagens which are formed after processing or cooking of foods, were not found in Ames Test specific literature; for instance a recently published study of Mosovska *et al.* (2010) investigated gentoxic and antimutagenic acitivities of extracts of pseudocereals in the Ames Test and came to the result that extracts of amaranth, sorghum and Japanese millet were effective to inhibit the mutagenic response to AFB_1. Several studies on the behaviour of AFB_1 were also published in cell studies, therefore, in the future, according to the literature currently available, mechanistic studies along the field of bile pigments are needed.

4.1.2. TA 102

4.1.2.1. Mutagenicity induced by AFB_1 100µg/ml +S9

Antimutagenic effects of BR, BV, BRDT and PRO were tested against AFB_1 in the presence of metabolic activation.

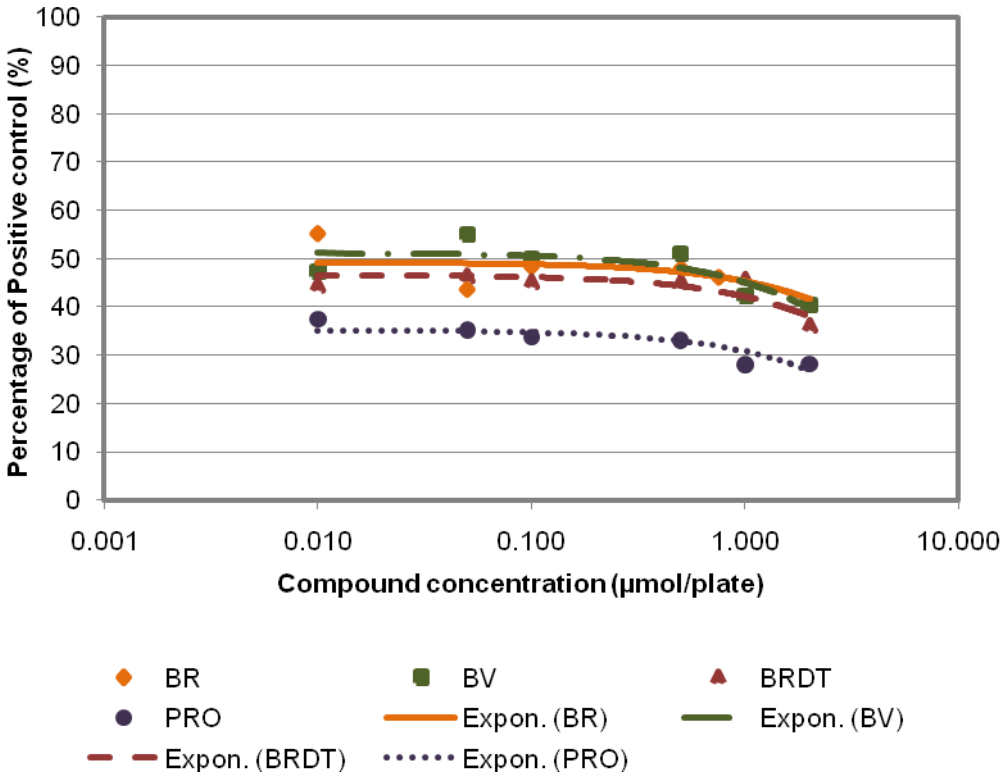

Figure 14 The modulatory effects of bilirubin (BR), biliverdin (BV), bilirubin-ditaurate (BRDT) and protoporphyrin (PRO) on AFB_1 induced mutagenicity in *TA 102*

The inhibition curves of all pigments passed the 50 percent line, except BR 0.01, BV 0.5 and BV 0.05. Therefore a highly antimutagenic effect was observed in this approach. Again the greatest antimutagenic effect was clearly showen by PRO in all tested concentrations as demonstrated in figure 14.

Compared to the positive control, a highly significant reduction of colony formation of all tested concentrations of BR, BV, BRDT and PRO was observed ($p<0.01$).

In the Tukey- test, all tested concentrations of BR, BV, BRDT and PRO showed heterogenic variances compared to the positive control.

4.1.2.2. Mutagenicity induced by TNFone 0.07 mg/ml

Since TNFone needs no activation, BR, BV, BRDT and PRO were tested without addition of the rat liver homogenate.

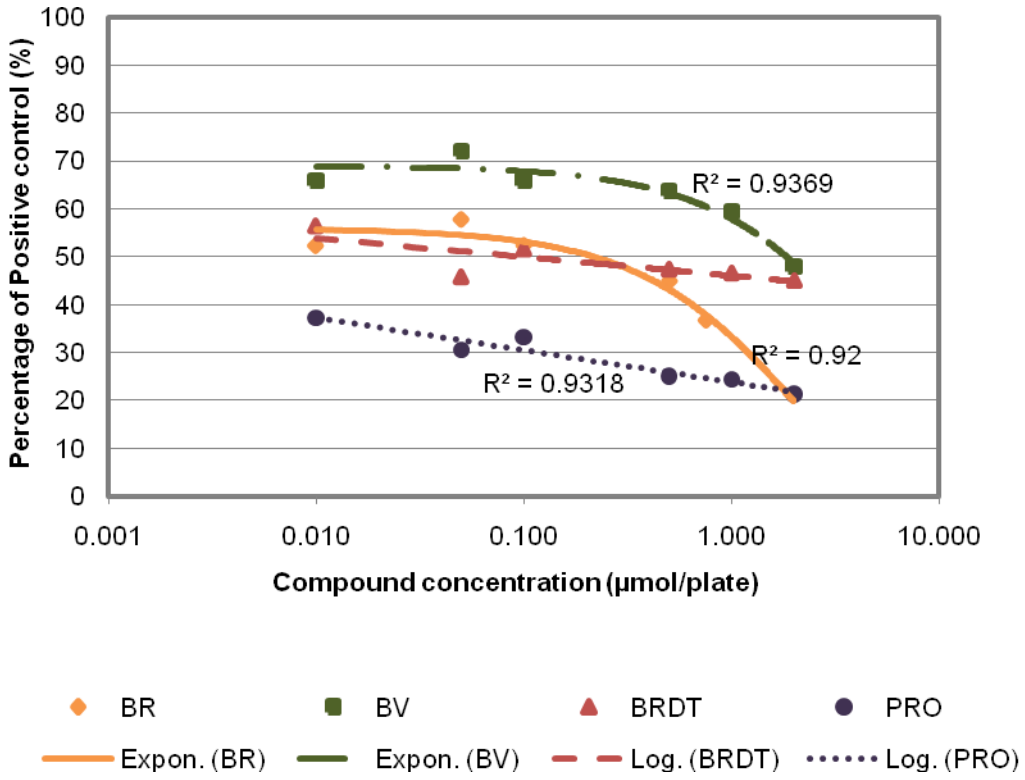

Figure 15 The modulatory effects of bilirubin (BR), biliverdin (BV), bilirubin-ditaurate (BRDT) and protoporphyrin (PRO) on TNFone induced mutagenicity in *TA 102*

All four compounds illustrated antimutagenic effects within the test regimen. Highest tested concentrations of BR (0.75; 0.5; 0.1), BV (2.0) and BRDT (2.0; 1.0; 0.5) passed the 50 percent line. Once more, PRO was the most effective substance; all tested concentrations passed the 50 percent line and were observed to prevent TNFone- induced mutagenicity, as depicted in figure 15.

