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DISSERTATION

Titel der Dissertation

„*In vitro* anti-mutagenic, antioxidant and anti-genotoxic properties of bile pigments and their derivatives“

Verfasserin

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"So once you do know what the question actually is,

you' ll know what the answer means."

Douglas Adams, The Hitchhiker's Guide to the Galaxy

I. ABSTRACT

Primary bile pigments including bilirubin and biliverdin as well as structurally related tetrapyrroles have been explored for decades, more closely however only during the recent years. Initially exclusively discussed as being toxic waste products of haem catabolism, bile pigments are increasingly considered as being beneficial in terms of health promotion and disease prevention. Data from *in vivo* studies suggest vasoprotective, anti-inflammatory, anti-viral and anti-cancer properties, and *in vitro* research attests anti-genotoxic and anti-apoptotic activity to these compounds. Despite such promising reports, underlying mechanisms of action remain essentially unknown and are a matter or current research. Especially the prevention of worldwide increasingly chronic disorders such as cardiovascular disease and cancer is of great medical and economic importance, and thus fundamental *in vitro* research in this field is importantly required. Especially testing endogenous compounds such as bilirubin in that regard and estimating the pigments' underlying mechanisms of action *in vitro*, represent meaningful approaches towards deflecting potential physiological effects. Therefore, the aim of this study was *1)* to enlarge the existing body of evidence on *in vitro* anti-mutagenic, antioxidant and anti-genotoxic properties with data on newly tested tetrapyrroles, and *2)* to explore possible underlying mechanisms of action *in vitro*. The study was performed within the framework of the FWF project *"The physiological relevance of bile pigments"* (grant number P21162-B11), and focused on *in vitro* cell- and bacterial culture models.

In summary, the majority of the tested bile pigments (especially protoporphyrin and the bilirubinoids) acted in an anti-genotoxic manner against synthetic (2,4,7-trinitro-9H-fluoren-9-one), food-borne (aflatoxin B1 and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and pro-oxidant (tertiary-butyl hydroperoxide) mutagens in the *Salmonella* reverse mutation assay. The obtained results specifically implicate protoporphyrin as a potent anti-mutagen in both bacterial tester strains, whose activity was most likely based on structural mutagen interaction with synthetic

and food-borne mutagens. Especially bilirubin, biliverdin and bilirubin ditaurate showed moderate to high anti-oxidant properties in the performed antioxidant capacity assays versus trolox and iron, and for the first time *in vitro* TEAC and ORAC antioxidant capacities were reported for urobilin, stercobilin and protoporphyrin. Especially these intestinally abundant compounds also induced DNA-damage in cancer cells, detected using the comet assay, which implies a toxic effect in the applied malignant cell lines HepG2 and Caco2. In summary, this outcome strongly supports existing data on anti-mutagenic/anti-oxidative activity and adds entirely novel evidence to known *in vitro* effects of bile pigments and derivatives.

Although results from *in vitro* studies cannot be directly transferred to the physiological condition, an antioxidant/anti-genotoxic behavior of tetrapyrroles could have implications for pathogenetic processes associated with prerequisite oxidative stress and/or mutagenesis. Such data represent the crucial basis for estimating potential effects of elevated bile pigment levels in the human organism.

II. PUBLICATIONS AND MANUSCRIPTS THIS THESIS IS BASED ON

Paper 1: **Bilirubin and related tetrapyrroles inhibit food-borne mutagenesis *in vitro* – implications for pathogenetic processes** (original research article; *under review*)

Paper 2: ***In vitro* antioxidant capacity and anti-genotoxic properties of protoporphyrin and structurally related tetrapyrroles** (original research article; *accepted*)

Paper 3: ***In vitro* physic-chemical properties of tetrapyrroles against TNFone-induced mutagenesis** (original research article; *prepared for submission*)

Paper 4: **Concentration-dependent bacterial bile pigment absorption and anti-genotoxic compound effects *in vitro*** (short communication; *accepted*)

Paper 5: ***In vitro* DNA-damaging effects of intestinal and related tetrapyrroles in human cancer cells** (original research article; *under review*)

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A list of further publications as well as oral and poster presentations can be found on page 67.

III. CONTENTS

IV. ABBREVIATIONS

AfB1:	Aflatoxin B1
αTOS:	α-tocopherol succinate
ATP:	Adenosine tri-phosphate
BLVRA:	Biliverdin reductase
BMI:	Body mass index
BP(s):	Bile pigment(s)
BR:	Unconjugated bilirubin IXα
BRDT:	Bilirubin conjugate (ditaurate)
BR-DME:	Bilirubin dimethyl ester
BV:	Biliverdin IXα
BV-DME:	Biliverdin dimethyl ester
CD:	Circular dichroism technique
CL:	Chlorine e4
EtBr:	Ethidiumbromide
FACS:	Fluorescence-activated cell sorting
Hb:	Haemoglobin
HMOX-1:	Haem oxygenase 1
HPLC:	High performance liquid chromatography
HSA:	Human serum albumin
IR:	Infrared
LDL	Low density lipoproteins
LMA:	Low melting point agarose
MRP2:	Multidrug resistance protein 2
NADPH/NADP$^+$:	Reduced/oxidized nicotinamide adenine dinucleotide phosphate
NMA:	Normal melting point agarose
PhIP:	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
RO(N)S:	Reactive oxygen (and nitrogen) species
PRO:	Protoporphyrin IX
rcf:	Relative centrifuge force
rpm:	Revolutions per minute
RT:	Room temperature (25 °C)
SB:	Stercobilin
SBG:	Stercobilinogen
SCGE:	Single cell gel electrophoresis (assay)
S. typhimurium/Salmonella:	*Salmonella typhimurium*
TAC:	Total antioxidant capacity
TA98/TA102:	*Salmonella typhimurium* strains TA98 and TA102
TCM:	Traditional Chinese Medicine
T2DM:	Type 2 diabetes mellitus
***tert*-BOOH:**	*Tertiary* butyl hydroperoxide
TNFone:	2,4,7-trinitro-9H-fluoren-9-one
TP(s):	Tetrapyrrole(s)
UB:	Urobilin
UBG:	Urobilinogen
UGT1A1:	Uridine diphosphate glucuronosyl transferase 1 family
VCD:	Vibrational circular dichroism technique

1. INTRODUCTION

1.1. Occurrence and accumulation of bile pigments and tetrapyrroles in the human organism

Bile pigments (BPs) are found in the reticuloendothelial system where the catabolism of haem takes place [1,5]. In specific, mostly unconjugated bilirubin (BR; without glucuronates) circulates in the plasma, the majority of which tightly bound to albumin[6], and is excreted by the liver [1], to eventually enter the intestine. In the human body, biliverdin (BV) is rapidly metabolized to BR [7]. Thus BV cannot be measured unambiguously in the plasma [8]. Bile pigments in the broadest sense are eliminated from the body *via* urine and faeces at a daily estimate of 300 mg [9]. Detailed aspects of BP metabolism are specified in section 1.3.3.

1.2. Aspects of tetrapyrrole chemistry – structural characteristics and properties

Bile pigments such as BR and BV as well as their structurally related derivatives belong to the porphyrin group of molecules [10], which represent tetrapyrrolic, dicarboxylic acids carrying extended double-bond and π-electron systems. Due to their common basic structure, this group of molecules is summarized as "tetrapyrroles" (TPs). Based upon their structural properties, either direct structural interactions with molecules (*e. g.* stacking or non-/covalent complex formation with potential mutagens) [5,11] and/or an antioxidant activity through the donation of electrons[12] or hydrogen (H) atoms, have been hypothesized as underlying mechanisms of anti-genotoxic action. Furthermore, free NH-groups [13] within the molecules have been discussed as being crucial factors determining the compounds' antioxidant activity. However, the exact roles structural properties and functional groups attached to the individual molecules play in terms of the pigments' multidimensional activity remain vastly unknown. The fact that 10.000-fold lower BR concentrations prevented hydrogen peroxide toxicity,

implied some kind of BR regeneration, and resulted in the postulation of a redox-cycle between BR and BV, published by Barañano *et al.*, Doré *et al.* and Sedlak *et al.* [2-4,14], and involving the enzymatic biliverdin reductase system (NADPH/BLVRA). This cycle is based on the theory that BR upon its function as an oxidant is oxidized to BV, which is subsequently enzymatically reduced to BR, involving BLVRA. With reference to that it was reported that if BLVRA is functionally inactivated, the antioxidant and cytoprotective effects of the involved BPs are lost, leading to apoptotic or necrotic cell death [15]. However, the redox cycle's functional importance (oxidative conversion of BR to BV [16]) and its existence are discussed controversially today [4,17-19], since BR radicals can also be reduced non-enzymatically by interacting with molecular oxygen [2,4,20].

With reference to its antioxidant function, it is evident that BR scavenges singlet oxygen[21] and is a potent reducing agent for peroxides, in the presence of hydroperoxides [22,23]. Biliverdin however only carries an extended double-bond system, important for its antioxidant function, which is possibly based on the formation of a resonance-stabilized, carbon centered radical [24].

As mentioned in section 1.1., most of the extracellular abundant, mostly unconjugated BR in the human body is, because of its weak solubility in water, bound to the plasma protein albumin. This binding is thought to involve ion-pairing, H-bonding and π-interactions between the side chains of amino-acids and the pigment itself. This process fixes the molecule in an out-of-plane ("ridge-tile") conformation [22], which is expected to expose the reactive H-atoms at carbon atom 10 (C-10) for H-abstraction by radicals [24]. Albumin binding therefore facilitates BR's reductive capacity in the aqueous phase. The resulting increased antioxidant activity of albumin-bound BR can lead to a better protection of plasma proteins and lipids [24,25].

It is important to note that the ratio of bilirubin to albumin plays a crucial role in determining "free" bilirubin concentrations, since this concentration influences the degree to which bilirubin can diffuse into cells [26].

Despite its numerous polar groups, unconjugated BR yet is practically insoluble in water (approx. 70 nM [27]), which can be explained by internal hydrogen bonds (H-

bonds). Glucuronidation of the propionic acid carboxyls makes conjugated BR water-soluble and subsequently enables its urinary excretion [6]. Based on the poor solubility of unconjugated BR in the aqueous phase, its plasma concentration is limited to 7 nM [28].

1.3. Chemical structures of bile pigments and derivatives

1.3.1. Tested tetrapyrroles

Bile pigments and derivatives are tetrapyrrolic dicarboxylic acids which share porphyrin structure. As discussed in the previous paragraphs, the individual compounds carry different functional groups and side-chains and can be cyclic (fused) or linear (open-chain) in conformation. *Figure 1,* presents the chemical structures of the tested TPs*,* followed by specifications on the pigments' chemistry, in the chronological order of their physiological occurrence. Physiologically abundant TPs mainly include PRO, BV, un-/conjugated BR, SB and UB. The methyl esterified counterparts of BR and BV can be found as intermediate products, however are normally not physiologically abundant.