All tested concentrations of BR, BRDT and PRO were very effective in reducing His+ revertants and showed highly significant reductions compared to the positive control ($p<0.01$). For BV, only the 0.05 concentration was not significantly different.

Within testing homogeneity of variance, heterogeneity of variances was considered for all tested concentrations of BR, BV, BRDT and PRO.

Equal to our study, the recently published study of Bulmer *et al.* (2007) examined TNFone induced mutagenicity in *TA 102* related to BR, BRDT and BV. The results of Bulmer *et al*. showed that all tested bile pigments were antimutagenic in a dose dependent manner. Compared to our study, similar effects were detected and assumed antimutagenic effects in these series of tests were reconfirmed. All of our tested pigments (BR, BV, BR-DT and PRO) passed the 50 percent inhibition line.
Thus we can conclude that within our study we can confirm results by Bulmer *et al.* (2007) in their studies and, we contribute to the evident positive potential of these colourful compounds.

In our present study, a further series of tests were performed against the mutagenicity of AFB_1+S9 with the result of highly antimutagenic potential of BR, BV, BRDT and PRO. All substances passed the 50 percent inhibition line with highest effects for PRO.

4.2. Antioxidative Testing

4.2.1. TA 102

4.2.1.1. Antioxidative effects in tests with *t*-BOOH 0.75*10^-6 µmol/plate

In the presence and absence of metabolic activation, BR, BV, BRDT and PRO were tested for their efficacy to prevent oxidative damage induced by *t*-BOOH (see figure 16).

Without enzymatic activation, all four compounds showed an antioxidative trend; moreover PRO was able to pass the 50 percent inhibition line at the highest concentration tested.

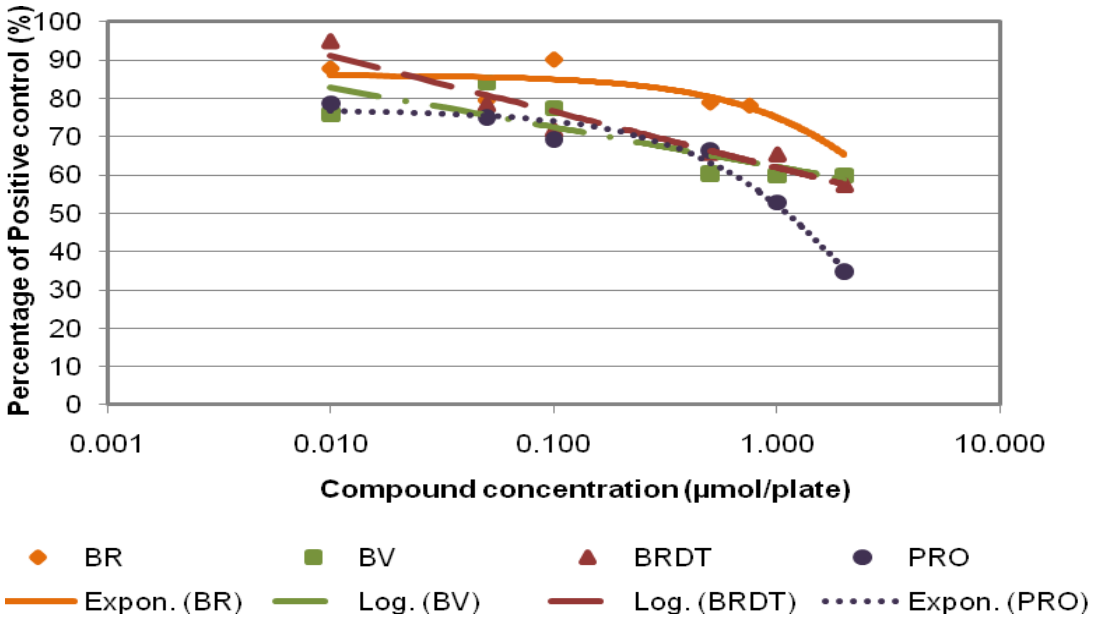

Figure 16 The modulatory effects of bilirubin (BR), biliverdin (BV), bilirubin-ditaurate (BRDT) and protoporphyrin (PRO) on *t*-BOOH induced antioxidativity in *TA 102*

Compared to the positive control, a highly significant reduction ($p<0.01$) of BV and BRDT was observed in following concentrations: 2.0; 1.0; and 0.5.

PRO reduced highly significant the numbers of His+revertants in all six tested concentrations ($p<0.01$), except the lowest one of 0.01.

Under this condition, BRDT and PRO inhibited the *t*-BOOH induced oxidation in a dose-dependent manner as seen in the figures below:

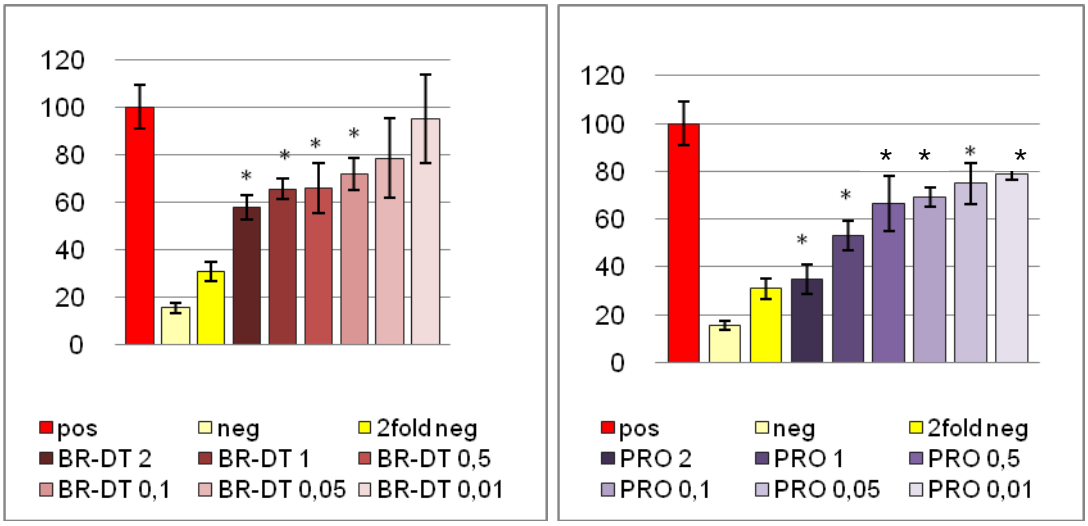

Figure 17 Colony counts after incubation with BRDT (red) and PROT (blue) in *TA 98* with *t*-BOOH; (positive control=100%)* significantly different from positive values

For both compounds the 2.0 concentration was significantly more efficient than the lowest concentration of 0.01 ($p<0.01$). Compared to the positive control tested homogeneity of variance showed significant values for BV and BRDT in concentrations 2.0, 1.0 and 0.5, as well for BRDT 0.1. PRO showed significant differences in all tested concentrations.

4.2.1.2. Antioxidative effects in tests with *t*-BOOH 0.75*10^-6 µmol/plate +S9

When metabolic activation was applied, the pigments lost their antioxidant effects. BRDT, BV and PRO showed an increase in the number of mutants and exceeded the 50 percent line.