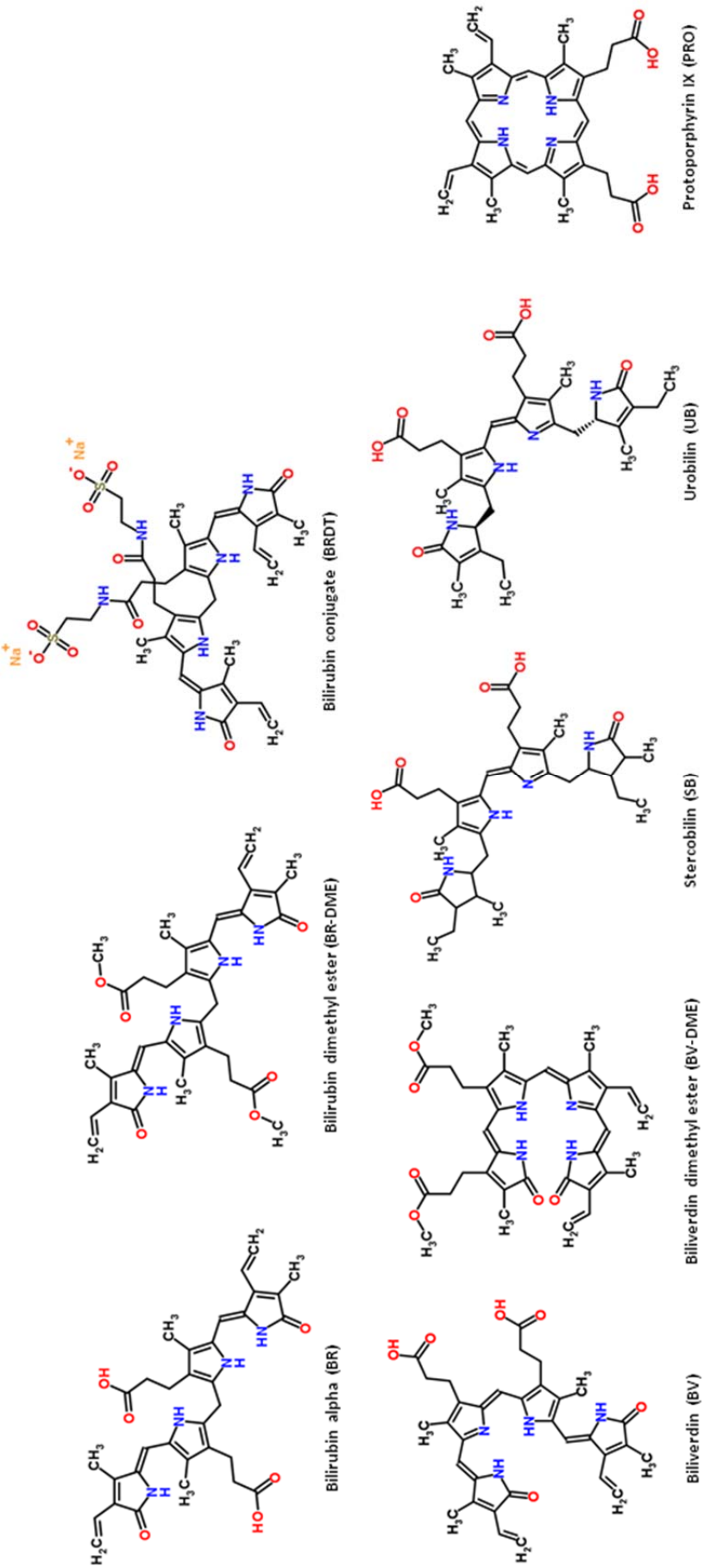

Figure 1. Chemical structures of the tested tetrapyrroles.

- **Protoporphyrin IX (PRO) – chemical basics**

Chemical name (IUPAC): 7,12-diethenyl-3,8,13,17-tetramethyl-21H,23H-Porphine-2,18-dipropanoic acid

Chemical formula: $C_{34}H_{34}N_4O_4$

Molecular weight (g/mol): 562.66

Protoporphyrin IX is the only naturally occurring isomer of the group of protoporphyrins, and represents the direct precursor of haem. Like haem, it is red in color and contains four methyl, two propionic and two vinyl side chains. Protoporphyrin is produced by oxidation of the methylene bridge of protoporphyrinogen, involving the enzyme protoporphyrinogen oxidase. The molecule's macrocycle (fused porphyrin ring) represents the chromophore of haem [29], and is synthesized after elimination of an iron molecule from hematin, representing a trivalent-iron derivative of haem [1].

- **Biliverdin IXα (BV) – chemical basics**

Chemical name (IUPAC): 3-[2-[(E)-[(5E)-3-(2-carboxyethyl)-5-[(4-ethenyl-3-methyl-5-oxo-pyrrol-2-yl)methylidene]-4-methyl-pyrrol-2-ylidene]methyl]-5-[(E)-(3-ethenyl-4-methyl-5-oxo-pyrrol-2-ylidene)methyl]-4-methyl-1H-pyrrol-3-yl]propanoic acid

Chemical formula: $C_{33}H_{34}N_4O_6$

Molecular weight (g/mol): 582.65

Green open-chain BV is formed when haem is degraded enzymatically. This process is accompanied by the loss of iron (Fe^{3+}), and a carbon atom in the form of CO, after $NADPH/H^+$-dependent oxidation at haem's α-methene bridge, involving the iso-enzyme haem oxygenase (HMOX-1/-2). This opens the porphyrin ring, and forms BV which consists of four linearly connected pyrrole rings [1].

- **Biliverdin dimethyl ester (BV-DME) – chemical basics**

Chemical name (IUPAC): methyl 3-[2-[(E)-[3-(3-methoxy-3-oxo-propyl)-4-methyl-5-[(E)-(3-methyl-5-oxo-4-vinyl-pyrrol-2-ylidene)methyl]pyrrol-2-ylidene]methyl]-4-methyl-5-[(E)-(4-methyl-5-oxo-3-vinyl-pyrrol-2-ylidene)methyl]-1H-pyrrol-3-yl]propanoate

Chemical formula: $C_{35}H_{38}N_4O_6$

Molecular weight (g/mol): 610.70

This green-colored molecule is (synthetically) formed through esterification of BV with methyl groups (methanol). Its characteristics are strong intramolecular H-bonds, which are to be found between the molecule's NH- and ester groups [13].

- **Unconjugated bilirubin IX*α* (BR) – chemical basics**

Chemical name (IUPAC): 3-[2-[3-(2-Carboxy-ethyl)-4-methyl-5-(3-methyl-5-oxo-4-vinyl-1,5-dihydro-pyrrol-2-ylidenemethyl)-1H-pyrrol-2-ylmethyl]-4-methyl-5-(4-methyl-5-oxo-3-vinyl-1,5-dihydro-pyrrol-2-ylidenemethyl)-1H-pyrrol-3-yl]-propionic acid

Chemical formula: $C_{33}H_{36}N_4O_6$

Molecular weight (g/mol): 584.66

Bilirubin IX*α* is an isomer of BR. Orange colored BR is a linear, open-chain tetrapyrrole that consists of four covalently bound pyrrole rings [30]. The presence of multiple polar groups (*e. g.* the two propionic acid side chains and amino groups) make it highly lipophilic, yet it dissolves poorly in most lipid solvents. Hydrogen bond-breaking solvents (*e. g.* DMSO) most effectively dissolve BR [31], however they lower its affinity for albumin. Unconjugated BR is also referred to as "indirect BR".

- **Bilirubin ditaurate (BRDT) – chemical basics**

Chemical name (IUPAC): 2-aminoethanesulfonic acid; 3-[2-[[3-(2-carboxyethyl)-4-methyl-5-[(Z)-(4-methyl-5-oxo-3-vinyl-pyrrol-2-ylidene)methyl]-1H-pyrrol-2-yl]methyl]-4-methyl-5-[(Z)-(3-methyl-5-oxo-4-vinyl-pyrrol-2-ylidene)methyl]-1H-pyrrol-3-yl]propanoic acid

Chemical formula: $C_{37}H_{44}N_6Na_2O_{10}S_2$

Molecular weight (g/mol): 842.89

This yellow colored open-chain molecule, also referred to as "taurobilirubin" [32], represents the synthetic analogue to physiologically abundant conjugated ("direct") BR, which in the human body is responsible for a balanced BR threshold. Bilirubin glucuronides are natural human metabolites of BR, generated in the liver by uridine diphosphate (UDP-) glucuronosyltransferase 1A1 (UGT1A1), forming *β*-1-*o*-glycosidic bonds. Glucuronides of BR are formed through esterification of BR's propionic acid groups with monosaccharides (D-glucose, D-xylose), and uronic acid [33]. The resulting molecule is highly water-soluble based on the cleavage of H-bonds between the propionic-, amino- and lactam groups. Physiological conjugated BR is labile under *in vitro* conditions, and therefore has to be used in the form of the synthetic conjugate BRDT in *in vitro* experiments.

- **Bilirubin dimethyl ester (BR-DME) – chemical basics**

Chemical name (IUPAC): methyl 3-[2-[[3-(3-methoxy-3-oxo-propyl)-4-methyl-5-[(Z)-(4-methyl-5-oxo-3-vinyl-pyrrol-2-ylidene)methyl]-1H-pyrrol-2-yl]methyl]-4-methyl-5-[(Z)-(3-methyl-5-oxo-4-vinyl-pyrrol-2-ylidene)methyl]-1H-pyrrol-3-yl]propanoate

Chemical formula: $C_{35}H_{40}N_4O_6$

Molecular weight (g/mol): 612.73

Orange coloured BR-DME is (synthetically) formed through esterification of BR and methyl groups (methanol). Involving esterases and UDP-glucuronosyltransferase, open-chain BR-DME ester is converted to BR conjugate, which can be excreted from the body [34,35].

- **Urobilin (UB) – chemical basics**

Chemical name (IUPAC): 3-[2-[(Z)-[3-(2-carboxyethyl)-5-[[(2S)-4-ethyl-3-methyl-5-oxo-1,2-dihydropyrrol-2-yl]methyl]-4-methyl-pyrrol-2-ylidene]methyl]-5-[[(2S)-3-ethyl-4-methyl-5-oxo-1,2-dihydropyrrol-2-yl]methyl]-4-methyl-1H-pyrrol-3-yl]propanoic acid

Chemical formula: $C_{33}H_{42}N_4O_6$

Molecular weight (g/mol): 590.71

By the intestinal microflora, BR is reduced to an uncolored pigment, named urobilinogen (UBG) [36], to which orange colored open-chain UB represents an oxidation product, that is excreted *via* the urine and accounts for its coloration.

- **Stercobilin (SB) – chemical basics**

Chemical name (IUPAC): 3-[2-[(Z)-[3-(2-carboxyethyl)-5-[[(2S,3R,4R)-4-ethyl-3-methyl-5-oxo-pyrrolidin-2-yl]methyl]-4-methyl-pyrrol-2-ylidene]methyl]-5-[[(2S,3R,4R)-3-ethyl-4-methyl-5-oxo-pyrrolidin-2-yl]methyl]-4-methyl-1H-pyrrol-3-yl]propanoic acid

Chemical formula: $C_{33}H_{46}N_4O_6$

Molecular weight (g/mol): 594.74

This molecule represents the final product of the intestinal BR degradation [37]. In the colon, BR is fermented by the microflora. After enzymatic elimination of glucuronates through the enzyme *β*-glucuronidase this process yields stercobilinogen (SBG).

Following dehydration of the methylene group, brown SB is oxidatively formed [1], giving the distinct coloration of the faeces.

1.3.1.1. Applied mutagens

To consider different modes of antioxidant and anti-mutagenic action of TPs *in vitro* (*e. g.* structural interactions/complexation, anti-oxidant action *etc.*), various mutagens *(Figure 2)* were utilized in the *Salmonella* reverse mutation, SCGE/comet and flow cytometry assays.

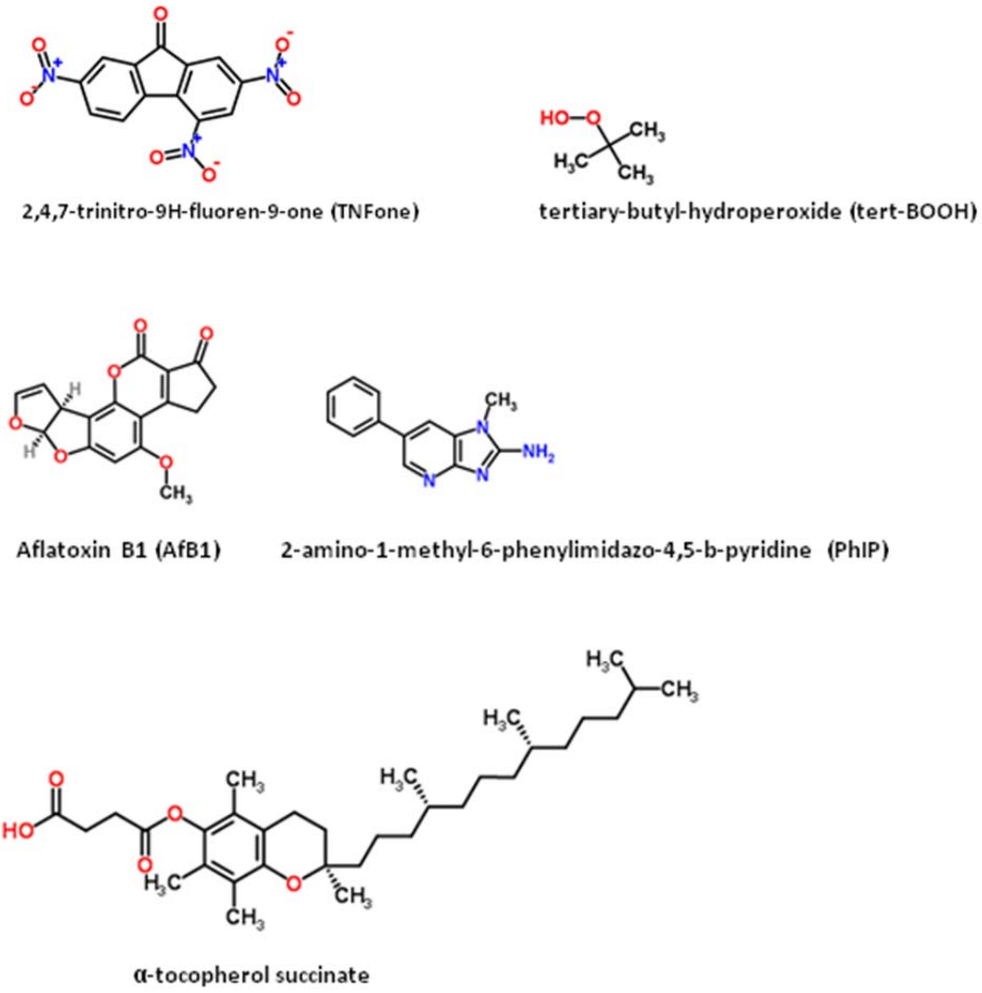

Figure 2. Chemical structures of the applied mutagens/positive controls.

1.3.2. Historical aspects of bile pigments and associated research

From centuries ago until present, salts of BR have been used in Traditional Chinese Medicine (TCM) to treat and cure diseases and conditions associated with acute or chronic inflammation [38]. Originally, pulverized gallstones *(calculus bovis)* of the Asian cattle breed *"Bos Taurus Domesticus Gmelin"* (fam. *bovidae*) were used, whereas today mostly the synthetic form is applied, for normalizing gall bladder function and treating inflammation and fever [39]. Recently, also data on immune-modulating and anti-carcinogenic effects of *calculus bovis* have been published [40].

By the academic medicine and the scientific community however, BR was for long considered useless at its best or toxic at its worst [41], and elevated BR plasma concentrations were exclusively considered in connection to disease. It is only since the late 1990s that the scientific opinion on BR and related BPs has shifted. Due to growing evidence on anti-mutagenic and antioxidant effects *in vitro* [5,42-46] and a recently evident anti-atherogenic *in vivo* action in the broadest sense [47-49], BR and its derivatives are now regarded as being beneficial in terms of health promotion. Elevating circulating BR levels[50] could represent a promising approach towards preventing or curing inflammatory diseases such as coronary heart disease [25,47,48,51] and other oxidative stress-mediated disorders, including diabetes and cancer [52,53]. *In vitro* and *in vivo* properties of BPs are described in sections 1.3.5. and 1.3.6.

1.3.3. Aspects of the human bile pigment metabolism

Bilirubin and BV are produced within the enzymatic haem catabolism, which begins inside macrophages of the spleen. Hemoglobin (Hb) is released from senescent red blood cells, as well as from non-erythroid hemoproteins (*e. g.* cytochrome systems), representing the two major sources of organic haem for the production of BPs [54]. Haem to which PRO represents the direct precursor, is degraded involving the rate-limiting iso-enzyme complex haem oxygenase (HMOX-1/-2), which by means of its function helps to maintain the cellular level of BR [27]. As a reaction to elevated oxidative

stress, HMOX-1 expression is increased, which should protect from the deleterious effects of free (pro-oxidant) haem [55]. From the oxidative HMOX-1 reaction, equimolar amounts of BV, carbon monoxide (CO) and trivalent-iron (Fe^{3+}) are liberated. Subsequently, BR is enzymatically produced from BV, involving the reductive enzyme NADPH/$NADP^+$ BLVRA, which is highly expressed in the cytosol of most eukaryotic cells[27].

Due to BR's strong affinity for albumin [22,28] and its limited solubility in the aqueous phase, most of the formed unconjugated BR is bound to albumin in the plasma and transported to the liver, where it is taken up into hepatocytes *via* active and passive modes of absorption [56]. From there, BR is shifted to the endoplasmic reticulum involving glutathione-S-transferase, where glucuronic acid conjugates (esterification of propionic acid side-chains with mono- and mainly diglucuronides) are formed by UGT1A1 [1,57]. The process of conjugation changes lipophilic BR to water-soluble BR [56], and thereby enables its urinary excretion. Under normal circumstances, the majority of plasma BR is unconjugated [6]. This is important, since large amounts of conjugated BR in the plasma could bind irreversibly to albumin, generating *δ*-BR and thereby forming a potentially toxic non-excretable complex [6].

Multidrug resistance protein 2 (MRP2) ATP-dependently transports BR into the bile caniculi [58], and BR conjugates are finally released into the duodenum *via* the bile duct. Upon entry into the intestinal tract, unconjugated BR is formed by bacterial enzymes including *β*-glucuronidase. Parts of the product are excreted, large parts of it are re-absorbed *via* entero-hepatic recirculation (approx. 70 %) [59,60]. Remaining pigments undergo a series of reductions involving the intestinal microflora, and leading to the formation of UB and SB [61,62]. The BR metabolites, urobilinogen (UBG) and urobilinoids (UB, SB and their intermediate forms; resembling BR), are exclusively found in the intestinal- (UB and SB) and the urinary tract (UB) [1] and giving the distinct coloration of urine and faeces. Bilirubin plasma levels (usually between 0.5 and 1 mg/dl or 8.5 and 17 µM [1]), are determined by the relative amounts at which the compound enters and leaves the circulation [1]. The daily endogenous BR production amounts to

approximately 4 mg/kg body weight, and a total of about 300 mg is excreted *via* urine and faeces every day [24]. As mentioned, also the direct haem precursor PRO accumulates in the intestinal tract and is excreted in the faeces at concentrations of 0.2 – 14.3 nmol/g (or 0.113 – 8.05 µg/g) [63]. The usual daily excretion of UBG in the faeces (mainly SB) ranges between 40 and 280 mg and from 0 to 3.5 mg (mainly UB) in the urine [61].

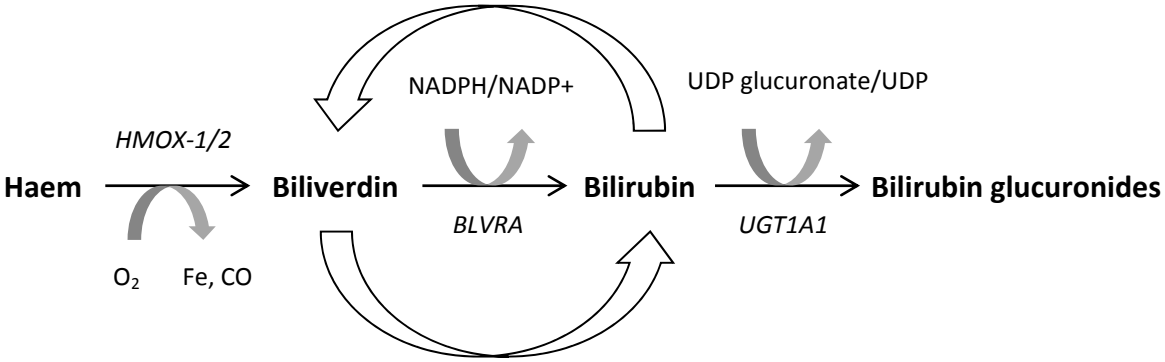

Figure 3. **Schematic bile pigment metabolism – from haem to BR.** Hemoglobin is split to yield globin and hematin, a hydroxide of the trivalent-iron derivative of haem *(not illustrated)*. Haem is enzymatically converted to BV by removing iron, and by oxidation at its *α*-methene bridge, with loss of a carbon atom in the form of CO. This opens the porphyrin ring, and forms the open-chain, linear tetrapyrrole BV, which yields BR after enzymatic reduction of BV's central methene bond [1]. In the liver, BR is conjugated to enable excretion, involving the enzyme UGT1A1. A redox-cycle between BR and BV has been proposed [2-4].

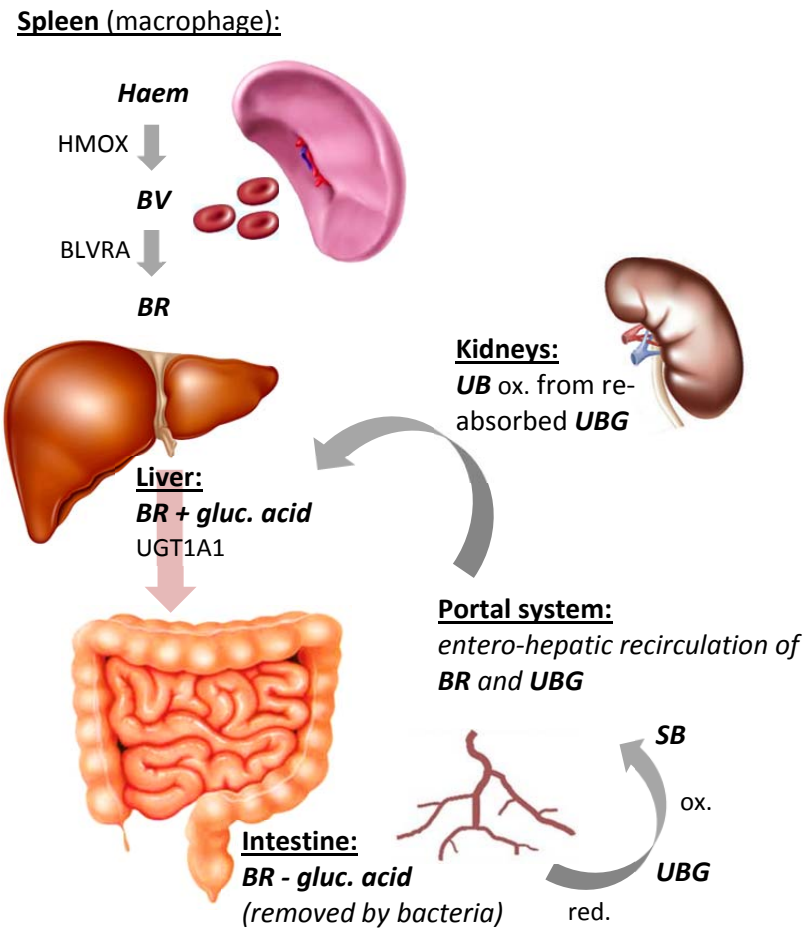

Figure 4. **Schematic bile pigment metabolism – from BR to SB and UB.** Most of the formed BR is bound to plasma albumin and transported to the liver, where albumin is removed, and glucuronidation occurs, to form conjugated BR. Conjugated BR can be excreted *via* the urine. Some of it however, enters the intestinal tract where bacterial esterases cleave the *β*-1-*o*-acyl glycosidic bonds. Through reduction, the intestinal flora further produces UBG which in parts is oxidized, and finally forming SB that is excreted in the faeces. Through entero-hepatic recirculation, BR (70 %) and UBG are transported back to the liver. Following oxidation, the formed UB is excreted *via* the urine [1].