PRO was the compound with the highest increase; all tested concentrations passed the 100 percent line, except the 0.01 concentration. PRO 1.0 was more than 20 percent higher than the positive control. No effect was shown for BR.

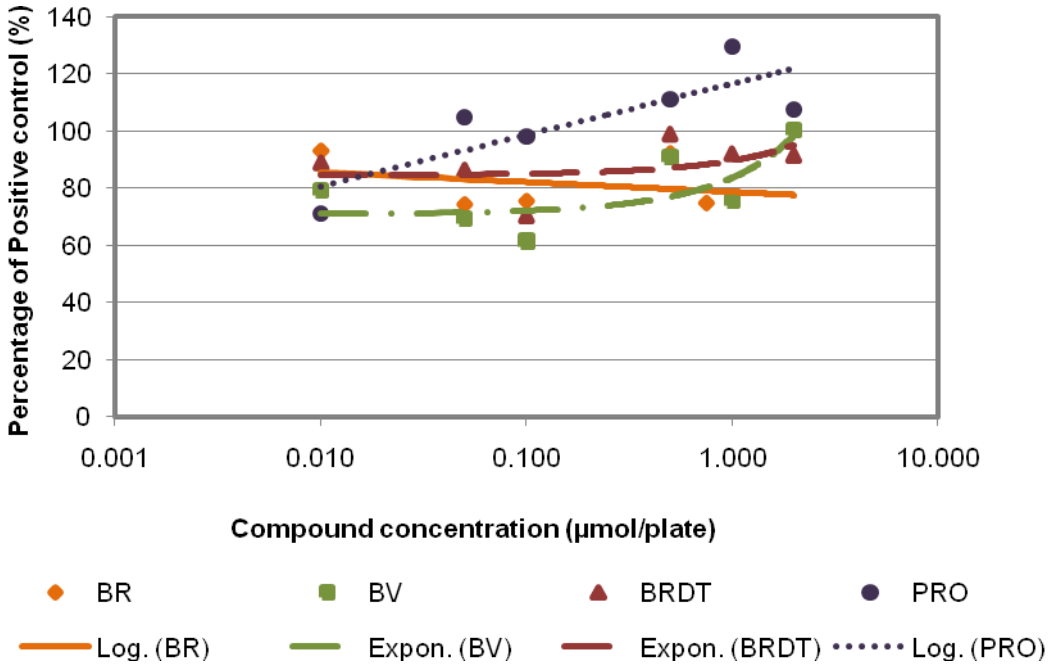

Figure 18 The modulatory effects of bilirubin (BR), biliverdin (BV), bilirubin-ditaurate (BRDT) and protoporphyrin (PROT) tested with *t*-BOOH + S9

Only BV 0.1 showed significant differences to the positive control ($p<0.05$).
No significant differences of BR, BRDT and PRO were found.

Bulmer *et al.* (2007) investigated the potential of BR, BV and BRDT in reducing *t*- BOOH induced oxidation in *TA 102*. This study was a premiere; investigating the effects of bile pigments under such condition has never been done before according to literature. The outcome was that BR and BV showed a dose-dependent inhibitory effect of mutagenicity in the absence of the rat liver homogenate; for BRDT, no effect was confirmed.
However, the here presented findings differ from the study of Bulmer *et al.* (2006). Our results showed that all tested substances (BR, BV, BRDT, PRO) indicated an antioxidative trend, BRDT and PRO in a dose dependent manner. But only PRO showed the clearest antioxidant effect and passed the 50 percent inhibition line at all tested concentrations in the TA 98 test against induced mutations of PhIP and TNFone, as well in the TA 102 test against induced mutagenicity of AFB_1 and TNFone.

Bulmer *et al.* (2007) also investigated antioxidative effects in the presence of metabolic activation and similar results were found. Summing up the results of Bulmer *et al.,* BR and BV inhibited mutagenicity induced by *t*-BOOH in a dose dependent manner; BRDT showed no effect. These findings were contrary to our results, because we found no antioxidative effect in this approach

Due to the fact that the study of Bulmer *et al.* (2007) and the here presented study found different results, further research work is needed to fill the lack of knowledge and to produce clearly evidenced data in the future.

4.3. Concluding considerations of antimutagenic/ antioxidative testing

Bulmer *et al.* (2008) reviewed in their paper that the antimutagenic effects of bile pigments were mainly dependent on the tested mutagen.

Tables 8-10 are summarizing our findings of *TA 98* and *TA 102:*

	AFB_1 (+S9)	**TNFone**	**PhIP (+S9)**
Bilirubin	-	↓	-
Biliverdin	-	-	↓↓↓
Bilirubin-ditaurate	-	↓	-
Protoporphyrin	-	↓↓↓	-
	- ID_{50} not calculable ↓ 50% inhibition of positive control at 2.0 µmol/plate (0.75 µmol/plate for BR) ↓↓ 50% inhibition of positive control between 1.0 and 0.1µmol/plate and at 0.01 µmol/plate ↓↓↓ 50% inhibition of positive control at each tested concentration		

Table 8 Modulatory effects of bilirubin, biliverdin, bilirubin-ditaurate, protoporphyrin in *Salmonella typhimurium* strain *TA98*

	AFB_1 (+S9)	TNFone	*t*-BOOH (-S9)	*t*-BOOH (+S9)
Bilirubin	-	↓	-	↑
Biliverdin	↓	-	-	-
Bilirubin-ditaurate	-	↓	-	-
Protoporphyrin	-	↓↓↓	↓	↑

- ID_{50} not calculable
↓ 50% inhibition of positive control at 2.0 µmol/plate (0.75 µmol/plate for BR)
↓↓ 50% inhibition of positive control between 1.0 and 0.1µmol/plate and at 0.01 µmol/plate
↓↓↓ 50% inhibition of positive control at each tested concentration
↑ no inhibition of positive control

Table 9 Modulatory effects of bilirubin, biliverdin, bilirubin-ditaurate and protporphyrin in *Salmonella typhimurium* strain *TA102*

Due to the weak solubility of bile pigments in the lab, Bulmer *et al.* (2007) suggested that it would be worth to try albumin-bound BR instead of BR alone. Albumin-bound BR is a more water-soluble complex which makes it easier to investigate higher concentrations of BR.

Evidently, in both series of tests in *TA 98* and *TA 102* PRO showed the highest antimutagenic effects, almost always in a dose dependent manner. These results should be seen as a motivation for researchers to investigate this substance more detailed in future.

Finally, the following table shows our results, for the ID_{50} and $ID_{0.75}$ inhibition:

Strain	Mutagen	S9	Compound	ID_{50} (µmol/plate)	$ID_{0.75}$ (%)
TA 98	PhIP 0.1µmol/ml	+	BR	-	36
			BV	0.011	19
			BRDT	-	32
			PROT	-	32
TA 98	TNF one 0.0001 mg/ml	-	BR	0.153	31
			BV	-	-
			BRDT	0.434	46

			PROT	0.000010	9
TA98	AFB_1 35 ug/ml	+	BR	-	-
			BV	-	-
			BRDT	-	-
			PROT	-	-
TA 102	AFB_1 100 µg/ml	+	BR	-	46
			BV	0.184	47
			BRDT		43
			PROT		32
TA 102	TNF one 0.07 mg/ml	-	BR	0.216	38
			BV	-	-
			BRDT	0.096	46
			PROT	0.00012	25
TA 102	BOOH 0.75*10^-6 µmol/plate	-	BR	-	-
			BV	-	-
			BRDT	-	-
			PROT	1.119	57
TA 102	BOOH 0.75*10^-6 µmol/plate +S9	+	BR	0.223	-
			BV	-	-
			BRDT	-	-
			PROT	0.00020	-

Table 10 The modulatory effects of BR (bilirubin), BV (biliverdin), BRDT (bilirubin-ditaurate) and PROT (protoporphyrin) on genotoxicity in *TA 98* and *TA 102 Salmonella typhimurium* strains. ID $_{50}$, dose of compound required for 50% inhibition; ID $_{0.75}$, percent inhibition at 0.75 µmol/plate

4.4. Mutagenic Testing

Mutagenic testing was performed in order to support our tested anitmutagenic/ antioxidative results of BR, BV, BRDT and PRO and further to investigate whether the tested bile pigments do have mutagenic activity. The test procedure was identical to the antimutagenic test series, except that no positive control was added to the test tubes.

In general, it can be concluded, that all results in the mutagenic testing procedure were nearby the 100 percentage line. Neither significant increases nor obvious reductions were observed after testing the mutagenicity/oxidative behaviour of these compounds.

A comprehensive overview about all tested values and counted single revertant numbers are listed in chapter nine (Appendix).

4.4.1. TA 98

Within the test procedure, BR, BV, BRDT and PRO were tested against the mutagens PhIP, TNFone and AFB_1.

For all mutagens, all tested compounds were highly significant lower than the double negative control ($p<0.01$).

4.4.2. TA 102

Bacterial strain was tested against TNFone, AFB_1 +S9 and *t*-BOOH with and without metabolic activation. Equally to the mutagenic testing in TA 98, for all mutagens, all tested compounds were significantly lower compared to the double negative control ($p<0.01$).

5. CONCLUSION

In general, the area of bile pigments is a relatively new field of research which became more and more attractive in the last two decades. Although published data is still rare, the theory of a physiological relevance of bile pigments is growing in the past years with the suggestion that bile pigments possess preventative potential, including anti-complement, anti-inflammatory anti-viral, antimutagenic and antioxidative properties, as well antioxidative capacity towards oxidation of lipids and proteins [BULMER et al., 2008].
The thesis at hand deals with the potential protective and beneficial role of bile pigments and is especially focused on the theory of possible antimutagenic and antioxidative properties of bilirubin, biliverdin, bilirubin-ditaurate and protoporphyrin. In recently published studies (*e.g*. BULMER, 2008; BULMER *et al.*, 2007; STOCKER, 2004), these antimutagenic and antioxidative effects of bile pigments were already found.

Aim of this thesis was to support these effects. Therefore, the *Salmonella* Ames test was used as test model, which is a popular method to detect relative simply mutagens/antimutagens. Antimutagenic and antioxidative properties of bilirubin, biliverdin, bilirubin-ditaurate and protoporphyrin were tested in different concentrations (2.0- 0.01) with selected groups of mutagens (TNFone, PhIP, *t*-BOOH, AFB_1) in presence and absence of metabolic activation. For the test procedure, *Salmonella typhimurium* indicator strains *TA 98* and *TA 102* were used. The experiments were aimed to investigate antimutagenic properties in both tester strains; possible antioxidative properties were investigated with and without the S9 mix by using *TA 102* as tester strain for *t*-BOOH induced oxidative mutations.

In general, our test results showed different antimutagenic and antioxidative effects and tendencies in both bacterial strains. Therefore, we were able to confirm the theory of the antimutagenic and antioxidative potential of these specific bile pigments.

Reconsidering the test series with *TA 98* against PhIP and TNFone induced mutagenicity, there is every indication of antimutagenic effects in both test. All four substances significantly inhibited PhIP induced mutagenicity compared to the positive control in all tested concentrations, PRO and BV in a dose dependent manner. The TNFone test resulted in a significant reduction of His+revertants of all PRO concentrations. The same significant reduction was true for highest tested doses of BR (0.75; 0.5) and BRDT (2.0; 1.0). A dose-dependency was shown for BR, BRDT and PRO. Under this condition we could confirm published data of Bulmer *et al.* (2007) because they showed as well a dose dependent manner of BR and BRDT against TNFone induced mutagenicity. Taken these two tests together, the greatest antimutagenic effect was observed by PRO, which passed plainest the 50 percent inhibition line. Since similar tests of PRO of this condition could not be found in literature, further test series are needed to confirm these promising results.
Unexpected and uncommonly high values were shown in the *TA 98*-test against AFB_1 in presence of metabolic activation. This non expected increase in mutagenicity was confirmed in two test reruns. Comparable results cannot be found in literature. Results of test procedures with bacterial strain *TA 102* against AFB_1+S9 and TNFone induced mutagenicity suggested also antimutagenic effects. Compared to the positive control, all four test substances showed significant reductions of His+revertants in the test series against AFB_1 induced mutagenicity. The same was true for BR, BV (except the 0.05 concentration) and PRO in the TNFone test. Interestingly, ineffectiveness of test samples became apparent in the *TA 102*-test with *t*-BOOH+S9, while the test without metabolic activation showed antioxidative trends of all compounds. Therefore we can conclude that the S9 mix has an effect on bile pigments to neutralize antioxidative effects.

These findings give interesting insights in possible modes of action of bile pigments, but no clear statement about the impact in the living organism can be made. Our findings can be considered as important observations contributing to

the indicated possible protective physiological role of bile pigments and the understanding and the mechanism behind the bile pigments.

In future, it must be the aim to stimulate further research work in this area to confirm already published studies and to gain information of the functionality of bile pigments more detailed. The clinical use of BR as a possible therapeutical agent to prevent several diseases is imaginable and must be the long-time objective for scientists.

6. SUMMARY

The objective of the present sub-study of an FWF project was to investigate possible antimutagenic and antioxidative effects of bile pigments.
The *Salmonella* /microsome mutagenicity assay was used as well established model to identify mutagenic potential of chemicals and drugs.

Biliverdin, bilirubin-ditaurate and protoporphyrin were tested in six (2.0; 1.0; 0.5; 0.1; 0.05; 0.01), BR was used in five (0.75; 0.5; 0.1; 0.05; 0.01) concentrations in the presence and absence of S9. Different groups of mutagens were used (TNFone, AFB_1 and PhIP) as known mutagens to test the antimutagenic potential of these selected pigments; *t*-BOOH was used to gain information about their antioxidative potential.
In general, our results indicate that all substances possess antimutagenic properties in the chosen range of concentrations, except mutagenicity induced by AFB_1 in the bacterial strain *TA 98* which showed an uncommonly and indefinably pronounced increase of mutagenicity in the presence of S9.
More precisely, all four compounds tested with PhIP+ S9 and TNFone showed antimutagenic tendencies and effects. The highest effect was clearly apparent in PRO. In both experiments all four tested substances were able to reduce the induced mutagenicity. Antioxidative effects of all four compounds were confirmed tested against *t*-BOOH. In the presence of metabolic activation, interestingly, no effect was observed in all of the tested pigments.