1.3.4. Diseases and conditions related to impaired bilirubin metabolism and elevated bilirubin plasma levels

Jaundice and cholestasis both result in yellow coloration of the skin, mucous membranes, and sclera, however the conditions should be clearly distinguished from one another. Cholestasis refers to a decreased flow rate of the bile (*e. g.* due to obstruction of bile caniculi), whereas jaundice can have multiple underlying causes including hepatitis, gallstones, pancreatitis, hemolysis as well as existent genetic non-hemolytic conditions of hyperbilirubinaemia [64]. Thus, jaundice can either be acquired, or hereditary. For an initial differential diagnosis, different fractions of BR can be measured. In the clinic, circulating total BR levels are routinely measured, representing the sum of both unconjugated (indirect) and conjugated (direct) BR. Besides, conjugated BR can be measured individually, which is then subtracted from total measures, to yield the unconjugated fraction. Elevation of conjugated BR can be a sign of a lowered elimination, *e. g.* due to gallstones obstructing the bile ducts. An increase of unconjugated BR in contrast, may be the result of lowered enzyme activities, as are present in inherited forms of hyperbilirubinaemia.

Under physiological circumstances, circulating plasma BR concentrations in humans range between 8.5 and 17 µM [1] (1 – 5 µM conjugated), the major part of which being albumin-bound [65]. Inherited chronically elevated circulating total BR levels can result in specific harmless conditions or harmful/severe diseases followed by a plethora of symptoms, since free, unconjugated BR is toxic to various cell types, in specific neurons[6,66,67]. The crucial factor determining disease severity and (neuro-)toxicity is the activity of the iso-enzyme UGT1A1, which is based on mutations in its gene promoter region. Depending on the enzyme's remaining activity, conditions of hyperbilirubinaemia do or do not require treatment. If required, BR-associated disorders can be controlled by applying certain medications (*e. g.* phenobarbital), phototherapy, and if necessary can be cured by liver transplantation [68]. The following paragraphs list the most commonly known conditions of inherited hyperbilirubinaemia, followed by neonatal hyperbilirubinaemia as an important

example for non-inherited BR-associated disorders, and protoporphyria as an example of impaired PRO metabolism.

- Gilbert's syndrome

Autosomal recessive Gilbert's syndrome occurs at a mean prevalence of 3 – 10 % in the general world population, in which men are more frequently affected than women [69]. The condition is characterized by total serum BR levels of up to a maximum of 6 mg/dl or 103 µM (90 % of which unconjugated), based on a mutation in the promoter region of UGT1A1 and leading to a lowered enzyme activity of about 30 % that of not affected subjects [70]. The UGT1A1 gene carries a TA repeat promoter polymorphism which is located 41 nucleotides upstream of the translational start site adjacent to a putative TATA box [71]. The polymorphism includes five to eight TA repeats, with six repeats representing the most common allele [72]. The condition is more or less free of symptoms and liver morphology in affected subjects is normal. Serum BR levels can be elevated by fasting [73], which besides genetic analysis also represents an important diagnostic tool. Due to the fact that today moderately elevated BR levels are discussed as being beneficially involved in health promotion, persons with Gilbert's syndrome could be better protected from heart diseases, cancers, inflammation and metabolic complications[12,25,47,49,74,75]. Therefore, today Gilbert's syndrome is mostly referred to as a condition rather than a disease.

- Crigler-Najjar syndromes I/II

The autosomal recessive disease is based on a total (type I: enzyme activity < 1 %) or partial (type II: enzyme activity approx. 10 %) deficiency in UGT1A1 activity. Type I can result in fatal kernicterus when unconjugated BR levels, typically greater than 20 mg/dl (342 µM) [76], are reached. As is the case in Gilbert's syndrome, however unnecessary in that mild condition, BR levels can be therapeutically lowered by administering phenobarbital, which induces microsomal enzymes, including UGT1A1. Less severe

type II is compatible with a normal life span and characterized by BR levels ranging between 6 and 20 mg/dl (103 – 342 µM) [73].

- Dubin Johnson syndrome

The autosomal recessive mostly asymptomatic Dubin Johnson syndrome, is characterized by increased un-/conjugated (total) BR levels (around 5 mg/dl or 86 µM; higher levels possible), based on the inability of hepatocytes to transport conjugated BR into the bile caniculi, which is due to a mutation in the MRP2 gene. The patients' hepatic morphology is normal, as is life expectancy, however functional liver parameters can be impaired [73].

- Rotor's syndrome

Autosomal recessive Rotor's syndrome represents a harmless condition of chronically elevated un-/conjugated (total) BR levels of less than 10 mg/dl [77] (171 µM). In contrast to Dubin Johnson syndrome, not the transport of BR into the bile caniculi, but the liver's capacity to store BR is decreased. Otherwise liver function is normal in Rotor patients [70].

- Neonatal hyperbilirubinaemia and kernicterus

This intrinsically non-inherited, but acquired condition, is due to a delayed development of UGT1A1 expression and activity, in addition to elevated hemolysis, both of which representing the most important causes of neonatal unconjugated hyperbilirubinaemia[6]. Plasma BR levels reach concentrations of up to 11.7 mg/dl (200 µM) and normalize after a week to settle at around 1.17 mg/dl (20 µM) [78,79]. This basically physiological condition during the first days of life can become fatal when BR-induced neuronal toxicity (kernicterus) occurs, which happens when the molar BR to

albumin ratio exceeds one [6], and unconjugated BR trespasses the blood-brain barrier. This can be the case when BR levels reach concentrations of above 340 µM and/or if inherited forms of UGT1A1 deficiencies are present [80]. Infants with high BR levels receive phototherapy in the blue-green spectrum (430 – 490 nm), which leads to photoisomerization of BR and creates more polar and water-soluble enantiomeric photobilirubins, that can be excreted in the bile without the necessity of conjugation[31].

- Impaired protoporphyrin metabolism

Another congenital (autosomal dominant) disorder of BP metabolism, however not directly related to BR but its metabolism in the broadest sense, are (besides other forms of protoporphyria), hepatic and erythropoietic conditions of protoporphyria. Both of which represent genetic disorders of haem biosynthesis, which cause severe photosensitivity against visible light in the case of erythropoietic protoporphyria, or mostly abdominal pain in the hepatic variant. In either of the two, protoporphyria is due to a deficiency in the enzyme ferrochelatase, responsible for converting PRO into haem, and therefore leading to PRO accumulation in tissues [81], and to an elevated faecal excretion of PRO. For normal PRO excretion see section 1.3.3.

1.3.5. Physiological importance and biological relevance of bilirubin

It is evident that BR and BV act as antioxidants *in vitro* (see section 1.3.6). Furthermore recent data from human trials strongly suggest that especially BR is of specific physiological importance and biological relevance in terms of preventing certain diseases. However, its exact mechanistic properties in the human organism remain uncertain, and further possible roles derivatives of BR could play in the body are vastly unknown. Therefore, during the recent years many groups have dedicated their work towards exploring BR's biological relevance and its protective potential. In the following paragraphs, established as well as suspected *in vivo* properties of BPs and derivatives are described.

1.3.5.1. Atherosclerosis and other cardiovascular diseases

In 1994 Schwertner and colleagues for the first time proposed a strong association of elevated BR levels and a lowered risk for coronary heart disease [82]. Today it is assumed that BR's functions as important antioxidant [14,48] and anti-inflammatory agent [49,83] in the human organism possibly form the main prerequisite for protection against atherosclerosis. The compound exerts its function by preventing the pathogenetic peroxidation of lipoproteins such as low density lipoproteins (LDL)/small dense LDL[84,85] and by protecting against plaque formation in the intima [82,86]. In that regard, several studies have reported that subjects with lower BR levels are at higher risk for coronary and atherosclerotic diseases [49,86-88], as well as for coronary artery calcification [89] and stroke [90]. In this context it has been *vice versa* reported that elevated BR provides coronary protection in terms of lowering the atherosclerosis risk [82,91] and the severity of existing disorders of the circulatory system [92]. With reference to that it has been furthermore proposed that based on HMOX-1 overexpression in mice, ischemic reperfusion injury could be prevented [93], and the infarct size reduced in rats [94].

It is commonly known that weight loss in overweight subjects represents a crucial step to reduce the overall and in specific the coronary risk. However, recent studies show an inverse correlation of weight loss especially in abdominal obesity and an increase in serum BR levels [95]. Therefore it has been suggested that the health benefit of weight loss should be considered also in connection to BR's suspected health promoting effects [52]. These results have lately been confirmed by lowered BR levels in obese persons [96,97], and in the opposite, by lower BMI values in persons with moderately elevated BR levels [75].

In summary, lower BR levels which are based on various genetic and environmental factors (*e. g.* smoking, eating habits, enzyme expressions), seem to be an evident risk factor for atherosclerotic events and associated diseases [27]. However, since research in this field is fairly young and has so far mainly focused on exploring BR's biological potential, *in vivo* properties of other members of the haem catabolic pathway or

interrelation of these and their effects on cardiovascular protection are currently unknown [52]. Also very recently, controversy arose concerning BR's direct systemic cardioprotective effects. In a study by Stender *et al.,* commented by Johansen *et al.,* the statistical approach of Mendelian randomization and multifactorial adjustment for BR concentration was used, to estimate whether or not elevated BR levels in connection to the underlying UGT1A1 polymorphism were directly involved in the prevention of ischemic heart disease and myocardial infarction. Based on their analyses the authors concluded a non-causal relationship between BR and the occurrence of the aforementioned diseases, which would imply that the above mentioned polymorphism does not directly mediate cardioprotection. Indirect effects of elevated BR levels such as the prevention of oxidation (*e. g.* of blood lipids) were hypothesized instead. Taking into consideration BR's seemingly evident protective effects presented in the previous paragraphs, it currently remains unclear whether or not BR based on a UGT1A1 deficiency directly mediates disease prevention, or rather is a correlated biomarker [98].

1.3.5.2. Cancer

Numerous studies refer to an anti-cancer activity of BR *in vivo*. Lowered cancer risk for non-dermatological and colon cancer [99], breast cancer [100], as well as a lowered generalized cancer mortality [101] only to mention a few, have been reported in connection to elevated serum BR levels. In the study of Ching *et al.,* the relative risk for breast cancer was reported to be 0.5 for those subjects with the highest BR concentrations (> 7.5 µM), in comparison to the lowest concentration quartile of < 4.1 µM [100]. In a Belgian study published by Temme *et al.,* the adjusted relative risk for all-cause and cancer mortality was 0.73 and 0.42, respectively. The cancer mortality risk decreased with increasing serum BR levels [101]. In a US study by Zucker *et al.,* an increase in serum BR was associated with a reduced lifetime prevalence of gastrointestinal and colorectal cancer, with odd's ratios of 0.81 and 0.26 [53]. Furthermore, recently a protective role of the UGT1A1*28 polymorphism against

colorectal cancer was found by. Jiraskova *et al.*, who reported that cancer patients had lower BR levels compared to controls [102].

Albeit, potential anti-carcinogenic properties of related TPs *in vivo*, with the only exception of PRO, remain essentially unknown to date, and the majority of the published literature exclusively reports on *in vitro* data (see section 1.3.6.2 and paper 5). In either case, the exact underlying mechanisms of a possible anti-carcinogenic effect of BR (and its derivatives) remain unknown, however in summary the data suggest that higher BR levels predict lower cancer incidence, and that serum BR levels could therefore be a useful tool to estimate the cancer risk [27].

As mentioned one exception in terms of its scientific exploration with regard to anti-cancer effects is PRO. Due to its photosensitizing properties, it is successfully used in the clinical treatment of different forms of mainly skin cancer. After injection of 5-aminolevulinic acid (ALA), leading to increased PRO synthesis [103,104], light-excited PRO molecules selectively target cancer cells *via* different mechanisms, including mitochondrial depolarization and apoptosis induction, necrotic, or autophagic mechanism, followed by the induction of acute local inflammation, participating in the removal of dead cells [105].

1.3.5.3. Metabolic diseases – diabetes, dislipidemia, metabolic syndrome

It has been reported that BR positively influences glucose tolerance [106], diabetes-associated albuminurea [107], and the occurrence of the metabolic syndrome [108]. Furthermore, inverse correlations of elevated BR concentrations with multiple parameters specifying the general metabolic state (insulin resistance, apolipoprotein B, triglycerides, obesity and hypertension) were found and published by Ko *et al.,* Inoguchi *et al.*, and Papadakis *et al.*[106,109,110].

Referring to the metabolic state in diabetes and its connection to BR, several groups have suggested a relation between certain enzymes of BP metabolism and type 2

diabetes (T2DM), and associated complications [111-116]. The key antioxidant enzyme complex HMOX-1 has been shown to protect from the development of diabetes [52,117]. Underlying mechanisms of action include the enzyme's antioxidant function as well as a direct influence on glucose metabolism on the genetic level [118]. Furthermore, HMOX-1 has been shown to stimulate insulin production in animals, and induction of HMOX-1 has been shown to be associated with improved insulin signaling and glucose metabolism [119-121].

In contrast, plasma HMOX levels were found to correlate with impaired glucose regulation, in which prerequisite elevated glucose levels as a causative factor possibly led to elevated oxidative stress [111]. *Vice versa*, high plasma glucose was found to induce HMOX-1 [116], fuelling the circle of elevated oxidative stress, and confirming the aforementioned results. In contrast, BLVRA reportedly could represent an important counter-regulatory factor to impaired glucose metabolism, in that it leads to improved insulin sensitivity [115], diminishes insulin signaling and reduces glucose uptake [113,114]. These properties of BLVRA could be used in future as a therapeutic approach towards reducing insulin resistance and the negative consequences of a pathologic glucose regulation (*e. g.* inflammation *via* generation of superoxide). In connection to that, there has been one further report that not only BLVRA but also BV itself (and possibly also BR) could significantly contribute to protection from diabetic complications (*e. g.* albuminurea), as has been found in mice [112].

1.3.5.4. Miscellaneous – anti-oxidation, ageing, clinical relevance

Data from the literature suggest, BR might exert a plethora of additional beneficial effects in the human organism, including anti-inflammation, direct intracellular effects *via* cell signaling and antioxidant function through the scavenging of overproduced ROS [52]. As mentioned in section 1.3.5.1., BR suppresses the oxidation of lipids, proteins and lipoproteins (mainly LDL) [14], its concentration in the human body is directly correlated to the total antioxidant capacity (TAC) of human serum [48], and BR is the confirmed first determinant of TAC in neonatal blood [122]. Also the process of ageing

has been related to elevated free radical concentrations in connection to lowered BR levels [123]. It has been proposed that BR might exert its antioxidant function at multiple stages in the circulation which include protection against lipoprotein peroxidation, against oxidation of membrane phospholipids, and enzyme inhibitions (metalloproteinases, known to trigger atherosclerotic plaque rupture) in the intima [27], as is discussed in section 1.3.5.1.

As underlying mechanisms of BR's antioxidant function *in vivo* which has recently been confirmed by Bulmer *et al.* [25], multiple potential candidate events come into consideration, and include increased HMOX-1 activity in tissues associated with elevated BR production, increased erythrocyte turnover or insufficient conjugation of BR and its subsequent accumulation, as is the case in Gilbert's syndrome for example. Since BR homeostasis depends upon a series of enzymes including HMOX-1/-2, BLVRA and UGT1A1, enzyme expression could have important impact on the disorders discussed in the previous paragraphs. For example HMOX-1 has been proposed as a more promising target for treating oxidative-stress-mediated diseases, rather than provoking a systemic increase in antioxidants through supplementation [16]. In summary, these results emphasize BR's antioxidant ability to lower the prevalence of oxidative stress-mediated disorders [47,52] such as neurodegenerative diseases [124]. Anti-inflammatory properties have been suggested in that respect (*e. g.* in demyelinating neuropathies such as multiple sclerosis)[125]. Besides, also rare reports on a possible anti-viral activity of BR *via* different hypothesized mechanisms exist [126,127].

Although BR's possible physiological importance was already reported in 1954 [128], it was only in the year 2007 that its clinical efficacy [129] and potential clinical application were proposed. There exists evidence from an animal trial, according to which BR and BV possibly prevent clinical complications such as sepsis and *post*-traumatic stress after surgery [130], however further information is still limited. Based on such reports and on BR's evident beneficial *in vivo* properties, discussions about enteral administration [50], supplementation, drug application and enzyme inductions [86,87,131] aiming to elevate circulating BR levels have been raised and are ongoing [123], although

direct systemic effects of elevated BR with reference to an underlying UGT1A1 polymorphism have recently been discussed controversially [98,132], which is described in section 1.3.5.1.

1.3.6. In vitro *properties of bile pigments*

1.3.6.1. Anti-mutagenic and antioxidant properties in vitro

The physiological importance of BPs such as BR is supported by reports on their *in vitro* antioxidant, anti-inflammatory, anti-complement [133] and anti-mutagenic potential. The majority of the published data however exclusively deals with anti-mutagenic and antioxidant effects of BPs and derivatives against multiple classes of mutagens in the *Salmonella* reverse mutation assay [5,11,42,134-136], and more rarely in cell culture studies[137-139]. Besides, also antioxidant effects have been discussed within the literature [5,140]. So far, mainly the core compounds un-/conjugated BR and BV as well as their more distantly related derivatives hemin, chlorophyll and chlorophyllin have been explored[42,134,135,137]. Specifications on the pigments' anti-mutagenic and antioxidant effects are presented and discussed in papers 1, 2, 3 and 4.

Antioxidant activities of BR *in vitro* other than those detected by using *Salmonella*, have been investigated utilizing physic-chemical methods and antioxidant capacity assays, which have also been applied in the present study (paper 2). Numerous publications[45,140,141], have reported that each molecule of BR was able to scavenge 1.9 molecules of peroxyl radicals, and that BV was comparatively more efficient by scavenging 4.7 molecules. Furthermore it was proposed that both BPs acted in synergy with membrane-bound α-tocopherol [24], and that they were able to scavenge one- and two-electron oxidants including singlet oxygen, peroxyl radicals and α-tocopheryl radicals [142], just to mention a few. Bilirubin was found to more effectively protect lipids from oxidation than water-soluble glutathione [14], and was thirty times more potent against LDL oxidation, compared to trolox [85]. Albumin-bound BR at physiological concentrations was reported to efficiently scavenge peroxyl radicals, and to protect fatty acids from oxidation [45].

1.3.6.2. Anti-carcinogenic properties in vitro

As mentioned, so far the scientific focus has mainly been directed towards *in vitro* anti-genotoxic properties of mostly BR and BV [5,140], with little attention focused on structurally related metabolites. Reports have been published that unconjugated BR induces cell cycle arrest, apoptosis and cytostasis *in vitro* in multiple cell lines [143], which is suggestive of a certain anti-carcinogenic activity of BR. Bilirubin also seems to play a role in defending against cancer by interfering with pro-carcinogenic signaling pathways, and therefore potently inhibits tumor cell proliferation [143]. Furthermore, BR has been shown to induce mitochondrial depolarization in colon cancer cells, leading to apoptosis in human gastric cancer cells and to arrest cell cycle [144]. Furthermore, some reports on anti-carcinogenic effects of mainly BR and PRO with and without prerequisite photodynamic excitation in cultured cells exist [41,143-147]. However, mechanistic specifications on the behavior of other BR metabolites on cancer cell biology are entirely lacking, with the exception of one report on UB, published by Rao *et al.* [41]. Despite the clinical use of PRO and its derivatives in the photodynamic treatment of mainly skin cancer [147,148], *in vitro* (comet) data and evidence on pro-apoptotic, anti-carcinogenic and anti-proliferative effects of PRO in different cell culture models are comparatively rare [146,147,149-151]. Also the DNA-damaging effects of BR in cancer cells have been investigated only once [41], by applying the single cell gel electrophoresis (SCGE/comet) assay. Reports on an *in vitro* anti-carcinogenic activity of TPs emphasize and support the compounds' physiological roles presented in section 1.3.5.2, and further aspects of *in vitro* anti-carcinogenic effects of TPs are described in paper 5.

2. AIMS OF THIS STUDY

- To test whether newly investigated TPs other than previously tested un-/conjugated BR and BV exert comparable or better anti-genotoxic effects *in vitro*.
- To assess their anti-mutagenic potential against various mutagens *in vitro*, by applying the *Salmonella* reverse mutation assay.
- To explore underlying mechanisms of anti-genotoxic action by testing structural interactions of BPs and mutagens, by using the techniques of (vibrational) circular dichroism.
- To investigate bacterial test compound absorption and its relationship with anti-genotoxic pigment properties.
- To assess possible anti-genotoxic/anti-carcinogenic TP effects by applying the single cell gel electrophoresis (SCGE/comet) assay, and thereby measuring DNA-damaging effects in human cancer cell lines.
- To test the pigments' potential to induce apoptosis and necrosis, uncouple respiratory chains/increase oxidative stress and induce cell cycle arrest in human cancer cells, by utilizing multiple flow cytometry assays.
- To assess antioxidant capacities of TPs *in vitro* by using the TEAC, ORAC and FRAP assays.

3. MATERIALS AND METHODS

3.1. Chemicals

All chemicals were purchased from Sigma Aldrich, Austria (unless otherwise noted), and were stored and used according to instructions. Specifications on the utilized chemicals can be found in the respective papers' materials and methods sections.

3.2. The Salmonella reverse mutation assay (papers 1 – 4)

The *Salmonella* reverse mutation assay, colloquially referred to as "Ames test" is an internationally well-accepted short-term bacterial assay used as a screening method to evaluate the mutagenic potential of new chemicals and drugs *in vitro,* and to detect a wide range of chemical substances that can provoke genetic damage, and lead to gene mutations.

Certain mutants of *Salmonella typhimurium (S. typhimurium)* are not capable of synthesizing the amino acid histidine. After adding a mutagenic substance, the bacteria revert to their wild type, which forms the technical basis for the assay. When *Salmonella* tester strains are grown on minimal glucose agar plates containing trace amounts of histidine, only those bacteria that revert to histidine-independence (his^+), are able to form colonies [152]. To classify a substance as genotoxic, the number of revertant colonies on the plates carrying the test compounds must exceed twice the number of colonies produced on the solvent control plates (negative control) [152].

In this study, *Salmonella* strains *TA98* and *TA102* were used to consider different mechanisms of mutations. The DNA repair proficient strain *TA102* detects cross-linking agents and can be reverted by mutagens which cause oxidative damage. Strain *TA98* reverts upon induction of frame-shift mutations. Prior to the tests being conducted, the strains were analyzed for their genetic integrity and spontaneous mutation rates in our laboratory [152].