To conclude, our results showed considerable antimutagenic and antioxidative effects, trends and tendencies. Therefore our results contribute to the theory that bile pigments possess positively modulating and beneficial properties *in vitro*. Our findings recommend further research work to get more detailed information on the preventative potential of bile pigments.

7. ZUSAMMENFASSUNG

Ziel der vorliegend Arbeit war es, die möglichen antimutagenen und antioxidativen Eigenschaften von Gallenfarbstoffe zu untersuchen und zu beweisen.

Als Methode wurde der Ames Test gewählt, ein international einheitliches, anerkanntes und populäres Testverfahren um Chemikalien, Pharmaka und Biozide hinsichtlich ihres anti-mutagenen/anti-oxidativen Potentials zu bewerten. Analysiert wurden die Substanzen Bilirubin, Biliverdin und Bilirubin-Ditaurat, ein synthetisches wasserlösliches Bilirubinconjungat, sowie Protoporphyrin, eine Vorläufersubstanz von Häm, mit und ohne metabolische Aktivierung in unterschiedlichen Konzentrationen. Biliverdin, Bilirubin-Ditaurat und Protoporphyrin wurden in sechs verschiedenen Konzentrationen getestet (2.0; 1.0; 0.5; 0.1; 0.05; 0.01), Bilirubin wurde in fünf verschiedenen Konzentrationen untersucht (2.0; 1.0; 0.5; 0.1; 0.05; 0.01).
Zu Analysezwecken kamen 2 verschiedene Bakterienstämmen von *Salmonella typhimurium* zum Einsatz. Als Mutagene wurden 2-4-7 trinitrofluenon (TNFone), Tertiär-Butylhydroperoxid (*t*-BOOH), Aflatoxin B_1 (AFB_1) und 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) gewählt.

Zusammengefasst lieferten die Tests folgende Resultate:
Alle vier getesteten Substanzen zeigten in den *TA 98* Tests mit PhIP und TNFone+S9 deutliche antimutagene Wirkung. Portoporphyrin zeigte in beiden Experimenten die stärksten antimutagenen Aktivitäten. Als deutlich mutagen im *TA 98* Test erwies sich jedoch AFB_1+S9. Trotz mehrmaliger Wiederholung dieser Testreihe bestätigten sich überraschenderweise diese ungewöhnlich hohen Werte. Weitere Analysen bestätigten ebenfalls das antimutagene Potential der getesteten Substanzen. In *TA 102* Testreihen mit AFB_1+S9 und TNFone zeigten alle Substanzen antimutagene Tendenzen und Effekte. Protoporphyrin zeigte abermals die stärksten antimutagenen Effekte.

Die antioxidativen Eigenschaften der Testsubstanzen wurde mit dem Mutagen *t*-BOOH mit und ohne metabolische Aktivierung untersucht. Konträre Resultate konnten beobachtet werden; während alle Substanzen ohne metabolische Aktivierung antioxidative Wirkung zeigten, waren in Testreihen mit metabolischer Aktivierung alle Substanzen inaktiv.

Zusammengefasst kann man sagen, dass diese Studie antimutagene und antioxidative Tendenzen und Effekte der Gallenfarbstoffe bestätigt hat. Um die physiologische Rolle sowie das Verständnis und die Funktionalität der Gallenfarbstoffe in Zukunft besser beurteilen zu können sind weitere Experimente auf diesem Gebiet sinnvoll um auch bereits bewiesene protektive Fähigkeiten der Gallenfarbstoffe zusätzlich zu bekräftigen.

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9. APPENDIX

9.1. Antimutagenicity tests with *TA 98*

9.1.1. Single revertant numbers: *TA 98* + TNFone 0.3*10^{-6} µmol/plate

Table 11

Sample		Concentration (µmol/plate)						
		2.0	**1.0**	**0.75**	**0.5**	**0.1**	**0.05**	**0.01**
BR	Assay I 15.4.09			84	116	192	232	277
				74	106	220	223	240
				80	103	217	228	260
	Assay II 22.4.09			51	54	83	111	126
				51	56	104	118	106
				40	85	89	125	108
mean				63	87	151	173	186
sd				18.3	26.5	65.5	60.3	80.9
BV	Assay I 15.4.09	248	159		286	211	203	211
		198	197		260	241	218	206
		153	214		210	212	225	188
	Assay II 22.4.09	81	89		61	95	120	100
		79	82		101	114	117	134
		62	98		76	100	96	120
mean		137	140		166	162	163	160
sd		75.4	58.0		98.5	66.0	58.2	47.7
BR-DT	Assay I 15.4.09	137	149		153	226	209	235
		105	127		201	203	230	227
		138	135		175	187	219	240
	Assay II 22.4.09	65	67		106	93	96	118
		60	73		80	97	121	128
		57	81		56	113	100	100
mean		94	105		129	153	163	175
sd		38.1	35.7		56.8	58.9	63.2	65.7
PROT	Assay I 15.4.09	11	16		17	25	18	30
		12	17		21	25	23	36
		14	17		22	20	16	28
	Assay II 22.4.09	18	18		35	49	55	107
		17	24		33	48	63	107
		19	27		44	49	64	98
mean		15	20		29	36	40	68
sd		3.3	4.5		10.3	14.0	23.1	40.0

		mean	*sd*
Pos	285/306/303/200/192/195	247	11.4
Neg	21/27/24/20/21/21/23/22	22	2.8
2xneg	42/54/48/40/42/42/46/44	44	5.5

9.1.2. Single revertant numbers: *TA 98* + AFB_1 $0.8*10^{-7}$ µmol/plate +S9

Table 12

Sample		Concentration (µmol/plate)						
		2.0	**1.0**	**0.75**	**0.5**	**0.1**	**0.05**	**0.01**
BR	Assay I 23.6.09			1696	1797	1682	1573	1313
				1794	1884	1427	1595	1567
				1763	1767	1752	1503	1736
	Assay II 6.7.09			1244	1549	1260	1160	1730
				1462	1488	1779	956	1702
				1316	1000	1412	1146	1761
mean				1546	1581	1552	1322	1635
sd				237.6	322.6	214.0	268.9	172.0
BV	Assay I 23.6.09	921	1700		1677	1457	1464	1563
		975	1645		1664	1637	1937	1564
		861	1510		1631	1992	1214	1542
	Assay II 6.7.09	1106	1250		1275	1728	1601	1632
		974	1517		1322	1384	1826	1547
		1130	1432		1636	1381	1760	1454
mean		995	1509		1534	1597	1634	1550
sd		104.7	160.0		183.9	239.3	264.9	57.2
BR-DT	Assay I 23.6.09	1075	1175		1433	1396	1547	1434
		817	1214		1527	1354	1673	1562
		924	1189		1484	1233	1654	1498
	Assay II 6.7.09	1107	1106		1678	1047	1309	1317
		819	960		1179	974	1061	1012
		858	974		1246	1467	1316	1165
mean		933	1103		1425	1245	1427	1331
sd		128.5	111.4		184.7	198.4	239.4	210.4
PROT	Assay I 23.6.09	264	258		358	399	521	458
		341	389		250	352	245	398
		442	254		353	383	563	528
	Assay II 6.7.09	462	337		242	365	287	353
		391	323		148	358	275	418
		157	323		108	332	254	345
mean		343	314		243	365	358	417
sd		115.9	51.1		102.6	23.6	144.3	68.8

		mean	*sd*
Pos	304/354/332/326/367/364/	341	24.7
Neg	36/39/46/39/36/33/27/39/31/31/43/	36	5.4
2xneg	72/78/92/78/72/66/54/78/62/62/86	73	10.7

9.1.3. Single revertant numbers: *TA 98* + PhIP 0.1*10^{-7} µmol/plate +S9

Table 13

Sample		Concentration (µmol/plate)						
		2.0	**1.0**	**0.75**	**0.5**	**0.1**	**0.05**	**0.01**
BR	Assay I 15.6.09			405	510	542	449	377
				385	383	368	379	370
				510	380	343	380	378
	Assay II 16.6.09			497	366	313	349	395
				363	354	615	379	580
				367	379	361	376	390
mean				421	395	424	385	415
sd				65.6	57.2	123.6	33.4	81.3
BV	Assay I 15.6.09		162		228	238	493	496
		117	150		248	350	469	487
		135	149		255	533	494	490
	Assay II 16.6.09	123	166		218	312	826	506
		132	183		275	437	390	573
		165	186		263	325	526	590
mean		134	166		248	366	533	524
sd		18.5	15.8		21.5	104.1	150.7	45.6
BR-DT	Assay I 15.6.09	259	374		473	367	405	369
		337	306		369	356	304	320
		295	443		418	343	318	325
	Assay II 16.6.09	293	370		483	333	472	362
		330	424		480	298	444	279
		354	367		533	424	366	340
mean		311	381		459	354	385	333
sd		35.1	48.3		57.4	41.9	67.6	32.7
PROT	Assay I 15.6.09	15	28		53	47	53	59
		17	30		21	41	68	60
		17	28		26	19	53	74
	Assay II 16.6.09	18	20		30	31	37	67
		13	28		29	35	45	69
		15	26		23	25	38	72
mean		16	27		30	33	49	67
sd		1.8	3.5		11.6	10.3	11.6	6.2

		mean	*sd*
Pos	1135/920/1143/1111/1238/1199	1124	110.4
Neg	36/33/35/34/30/38/47/40/43/31/32/37	36	5.1
2xneg	72/66/70/68/60/76/94/80/86/62/64/74	73	10.1

9.2. Antimutagenicity tests with *TA 102*

9.2.1. Single revertant numbers: *TA 102* + TNFone 0.2*10^{-7} µmol7plate

Table 14

Sample		Concentration (µmol/plate)						
		2.0	**1.0**	**0.75**	**0.5**	**0.1**	**0.05**	**0.01**
BR	Assay I 13.4.09			453	467	598	607	602
				425	440	561	583	575
				416	459	501	487	443
	Assay II 27.4.09			425	500	718	760	681
				382	564	615	738	521
				314	526	459	632	622
mean		403	493	575	635	574	403	493
sd		49.0	46.5	91.3	101.6	83.1	49.0	46.5
BV	Assay I 13.4.09	490	519		622	496	641	696
		497	517		629	679	708	731
		391	458		565	688	779	556
	Assay II 27.4.09	552	728		674	814	847	824
		623	762		833	799	879	857
		595	920		870	862	880	666
mean		525	651		699	723	789	722
sd		83.9	180.7		123.8	132.7	98.3	109.6
BR-DT	Assay I 13.4.09	476	387		519	523	360	519
		489	464		390	513	426	522
		465	406		439	440	517	575
	Assay II 27.4.09	507	537		629	556	594	718
		486	625		631	655	563	697
		533	645		504	702	548	685
mean		493	511		519	565	501	619
sd		24.2	109.8		97.9	97.0	90.0	91.2
PROT	Assay I 13.4.09	240	246		298	331	242	355
		302	253		335	336	326	402
		288	272		288	314	407	336
	Assay II 27.4.09	182	237		262	384	397	572
		196	234		240	397	363	463
		189	352		221	420	267	316
mean		233	266		274	364	334	407
sd		52.4	44.4		41.5	42.4	68.1	96.3

		mean	*sd*
Pos	983/968/972/1124/1429	1095	197.6
Neg	353/269/278/348/298/316/367/262/381	319	44.8
2xneg	706/538/556/696/596/632/734/524/762	638	89.5

9.2.2. Single revertant numbers: *TA 102* + AFB_1 $0.24*10^{-6}$ µmol/plate

Table 15

Sample		Concentration (µmol/plate)						
		2.0	**1.0**	**0.75**	**0.5**	**0.1**	**0.05**	**0.01**
BR	Assay I 15.6.09			627	603	488	463	825
				612	645	573	511	762
				660	679	657	445	496
	Assay II 15.6.09			615	679	739	533	698
				612	691	748	753	734
				527	496	623	747	849
mean				609	632	638	575	727
sd				44.1	74.0	99.6	139.0	126.5
BV	Assay I 15.6.09	595	526		714	568	866	691
		506	528		751	757	714	735
		591	449		503	647	649	503
	Assay II 15.6.09	482	604		631	659	711	620
		492	591		717	709	732	584
		530	652		724	617	682	609
mean		533	558		673	660	726	624
sd		49.5	71.9		92.7	66.8	74.7	81.6
BR-DT	Assay I 15.6.09	457	641		591	510	417	353
		505	668		586	438	534	491
		391	601		432	503	522	650
	Assay II 15.6.09	488	615		644	705	714	602
		536	677		647	727	773	806
		498	423		689	703	726	627
mean		479	604		598	598	614	588
sd		50.2	93.5		90.1	127.7	142.5	153.4
PROT	Assay I 15.6.09	306	291		365	457	424	409
		331	273		322	453	445	432
		293	229		357	464	462	428
	Assay II 15.6.09	393	533		563	442	515	513
		457	416		568	470	558	644
		452	472		445	392	382	543
mean		372	369		437	446	464	495
sd		72.6	122.2		107.6	28.3	63.4	90.2

		mean	*sd*
Pos	1456/1413/1553/1122/1142/1235	1320	178.6
Neg	397/364/342/387/377/382/327/313/369/324/ 297/251	344	43.4
2xneg	794/728/684/774/754/764/654/626/738/648/ 594/502	688	86.8

9.2.3. Single revertant numbers: *TA 102* + *t*-BOOH 0.75*10^{-6} µmol/plate

Table 16

Sample		Concentration (µmol/plate)						
		2.0	**1.0**	**0.75**	**0.5**	**0.1**	**0.05**	**0.01**
BR	Assay I 10.5.09			1979	2005	2171	1738	2294
				1648	1717	1883	1960	2419
				2276	1738	2806	1766	2348
	Assay II 11.5.09			2081	2280	2249	1902	2358
				1719	1831	2063	2299	1820
				1793	2031	2069	2035	1660
mean				1916	1934	2207	1950	2150
sd				239.4	214.6	318.3	205.1	323.9
BV	Assay I 10.5.09	1486	1333		1191	2282	1913	2033
		1426	1502		1531	2682	2292	1720
		1583	1218		920	1767	1983	1330
	Assay II 11.5.09	1194	1656		1810	1730	1929	1947
		1538	1662		1774	1343	2119	1998
		1567	1420		1640	1555	2104	2115
mean		1466	1465		1478	1893	2057	1857
sd		145.0	177.3		352.3	496.6	144.1	290.5
BR-DT	Assay I 10.5.09	1605	1597		1671	1550	1490	1949
		1487	1554		1392	1784	1639	1900
		1275	1581		1236	1923	1660	1898
	Assay II 11.5.09	1301	1785		1647	1555	1925	2841
		1340			1849	1845	2245	2757
		1437	1506		1881	1892	2555	2613
mean		1408	1605		1613	1758	1919	2326
sd		126.2	106.6		254.2	166.1	410.0	456.1
PROT	Assay I 10.5.09	937	1281		1441	1760	1550	1914
		840	957		1360	1618	1591	1885
		594	1186		1595	1548	1902	1870
	Assay II 11.5.09	994	1187		1468	1765	1986	1900
		975	1445		2130	1685	1990	1989
		773	1723		1762	1799	2003	2000
mean		852	1297		1626	1696	1837	1926
sd		152.0	262.2		284.0	97.5	209.9	54.9

		mean	*sd*
Pos	2113/2536/2641/2624/2329/	2449	224.9
Neg	318/346/256/395/420/413/406/432/398/374/375/396	377	49.8
2xneg	636/692/512/790/840/826/812/864/796/748/750/792	755	99.5