In the present study, the *Salmonella* reverse mutation assay was performed following Maron and Ames [153], and included a pre-incubation period of 25 min at 37 °C. This technique allows closer contact between bacteria, test compounds and positive controls. Some genotoxic and carcinogenic chemicals (*e. g.* poly-/heterocyclic amines such as AfB1 and PhIP), are biologically inactive unless they are metabolized to their active forms, or *vice versa*, mutagens are active but are inactivated whilst being metabolized. Since bacteria do not possess a cytochrome-based P450 metabolic oxidation system, a rodent activation system was applied, which consisted of a 9000 x g supernatant fraction of a liver homogenate from Aroclor 1254-treated rats (S-9 microsomal fraction; S9-mix), and was delivered to the test system in the presence of NADP and cofactors for NADPH-supported oxidation. The S9-mix consisted of 19.75 ml of $_{d}H_2O$, 25 ml 1 x PBS buffer, 0.5 ml $MgCl_2$ (0.85 M), 0.5 ml KCl (1.65 M) and 2 ml NADP (90.8 mM), 250 µl glucose-6-phosphate (1.08 M) and 2 ml of S9. The solution was mixed to form a homogenous solution, was kept on ice and used within one hour. Together with the test compounds, specific positive controls *(Table 1)* were used (with and without metabolic activation) to assess anti-genotoxic effects, and to confirm the reversion properties and specificity of each tester strain. Sterile DMSO served as a negative control.

Table 1. Positive controls/mutagens in use.

Mutagen	Description	Solvent	Assay	Metabol. activ. (S9)	Paper(s)
2,4,7-trinitro-9-fluorenone (TNFone; Vitas-M Labs, Russia)	Synthetic mutagen; fluorene derivative, non-planar polycyclic nitro-amine	DMSO	*Salmonella* reverse mutation assay	-	3
Aflatoxin B1 (AfB1)	Mycotoxin; polycyclic aromatic amine	DMSO	*Salmonella* reverse mutation assay	+	1
2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP; Toronto Research Chemicals, Canada)	Heterocyclic aromatic amine	DMSO	*Salmonella* reverse mutation assay	+	1
Tertiary-butyl hydroperoxide (*tert*-BOOH)	Oxidant	$_dH_2O$	*Salmonella* reverse mutation assay; comet assay	-/+	2, 5
Alpha-tocopherol succinate (α-TOS)	Vitamin E derivative; apoptosis inducer	$_dH_2O$	Flow cytometry (ROS, apoptosis/necrosis, cell cycle assay)		5

The experiments were performed in a dimly lit laboratory under sterile working conditions. 200 µl of BP solution (prepared in DMSO), 100 µl of mutagen solution (in DMSO or $_dH2O$), 500 µl of 1 x PBS or S9 mix, respectively and 100 µl of bacterial suspension (overnight culture, which had been prepared 12 hrs prior to the tests being conducted) were merged in sterile test tubes. After 30 min of incubation at 37 °C (on

shaker), 2 ml of molten top agar was added, the preparation was mixed and poured onto minimal glucose agar plates. Each concentration of every TP was tested in triplicate, positive and negative control six-fold. All experiments were repeated again on a different day.

3.3. Bile pigment solubility experiments and sample preparation (papers 1 – 5)

Prior to the tests being conducted, respective solubilities of BPs and related test compounds were analyzed. Based on preceding experiments of Bulmer *et al.* [5] and a series of solubility experiments which included supernatant analysis conducted photometrically at 380 (verdinic compounds) and 450 nm (rubinoid compounds), and HPLC analyses after high-speed centrifugation, the following TP concentrations were tested in the *Salmonella* reverse mutation assay: 0.01, 0.05, 0.1, 0.5, 1 and 2 µmol/plate. Due to its known weak solubility (approximately 70 nM in water [27], BR was investigated over a range of five concentrations including 0.01, 0.05, 0.1, 0.5 and 0.75 µmol/plate. For each test, solutions were freshly prepared in DMSO and were stored in lightproof and airtight containers throughout the experiments.

In the comet assay, tested BP concentrations were 0.5, 5 and 17 µM, with the highest concentration of 17 µM representing the tested border of solubility in the applied cell culture media. DMSO final concentrations per well did not exceed 0.1 %.

For the antioxidant capacity assays TEAC, FRAP and ORAC, TP stock solutions were prepared in DMSO at concentrations of 2.5, 0.75, and 0.25 mM for BV and BRDT, and at 0.75, 0.5 and 0.25 mM for the remaining compounds, due to solubility limitations. The tested concentrations in all of the assays were chosen to encompass the compounds' physiologically relevant concentration range.

3.4. HPLC analyses for assessing bacterial bile pigment absorption (paper 4)

Agar plates originally contained sample concentrations ranging from 2 or 0.75, to 0.01 µmol/plate (section 3.3.) in the top agar layer. After 48 hrs of incubation at 37 °C and 5 % CO_2, whole bacterial colonies were used for HPLC analyses of BP absorption. Briefly, all *S. typhimurium* colonies (≥ 1 mm in diameter) were harvested from one agar plate, and were lysed for 30 min in 40 µl of isocratic mobile phase (950 ml HPLC grade methanol, 50 ml HPLC grade water, 24.2 g n-diocylamine and 6.01 g glacial acetic acid per litre mobile phase). Supernatants were diluted therein at 1:4, and run on a Hitachi ELITE LaChrom HPLC, equipped with a Shimadzu SPD-M20A detector, over a C18 reverse phase column (5 micron, 250 x 4.6 mm) and an injection volume of 50 µl [154,155]. Oven temperature was set and kept constant at 35 °C, column pressure at 140 bar. Respective absorption maxima are specified in section 3.3.

3.5. Flow cytometry – FACS analyses (paper 5)

3.5.1. Apoptosis/Necrosis assay

The applied detection kit (r-phycoerythrin (PE) annexinV Apoptosis detection kit I, BD Pharmingen Austria) detects apoptosis and necrosis *via* flow cytometry. It requires the Ca^{2+}-dependent affinity of annexinV to phosphatidyl serine as well as the specificity of 7-aminoactinomycin-d (7-AAD) for guanine-cytosine DNA-base pairs. In viable cells phosphatidyl serine is localized on the inside of the cell, and opts out during the process of apoptosis. 1 x 10^6 cells/ml were washed in Ca^{2+}/Mg^{2+}-free 1 x phosphate buffered saline (PBS; PAA Austria) and re-suspended in binding buffer, were doubly labeled with PE-stained annexinV (ex_{max}: 496, em_{max}: 575 nm; FL-2), and 7-AAD (ex_{max}: 546, em_{max}: 647 nm; FL-3) and were incubated for 15 min in the dark prior to measurements. Intact cells react negative to both agents; early apoptotic cells react positive to annexinV, however negative to 7-AAD. In late apoptosis and necrosis, cells positively respond to both agents. Also entirely necrotic cells respond positive to 7-AAD, but negative to annexinV [156,157].

Apoptosis and necrosis represent two different types of cell death. Apoptosis – also referred to as programmed cell death – states a genetically determined, physiological "suicidal" program which differs significantly from necrosis [157]. Apoptosis which can be triggered in- or extrinsically, is a highly selective non-inflammatory process [158], characterized by nuclear condensation, decrease of mitochondrial membrane potential through the release of cytochrome c, caspase activation, cell shrinkage and fragmentation as well as by the final generation of apoptotic bodies. In living organisms, elevated as well as decreased apoptosis rates may result in malignancies, and maintaining the equilibrium between cell proliferation and cell death plays a crucial role in cell physiology. Therefore, tissue homeostasis is dependent on the balance between cell proliferation and cell death [159]. An imbalance can result in diseases connected to undesired apoptosis or cell growth. In healthy cells apoptosis has been linked to the activation of the tumor suppressor protein p53, whose expression in most tumors is down-regulated [159].

In contrast to apoptosis, necrosis is basically the passive "accidental" result of (external) cellular damage and stress, involving generalized inflammation alongside immunologic response and subsequent extensive tissue damage.

Apoptosis/necrosis assay – staining procedure:

1. Incubate 5 x 10^5 cells/ml (HepG2, Caco2) with BP and/or positive control (120 µM αTOS) in 24 wells containing 1.5 ml of respective cell culture media, carrying the test compound
2. Wash cells with 1 ml of cold 1 x PBS (800 rpm (85 rcf), 5 min), then remove supernatant
3. Re-suspend cells in 1 x binding buffer (1 x 10^6 cells/ml; 1 x binding buffer: mix 1 part of 10 x binding buffer with 9 parts of $_d$H2O)
4. Transfer 100 µl (1 x 10^6 cells/ml) to a 5 ml FACS tube
5. Add 5 µl of annexinV-PE and 5 µl of 7-AAD solutions
6. Carefully mix and incubate for 15 min at room temperature (RT) in the dark

7. Add 400 µl of 1 x binding buffer to each tube
8. Analyze within 1 hr on BD FACSCalibur®, annexinV (ex_{max}: 496, em_{max}: 575 nm; FL-2), and 7-AAD (ex_{max}: 546, em_{max}: 647 nm; FL-3)

3.5.2. Intracellular superoxides and (hydro)peroxides

Elevated intracellular concentrations of free radical and nitrogen species (RO(N)S) can impair cellular regulatory processes (*e. g.* respiratory chain-uncoupling in mitochondria) and lead to cell damage and apoptosis through the state of oxidative stress. To detect intracellular superoxides and (hydro-)peroxides in HepG2 and Caco2 cells, 10 µM final concentration of dihydroethidium (DHE) and 25 µM final concentration of dihydrofluorescein diacetate (DCFH-DA), both in DMSO, were used. DHE is cell-permeable and specifically reacts with O_2^- to form oxyethidium. This product interacts with nucleic acids and emits red fluorescence, which can be measured cytometrically (ex_{max}: 329, em_{max}: 373 nm; FL-2) [160]. When DCFH-DA enters the cell, it is cleaved by intracellular esterases to form dihydrofluorescein (DCFH), which is then rather unspecifically oxidized by peroxides to fluorescent 7'-dichlorodihydrofluorescein (DCF) (ex_{max}: 498, em_{max}: 522 nm; FL-1) [160]. After washing in Ca^{2+}/Mg^{2+}-free 1 x PBS and prior to measuring, 1 x 10^6 cells/ml were stained with DHE (in FACS tubes after harvesting) or with DCFH (in 24 wells prior to harvesting) and were left to incubate for 20 min in the dark [80].

<u>Detection of intracellular superoxides – staining procedure:</u>

1. Incubate 5 x 10^5 cells/ml (HepG2, Caco2) with BP and/or positive control (120 µM αTOS) in 24 wells containing 1.5 ml of respective cell culture media, carrying the test compound
2. Collect media supernatants in individual FACS tubes
3. Wash cells with cold 1 x PBS (1 ml per well) and detach by using accutase solution (140 µl per well, 5 min, 37 °C)

4. Detach cells from the bottoms of the wells by re-suspending in 700 µl/well of warm media and add to the respective FACS tubes
5. Centrifuge tubes at 600 rpm (64 rcf) for 4 min
6. Discard supernatants
7. Wash cell pellets with 1 ml of cold 1 x PBS per tube
8. Centrifuge tubes at 600 rpm (64 rcf) for 4 min
9. Discard PBS
10. Re-suspend cells in 1 ml of cold 1 x PBS per tube
11. Stain cells with 10 µM (final concentration) of DHE in 1 x PBS and leave to incubate for 20 min
12. Immediately run samples on BD FACSCalibur® (FL-2; ex_{max}: 329, em_{max}: 373/393 nm)

<u>Detection of (hydro-)peroxides – staining procedure:</u>

1. Incubate 5 x 10^5 cells/ml (HepG2, Caco2) with BP and/or positive control (120 µM αTOS) in 24 wells containing 1.5 ml of respective cell culture media, carrying the test compound
2. Collect media supernatants in individual FACS tubes
3. Wash cells with cold 1 x PBS (1 ml per well), followed by the addition of 1 ml of fresh 37 °C warm media per well
4. Add DCFH at a final concentration of 25 µM per well and leave to incubate (37 °C, dark) for 15 min
5. Discard media and detach cells from the bottoms of the wells by using accutase solution (140 µl per well, 5 min, 37 °C)
6. Detach cells from the bottoms of the wells by re-suspending in 700 µl/well of warm media and add to the respective FACS tubes
7. Centrifuge tubes at 600 rpm (64 rcf) for 4 min
8. Discard supernatants
9. Re-suspend cell pellets in 1 ml of cold 1 x PBS per tube

10. Centrifuge tubes at 600 rpm (64 rcf) for 4 min
11. Add 1 ml of cold 1 x PBS to each tube to re-suspend cell pellet
12. Immediately run samples on BD FACSCalibur® (FL-1; ex_{max}: 498, em_{max}: 522 nm)

3.5.3. Cell cycle assay

The assay measures fractions (percentages) of cells in the respective phases of the cell cycle (G0/1, S, G2/M), as well as apoptotic cells (sub G0/1), according to the fluorescence intensity which is based on the number of DNA copies in the cell. Propidium iodide (PI; ex_{max}: 535, em_{max}: 617 nm; FL-2) binds to DNA and RNA by intercalating between the bases with a stoichiometry of one PI molecule per 4.5 DNA base pairs, which enhances the molecule's fluorescence intensity [161]. After washing in cold 1 x PBS, 1 x 10^6 cells/ml were fixed with 75 % ice-cold absolute ethanol and were left to incubate for 30 min on ice. Cells were re-suspended in 500 µl of phosphate buffer (96 ml of 0.2 M Na_2HPO_4 + 4 ml of 0.1 M $C_6H_8O_7$), and after centrifugation 50 µM of 100 µg/ml RNase and 200 µl of 50 µg/ml PI (both in $_dH_2O$) were added [162,163].

<u>Cell cycle assay – staining procedure:</u>

1. Incubate 5 x 10^5 cells/ml (HepG2, Caco2) with BP and/or positive control (120 µM *α*TOS) in 24 wells containing 1.5 ml of respective cell culture media, carrying the test compound
2. Collect media supernatants in individual FACS tubes
3. Wash cells with cold 1 x PBS (1 ml per well), followed by the addition of 1 ml of fresh 37 °C warm media per well
4. Centrifuge tubes at 600 rpm (64 rcf) for 4 min
5. Discard supernatants and fix cells with 70 % ice-cold ethanol (approx. 250 µl added dropwise while gently vortexing)

6. Leave fixed cells to stand for a minimum of 30 min on ice (ethanol breaks apart cell membranes and denatures proteins – suspensions become cloudy)
7. Centrifuge tubes at 2500 rpm (267 rcf) for 5 min
8. Discard supernatants and re-suspend cells in 1 ml per tube of cold phosphate buffer
9. Centrifuge at 2500 rpm (267 rcf) for 5 min
10. Re-suspend each pellet in 1 ml of phosphate buffer and centrifuge again
11. Discard supernatants and stain DNA with 50 µl of 100 µg/ml RNase (in $_dH_2O$) per tube
12. Add 200 µl of 50 µg/ml PI solution (in $_dH_2O$) to each tube
13. Immediately run samples on BD FACSCalibur® (FL-2 or FL-3; ex_{max}: 550, em_{max}: 650 nm)

3.6. Antioxidant capacity assays (paper 2)

Antioxidants play a crucial role in preventing free radical formation and their action within the serum as well as in the cellular milieu. To assess the antioxidant and reducing power of structurally related TPs, the trolox equivalent antioxidant capacity test (TEAC), oxygen radical absorbance capacity assay (ORAC) and ferric reducing antioxidant potential assay (FRAP) were conducted. Due to differences in solubility, compound stock solutions were prepared in DMSO at concentrations of 2.5, 0.75, and 0.25 mM for BV and BRDT, and 0.75, 0.5 and 0.25 mM for the remaining compounds (see section 3.3.).

3.6.1. TEAC assay

The TEAC assay is a cupric ion reducing antioxidant capacity spectrophotometric method, which measures antioxidants at physiological pH. Trolox, a cell-permeable, water-soluble vitamin E derivative, is used to standardize and classify antioxidants,

based on their antioxidant power, measured and expressed as trolox equivalents. The assay measures hydrophilic, lipophilic and total antioxidant capacity based on the reduction of copper-II to copper-I. Prevention of radical production is directly proportional to the concentration of antioxidants in the sample.

20 µl of BP solution was mixed with 400 µl of 1 x PBS, 20 µl of metmyoglobin, 400 µl of 2,2'-azino-bis-(3-ethylbenzthiazolin-6-sulfonic acid) (ABTS) and 30 % H_2O_2 in disposable semi-micro cuvettes [164]. After six minutes of incubation at 37 °C, absorbance was measured on a Perkin Elmer Lambda 2 UV/VIS spectrophotometer at 570 nm [164]. To standardize the assay, trolox was tested at concentrations of 0.5, 1, 1.5, 2 and 2.5 mM.

3.6.2. ORAC assay

The ORAC assay is based on the inhibition of a peroxyl radical-induced oxidation which is triggered by thermal decomposition of azo-compounds (2,2'-azobis(2-amidino-propane) dihydrochloride, AAPH). It measures lipophilic, hydrophilic and total antioxidant capacities of substances. Over time, radicals generated from the thermal decomposition of AAPH are able to quench the fluorescent probe signal. Subsequent addition of an antioxidant stabilizes the fluorescence signal, depending on the antioxidant's capacity, which is expressed as trolox equivalents [165].

25 µl of test sample was mixed with 150 µl of 8.16 x 10^{-5} mM fluorescein disodium, dissolved in 75 mM phosphate buffer (10.96 g Na_2HPO_4 + 10.21 g KH_2PO_4/l $_dH_2O$). The pH was adjusted to 7.4, using KOH. 25 µl of 315 mM AAPH (1.92 g/10 ml phosphate buffer) was then added. 96-well plates were left to incubate in an FL 600 microplate fluorescence reader (FLUOstar Optima, Bio-Tek Instruments) for ten minutes at 37 °C. Fluorescence was measured at 520 nm every minute over a period of 35 min [165]. Trolox was tested as an external standard at concentrations of 2.5, 5, 10, 20, 30, 40 and 50 µM.

3.6.3. FRAP assay

In the FRAP assay, reduction of the ferric tripyridyltriazine complex (Fe^{3+}) to the ferrous complex (Fe^{2+}) is measured at 593 nm, at low pH. Change in absorbance is directly related to the total reducing power of the antioxidants present in the sample[166].

10 µl of test sample was mixed with 30 µl of $_dH_2O$ and 300 µl of FRAP reagent (25 ml of 300 mM Na-acetate trihydrate buffer, 2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazin in HCl, 2.5 ml of 20 mM Fe-III-hexahydrate) and was kept at 37 °C throughout the experiments. The change in absorbance was measured at 540 nm every 36 s over six minutes on an FL 600 microplate fluorescence reader (FLUOstar Optima, Bio-Tek Instruments). Fe-II sulphate heptahydrate served as an external standard substance at 50, 125, 250, 625, 750 and 1000 µM [166].

3.7. Bradford total protein analysis (paper 4)

As a reference parameter for bacterial protein content, the total protein in each sample (bacterial colonies) was measured photometrically [167], in which albumin was used as an external standard. Lysed sample supernatants (centrifuged at 14.000 rpm (1.493 rcf) for five minutes) were diluted at 1:30 in Bradford reagent in disposable semi-micro cuvettes and read on a Perkin Elmer Lambda 2 UV/VIS spectrophotometer at 595 nm, after 17 min of dark incubation. Bacterial TP absorption was calculated and expressed as absorbed TP (nmol), relative to the total protein content (mg) in the bacterial sample.

3.8. Single cell gel electrophoresis assay/comet assay (paper 5)

The alkaline single cell gel electrophoresis (SCGE) assay, colloquially referred to as “comet assay”, is a straight forward method detecting DNA single- and double strand breaks as well as alkali-labile sites. The assay developed by Östling and Johanson [168],

and modified by several groups [169-171] can be used to assess DNA damage in basically any eukaryotic cell. In brief, cells were embedded in agarose microgels on a microscope glass slide, were lysed and electrophoresed. Immediately before counting, the gels were fluorescently stained with ethidiumbromide (EtBr) and DNA-damage was evaluated by randomly counting 50 cells per gel.

As mentioned, the comet assay measures mainly DNA single- and double strand breaks in eukaryotic cells, embedded in 1 % normal melting agarose (NMA; Invitrogen Austria) fixed on agarose pre-coated microscope slides (1 % low melting agarose, LMA; Invitrogen Austria). After lysis at pH ten and 20 min of DNA unwinding, as well as subsequent 300 µM *tert*-BOOH treatment for positive controls, cells were exposed to a directed electric field (Electrophoresis CSL-10M40, Biozym Austria; 25 V, pH >13). After EtBr staining (20 µl of 20 µg/ml per gel), DNA migration was assessed using a fluorescent microscope (Zeiss Germany) equipped with a camera (Hitachi Austria) and the software Komet 5.5. (Andor Technology, Germany). Comet tail DNA content was quantified and expressed as percentages (% tail DNA). The applied method followed the protocols of Singh *et al.* [171], modified by Azqueta *et al.* [169], to measure both DNA single- and double strand breaks, summarized as DNA-damage. Human lymphocytes were replaced by cancer cells, using the same cell counts of 1 x 10^6/ml. Per compound and concentration, eight gels (two per slide; per compound and concentration) were prepared, six of which were randomly counted (50 cells/gel).

3.9. Physic-chemical studies (paper 3)

3.9.1. Circular and vibrational circular dichroism techniques (V/CD)

It is well established that under certain conditions BR and BV adopt chiral "ridge-tile"[172-175] and helical "lock-washer" [176] conformations, both of which are stabilized by intramolecular hydrogen bonds. The helical inversion equilibrium between the two enantiomeric M- (minus) and P- (plus) forms [172] is typical for these pigments in solution and causes the zero resultant optical activity of aqueous BP solutions. However, in the presence of chiral agents, the molecules preferably form one of the diastereoisomers

upon complexation with the chiral host [177,178], and become optically active. This enables to follow the subtle structural changes using circular dichroism (CD) spectroscopy [176,179]. The method refers to the differential absorption (attenuation) of left and right circularly polarized light. The vibrational variant of circular dichroism, vibrational circular dichroism (VCD), is a CD technique applied in vibrational spectra, and extends CD spectra into the infrared and near-infrared ranges. It follows the vibrational transitions that are localized in a molecule and can therefore determine the variation in three-dimensional molecular structures caused by biologically important interaction [180,181].

The CD spectra of BPs were measured in a quartz cuvette with an optical path length of 1 cm (Starna, USA), using a J-810 spectropolarimeter (Jasco, Japan). The final spectrum was obtained as an average of three accumulations. Spectra were corrected for baseline by subtracting the spectra of the corresponding solvents. Circular dichroism measurements were conducted at RT. For spectral measurements, BR, BV as their sodium salts, and chiral porphyrin chlorine e4 (CL) in double distilled water were used at concentrations of 1.2×10^{-5} M. The sodium salts of the pigments were prepared by freeze-drying as described previously [182]. Human serum albumin (HSA) was used as a chiral discriminator for BR and BV at a pigment/HSA molar ration of 1/1. The chiral derivative of PRO (CL) was chosen to follow the interaction with TNFone utilizing CD in the absence of a chiral discriminator. Vibrational CD spectra of CL with TNFone were recorded in DMSO d_6/D_2O (50:50) solution, at a concentration of 0.03 M with a resolution of 8 cm^{-1}, using a Fourier transform infrared (IR) spectrometer IFS-66/S (Bruker, Germany), equipped with a VCD/IRRAS module PMA37 (Bruker). A demountable cell with CaF_2 windows and Teflon spacer of a 50 µm pathlength was used. To interpret the VCD and IR spectra of CL with and without TNFone, a possible structure of the CL/TNFone complex was modeled (see paper 3, *Fig. 3*). Structure optimization was carried out by the HyperChem 8 software package [183], using the Amber 99 force field [184].