9.2.4. Single revertant numbers: *TA 102* + *t*-BOOH 0.75*10^{-6} µmol/plate +S9

Table 17

Sample		Concentration (µmol/plate)						
		2.0	**1.0**	**0.75**	**0.5**	**0.1**	**0.05**	**0.01**
BR	Assay I 30.6.09				1278	1338	849	1228
				1024	1325	1430	868	1233
				1199	1444	1102	921	1275
	Assay II 5.8.09			647	1028	645	879	1194
				917	935	777	1026	1257
				1030	1135	547	1199	1030
mean				963	1191	973	957	1203
sd				123.7	85.6	169.2	37.3	25.8
BV	Assay I 30.6.09	1093	891		1143	820	982	1121
		1114	903		1080	680	888	1017
		987	779		1190	578	946	640
	Assay II 5.8.09	1604	1129		1265	713	903	1045
		1366	1258		1138	818	1141	1200
		1609	901		1221	1154	524	1107
mean		1296	977		1173	794	897	1022
sd		68.1	68.4		55.2	121.5	47.4	253.1
BR-DT	Assay I 30.6.09	1150	904		973	841	1171	1024
		1140	932		917	687	1190	759
		938	1057		963	887	1103	773
	Assay II 5.8.09	1215	1556		1572	999	1174	1456
		1335	1247		1657	1028	974	1446
		1317	1439		1589	1007	1083	1432
mean		1183	1189		1279	908	1116	1148
sd		119.6	81.5		29.9	104.7	45.7	149.1
PROT	Assay I 30.6.09	1366	1946		1520	1382	1437	887
		1196	1763		1636	1632	1648	761
		1361	1895		1541	1462	1534	893
	Assay II 5.8.09	1421	1282		1488	984	996	754
		1470	1684		1055	1013	1392	1123
		1528	1474		1363	1111	1120	1097
mean		1390	1674		1434	1264	1355	919
sd		96.7	94.4		61.8	127.7	105.6	74.5

		mean	*sd*
Pos	1274/1056/953/1593/1372/1494/	1290	163.9
Neg	364/413/329/329/326/300/235/258/237/293/ 260/253	300	39.7
2xneg	728/826/658/658/652/600/470/516/474/586/ 520/506	600	79.4

9.3. Mutagenicity tests with *TA 98*

9.3.1. Single revertant numbers: *TA 98* + TNFone 0.3*10^{-6} µmol/plate

Table 18

Sample		Concentration (µmol/plate)						
		2.0		**0.75**		**0.01**	**0.005**	**0.001**
BR	Assay I 10.8.09			27		31		
				39		35		
				42		37		
	Assay II 11.8.09			37		30		
				46		42		
				36		42		
mean				38		36		
sd				6.4		5.2		
BV	Assay I 10.8.09	36				38		
		35				41		
		42				40		
	Assay II 11.8.09	31				44		
		25				39		
		57				32		
mean		38				39		
sd		11.0				4.0		
BR-DT	Assay I 10.8.09	42				37		
		42				28		
		52				31		
	Assay II 11.8.09	39				36		
		25				37		
		37				39		
mean		40				35		
sd		8.8				4.2		
PROT	Assay I 10.8.09	33				36	38	30
		37				41	38	43
		35				35	32	38
	Assay II 11.8.09	38				33	40	37
		32				38	42	37
		39				33	42	40
mean		36				36	39	38
sd		2.8				3.1	3.7	4.3

		mean	*sd*
Pos	392/404/403/426/398/449	412	21.5
Neg	31/50/36/31/48/33/22/28/42/34/38/51	37	9.1
2xneg	62/100/72/62/96/66/44/56/84/68/76/102	74	18.3

9.3.2. Single revertant numbers: *TA 98* + AFB_1 $0.8*10^{-7}$ µmol/plate +S9

Table 19

Sample		Concentration (µmol/plate)						
		2.0		**0.75**		**0.01**	**0.005**	**0.001**
BR	Assay I 12.8.09			31		26		
				36		38		
				32		45		
	Assay II 19.8.09			48		32		
				36		53		
				37		42		
mean				37		39		
sd				6.1		9.6		
BV	Assay I 12.8.09	33				39		
		31				33		
		30				22		
	Assay II 19.8.09	39				46		
		27				34		
		20				42		
mean		30				36		
sd		6.3				8.4		
BR-DT	Assay I 12.8.09	48				42		
		48				44		
		47				59		
	Assay II 19.8.09	46				42		
		47				51		
		45				47		
mean		47				48		
sd		1.2				6.6		
PROT	Assay I 12.8.09	43				46	30	48
		41				52	41	45
		47				48	38	47
	Assay II 19.8.09	39				38	48	48
		34				43	49	46
		42				56	49	44
mean		41				47	43	46
sd		4.3				6.4	7.7	1.6

		mean	*sd*
Pos	1056/1253/1251/1173/1259/1155	1191	79.9
Neg	39/37/49/48/46/48/39/27/25/30/49/42	40	8.7
2xneg	74/74/98/96/92/96/78/54/50/60/98/84	80	17.4

9.3.3. Single revertant numbers: *TA 98* + PhIP 0.1*10^{-7} µmol/plate +S9

Table 20

Sample		Concentration (µmol/plate)						
		2.0		**0.75**		**0.01**	**0.005**	**0.001**
BR	Assay I 12.8.09			31		26		
				36		38		
				32		45		
	Assay II 19.8.09			48		32		
				36		53		
				37		42		
mean				37		39		
sd				6.1		9.6		
BV	Assay I 12.8.09	33				39		
		31				33		
		30				22		
	Assay II 19.8.09	39				46		
		27				34		
		20				42		
mean		30				36		
sd		6.3				8.4		
BR-DT	Assay I 12.8.09	48				42		
		48				44		
		47				59		
	Assay II 19.8.09	46				42		
		47				51		
		45				47		
mean		47				48		
sd		1.2				6.6		
PROT	Assay I 12.8.09	43				46	30	48
		41				52	41	45
		47				48	38	47
	Assay II 19.8.09	39				38	48	48
		34				43	49	46
		42				56	49	44
mean		41				47	43	46
sd		4.3				6.4	7.7	1.6

		mean	*sd*
Pos	1117/1271/1255/1314/1274/1287	1253	69.5
Neg	39/37/49/48/46/48/39/27/25/30/49/42	40	8.7
2xneg	78/74/98/96/92/96/78/54/50/60/98/84	80	17.4

9.4. Mutagenicity tests with *TA 102*

9.4.1. Single revertant numbers: *TA 102* + TNFone 0.2*10^{-7} µmol/plate

Table 21

Sample		Concentration (µmol/plate)						
		2.0		**0.75**		**0.01**	**0.005**	**0.001**
BR	Assay I 17.8.09			312		584		
				202		598		
				403		600		
	Assay II 18.8.09			408		456		
				338		364		
				289		214		
mean				325		469		
sd				77.1		157.0		
BV	Assay I 17.8.09	494				579		
		487				512		
		361				364		
	Assay II 18.8.09	460				536		
		482				537		
		493				552		
mean		463				513		
sd		51.4				76.4		
BR-DT	Assay I 17.8.09	401				377		
		418				461		
		445				518		
	Assay II 18.8.09	472				446		
		519				407		
		420				430		
mean		446				440		
sd		43.6				48.4		
PROT	Assay I 17.8.09	543				447	533	347
		452				376	517	472
		427				553	484	469
	Assay II 18.8.09	456				527	572	586
		498				444	516	517
		453				435	555	458
mean		472				464	530	475
sd		41.9				65.1	31.2	78.5

		mean	*sd*
Pos	1217/1185/1245/1233/1307/1259	1241	41.2
Neg	460/473/450/422/417/389/333/399/400/407/ 456/447/	421	39.2
2xneg	920/946/900/844/834/778/666/798/800/814/ 912/894	842	78.3

9.4.2. Single revertant numbers: *TA 102* + AFB_1 $0.24*10^{-6}$ µmol/plate +S9

Table 22

Sample		Concentration (µmol/plate)						
		2.0		**0.75**		**0.01**	**0.005**	**0.001**
BR	Assay I 19.8.09			257		401		
				280		472		
				275		421		
	Assay II 20.8.09			201		322		
				234		394		
				244		399		
mean				249		402		
sd				29.2		48.4		
BV	Assay I 19.8.09	361				486		
		341				499		
		316				383		
	Assay II 20.8.09	433				303		
		373				318		
		335				253		
mean		360				374		
sd		41.0				101.1		
BR-DT	Assay I 19.8.09	463				362		
		498				481		
		522				414		
	Assay II 20.8.09	552				417		
		566				397		
		546				426		
mean		525				416		
sd		38.6				39.0		
PROT	Assay I 19.8.09	343				372	424	448
		358				417	376	397
		366				403	373	483
	Assay II 20.8.09	375				395	414	342
		469				400	363	347
		328				399	435	404
mean		373				398	398	404
sd		49.8				14.7	30.4	55.3

		mean	*sd*
Pos	1768/1413/1315/1118/1475/1358	1408	214.2
Neg	367/419/421/374/463/429/352/397/356/397/387/335/	391	37.1
2xneg	734/838/842/748/926/858/704/794/712/794/774/670	783	74.2

9.4.3. Single revertant numbers: *TA 102* + *t*-BOOH 0.75*10^{-6} µmol/plate

Table 23

Sample		Concentration (µmol/plate)						
		2.0		**0.75**		**0.01**	**0.005**	**0.001**
BR	Assay I 17.8.09			312		584		
				202		598		
				403		600		
	Assay II 18.8.09			408		456		
				338		364		
				289		214		
mean				325		469		
sd				77.1		157.0		
BV	Assay I 17.8.09	494				579		
		487				512		
		361				364		
	Assay II 18.8.09	460				536		
		482				537		
		493				552		
mean		463				513		
sd		51.4				76.4		
BR-DT	Assay I 17.8.09	401				377		
		418				461		
		445				518		
	Assay II 18.8.09	472				446		
		519				407		
		420				430		
mean		446				440		
sd		43.6				48.4		
PROT	Assay I 17.8.09	543				447	533	347
		452				376	517	472
		427				553	484	469
	Assay II 18.8.09	456				527	572	586
		498				444	516	517
		453				435	555	458
mean		472				464	530	475
sd		41.9				65.1	31.2	78.5

		mean	*sd*
Pos	1184/1564/1566/1362/1566/1597	1473	165.4
Neg	460/473/450/422/417/389/333/399/400/407/456/447	421	39.2
2xneg	920/946/900/844/834/778/666/798/800/814/912/894	842	78.3

9.4.4. Single revertant numbers: *TA 102* + *t*-BOOH 0.75*10^{-6} µmol/plate +S9

Table 24

Sample		Concentration (µmol/plate)						
		2.0		**0.75**		**0.01**	**0.005**	**0.001**
BR	Assay I 19.8.09			257		401		
				280		472		
				275		421		
	Assay II 20.8.09			201		322		
				234		394		
				244		399		
mean				249		402		
sd				29.2		48.4		
BV	Assay I 19.8.09	361				486		
		341				499		
		316				383		
	Assay II 20.8.09	433				303		
		373				318		
		335				253		
mean		360				374		
sd		41.0				101.1		
BR-DT	Assay I 19.8.09	463				362		
		498				481		
		522				414		
	Assay II 20.8.09	552				417		
		566				397		
		546				426		
mean		525				416		
sd		38.6				39.0		
PROT	Assay I 19.8.09	343				372	424	448
		358				417	376	397
		366				403	373	483
	Assay II 20.8.09	375				395	414	342
		469				400	363	347
		328				399	435	404
mean		373				398	398	404
sd		49.8				14.7	30.4	55.3

		mean	*sd*
Pos	1452/1649/1410/1508/1548	1513	92.3
Neg	367/419/421/374/463/429/352/397/356/397/ 387/335/	391	37.1
2xneg	734/838/842/748/926/858/704/ 794/712/794/774/670	783	74.2

10. CURRICULUM VITAE

Personal Information

HUBER Hedwig
Manhartstraße 40, 2000 Stockerau (Austria)

Born on April, 24th 1985 in Stockerau (Austria)

Work experience/ job-related internships

10/2007-12/2007	Six-week practical training **IMSB Austria, Department of Nutrition** 2345 Brunn am Gebirge, Lower Austria
06/2008	Four-week practical training **Medical University of Vienna**, Department of Nutrition and Prevention, University Hospital of Pediatrics, 1090 Vienna
7/2008	Four-week practical training **Consumer advocacy group in Austria (VKI)**
11/2008-1/2009	Eight-week employee, research assistant **Medical University of Vienna**, Department of Nutrition and Prevention, University Hospital of Pediatrics, 1090 Vienna
2/2010-3/2010	Seminar conducted by a postgraduate: Nutrition ecology **Department of Nutritional Sciences**,1090 Vienna
since 1/2008	Self –emplyment for the company xx-well.com AG, 10115 Berlin, Germany

Education

1991- 1995	**Primary school** Schulweg 4, 2000 Stockerau, Lower Austria
1995- 1999	**Secondary modern school** Schulweg 4, Stockerau, Lower Austria
1999- 2004	**Federal Secondary Collage for Economic Profession** HBLA 5 Mühlgasse, 2020Hollabrunn, Lower Austria

Course of studies

Oct. 2004- July 2010	**Nutritional Science at the University of Vienna**