3.10. Statistical analyses (papers 1 – 5)

All data were analyzed using IBM SPSS (versions 17.0 – 19.0) for Microsoft Windows. Data were tested for normal distribution applying the Kolmogorov-Smirnov test and histograms. Parametric statistical analyses (ANOVA), followed by the Scheffé *post hoc* test for homogenous or Dunnet T3 test for non-homogenous variances, were performed on normally distributed data and corresponding non-parametric tests (Kruskal-Wallis U-test) were used for skewed data. A p-value ≤ 0.05 was considered significant (papers 1, 2, 3, 4, 5). $IP_{0.5}$ values (percentage of positive control inhibition at 0.5 µmol TP plate concentration) were used to assess the orders of effectiveness in *S. typhimurium* tests and were determined using the software Derive 6 (Texas Instruments Inc.; papers 1, 2, 3). Linear regression slope calculations were completed using Microsoft Excel 2007, to determine the orders of TP effectiveness in the antioxidant capacity assays (paper 2). Relationships between variables (papers 4, 5) were assessed by performing bivariate correlations (Pearson for parametric, and Spearman rho for non-parametric data; papers 4, 5).

4. RESULTS SUMMARY AND CONCLUSIONS OF ALL PAPERS

4.1. Paper 1: **Bilirubin and related tetrapyrroles inhibit food-borne mutagenesis *in vitro* – implications for pathogenetic processes**

(original research article; *under review*)

Together with BR and BV, additional TPs that are concentrated within the gut, were tested for their anti-genotoxic effects in the *Salmonella* reverse mutation assay, in the presence of the food-borne mutagens AfB1 and PhIP. Bilirubin- and biliverdin dimethyl esters, naturally abundant un-/conjugated BR, BV, PRO, UB and SB were tested within physiologically relevant concentrations (0.01 – 2 µmol/plate; 3.5 – 714 µM). In the presence of metabolic activation (S9), the test compounds and mutagens were added to *Salmonella typhimurium* strains *TA102* and *TA98,* to consider different mechanisms of mutagenesis. Most TPs were clearly anti-mutagenic against both mutagens, with PRO being the most effective compound against AfB1 in strain *TA102* and against PhIP in *TA98*, followed by the bilirubinoids. Within the *TA98*-AfB1 condition, UB was the most effective compound. In all other experiments, the intestinal compounds UB and SB were moderately active. In strain *TA98* against AfB1, BR and BV exerted pro-mutagenic responses, which were likely due to their competition with reactive mutagens for glucuronidation and detoxification. This study reports that intestinally abundant TPs prevent genotoxicity induced by poly-/heterocyclic amines, which could be specifically important for (gut) health. The TPs' mechanisms of anti-genotoxic action might include either direct interaction with mutagens (relevant to strain *TA98* data) or *via* their antioxidant effects (in strain *TA102*).

The data furthermore suggest unconjugated BR and BV could increase the risk for frame-shift mutation in the presence of AfB1, by competing for phase-II detoxification.

4.2. Paper 2: *In vitro* antioxidant capacity and anti-genotoxic properties of protoporphyrin and structurally related tetrapyrroles

(original research article; *accepted*)

A series of newly tested (physiologically relevant) TPs were investigated in the *Salmonella* reverse mutation assay and multiple antioxidant capacity assays (TEAC, ORAC, FRAP) and were compared to already tested, biologically relevant BR and BV. In the absence of metabolic activation (-S9) the order of effectiveness in the *Salmonella* reverse mutation assay based on $IP_{0.5}$ values was PRO > BV > BRDT > BR > BV-DME > SB > BR-DME > UB. In the presence of S9 the anti-mutagenic effectiveness was reversed, suggesting UB > BV-DME > BR-DME > SB > BV > BR > BRDT > PRO. In the antioxidant capacity assays most TPs showed moderate to high antioxidant activities in comparison to trolox and Fe^{2+} with BR, BRDT and BV being specifically active. The only exceptions were UB and SB which possessed no FRAP ability, however, retained moderate ORAC and TEAC activity.

These data are the first to indicate that UB and SB act as antioxidants by donating hydrogen atoms to incumbent radical species. This study's findings show evidence of a strong antioxidant behavior of the TPs BR, BV and BRDT in the *Salmonella* reverse mutation assay, and present the first data on an antioxidant activity of BR-/BV-DME, UB, SB and PRO *in vitro*. Although *in vitro* results cannot be directly transferred to the *in vivo* condition, the data provide preliminary evidence to suggest a possible physiological role of structurally related, partially oxidized TPs, in preventing oxidative DNA damage within the gut. Therefore these compounds could be beneficial if administered enterally for therapeutic means [50].

4.3. Paper 3: *In vitro* physic-chemical properties of tetrapyrroles against TNFone-induced mutagenesis

(original research article; *prepared for submission)*

To explore the physic-chemical background of TP/mutagen interaction, molecular systems of CL e6 porphyrin, BR and BV with TNFone were tested using (V)CD techniques, which consistently revealed suprastoichiometric anti-mutagenic effects of the considered compounds. Addition of TNFone to CL e6 porphyrin, BR/albumin and BV/albumin led to a substantial change in pigment spectral characteristics, providing evidence for tight TP/mutagen interactions especially in the case of protoporphyrin's model substance. These results are reflected in the bacterial model, in which the majority of compounds significantly attenuated TNFone-induced mutagenesis. Protoporphyrin showed the highest anti-genotoxic activity of those compounds tested, followed by the rubinoids (BR, BRDT, BR-DME) and verdins (BV, BV-DME).

This study supports the hypothesis on a strong anti-genotoxic behavior of the tested tetrapyrrolic compounds. In that respect, the obtained novel evidence supports existing data on an anti-mutagenic activity of BR and BV and possible underlying physic-chemical events. Especially PRO continuously showed a remarkable anti-mutagenic potential in the trials and strong underlying PRO/TNFone structural interactions were newly revealed. Bilirubin was found to be the most effective among those compounds with known biological relevance. These data form the crucial basis for further interaction studies which might account for known potent biological effects of BPs.

4.4. Paper 4: **Concentration-dependent bacterial bile pigment absorption and anti-genotoxic compound effects *in vitro***

(short communication; *accepted*)

This study is the first to report on bacterial BP absorption and its relationship with observed anti-mutagenic effects. When exposed to mutagens, extracellular (plate) BP concentrations were negatively correlated to genotoxicity. Furthermore, anti-mutagenic testing in *Salmonella* strain *TA98* revealed that BV and BR absorption was more strongly related with anti-mutagenesis, when compared to the anti-mutagenic effect relative to plate concentrations. HPLC analyses confirmed that bacterial BP absorption was concentration-dependent. Plate BP concentrations were inversely associated with genotoxicity of all tested mutagens, irrespective of strain and test conditions. However, protection against frame-shift mutation in strain *TA98* most strongly depended on the bacterial absorption of BR and BV, which indicates that BPs can protect by intercepting mutations extracellularly and specifically inhibit frame-shift mutations intracellularly. This implicates anti-cancer activity of BPs, since specifically intestinal cancers reportedly are frequently based on frame-shift mutations [185,186].

4.5. Paper 5: ***In vitro*** **DNA-damaging effects of intestinal and related tetrapyrroles in human cancer cells**

(original research article; *under review*)

Epidemiological studies report a negative association between circulating bilirubin concentrations and the risk for cancer and cardiovascular disease. Furthermore, few data suggest TPs exert anti-carcinogenic effects *via* induction of cell cycle arrest and apoptosis. To further investigate whether TPs provoke DNA-damage in human cancer cells, they were tested in the single cell gel electrophoresis assay (SCGE). Eight TPs (un-/conjugated BR, BV, BR-/BV-DME, UB, SB and PRO were added individually to cultured Caco2 and HepG2 cells to measure their effects on comet formation (% tail DNA). Flow cytometric assessment (apoptosis/necrosis, cell cycle, intracellular radical species generation) assisted in revealing underlying mechanisms of intracellular action. Cells were incubated with TPs at concentrations of 0.5, 5 and 17 µM for 24 hrs. Addition of 300 µM *tert*-BOOH to cells served as a positive control. Tetrapyrrole incubation mostly resulted in increased DNA-damage (comet formation) in Caco2 and HepG2 cells. Test compounds that are concentrated within the intestine, including PRO, UB and SB, led to significant comet formation in both cell lines, implicating the compounds in inducing DNA-damage and apoptosis in cancer cells found within organs of the digestive system. In summary, this study revealed substantial DNA-damaging effects of tetrapyrrolic compounds in human cancer cells, particularly for PRO, UB and SB in HepG2 and for PRO in Caco2 cell lines. From this evidence, a chemopreventive effect of the compounds might exist within the liver and intestine. Possible underlying mechanisms of DNA-damage (*e. g.* including ROS formation), leading to cell cycle arrest and subsequent apoptosis induction might be responsible for the toxicity observed in malignant cells.

Subsequent future experiments could focus on clarifying the effects of TPs also in non-malignant cells as well as the pigments' DNA-damaging potential in a range of various other cancer cell lines. Furthermore, exploring the effects of TPs also on chromosomal stability (*e. g.* prevention of micronuclei formation) could represent a valuable future scientific approach.

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7. INDEX OF FIGURES AND TABLES

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9. DEUTSCHE ZUSAMMENFASSUNG (GERMAN SUMMARY)

Gallenpigmente und ihre Derivate sind seit Jahrzehnten Gegenstand wissenschaftlicher Forschung, spezifischer und fokussierter allerdings werden sie erst seit wenigen Jahren untersucht. Wurden die Verbindungen zu Beginn hauptsächlich als unnütze oder gar toxische Neben- und Endprodukte des Häm-Katabolismus betrachtet, werden sie heute zunehmend in Zusammenhang stehend mit Gesundheitsförderung und Krankheitsprävention gesehen. Daten aus *in vivo* Studien legen vasoprotektive, anti-inflammatorische, anti-virale und anti-cancerogene Eigenschaften nahe, und *in vitro* Experimente attestieren Gallenpigmenten anti-genotoxische und anti-apoptotische Aktivität. Trotz solch vielversprechender Resultate bleiben exakte zugrundeliegende Wirkmechanismen weitestgehend unbekannt, und sind Gegenstand aktueller Forschungen.

Speziell die Prävention weltweit zunehmender kardiovaskulärer Erkrankungen und Krebs, ist von großem medizinischem sowie ökonomischem Interesse, und unterstreicht die Wichtigkeit fundamentaler *in vitro* Grundlagenforschung in dem Bereich. Speziell die Erforschung natürlicher, endogen synthetisierter Verbindungen wie beispielsweise des Bilirubins bezüglich seiner Wirkmechanismen, stellt eine Voraussetzung dar um mögliche gesundheitlich vorteilhafte physiologische Effekte moderat erhöhter Bilirubinspiegel abzuschätzen. Daher war es das Ziel dieser Studie *1)* bestehende Daten zu *in vitro* anti-mutagenen, anti-oxidativen und anti-genotoxischen Effekten um bis dato ungetestete Tetrapyrrole zu erweitern, und *2)* zugrundeliegende Wirkmechanismen ihrer Effekte zu erforschen.

Die Studie wurde im Rahmen des FWF-Projekts *„The physiological relevance of bile pigments“* (Projekt Nr. P21162-B11) durchgeführt, und konzentrierte sich auf *in vitro* Bakterien- und Zellkulturmodelle.

Zusammenfassend zeigte die Mehrzahl der getesteten Gallenpigmente und Derivate anti-genotoxische Effekte gegen eine Vielzahl unterschiedlicher Mutagene im *Salmonella* Assay. Weiters bestätigte sich ein vermutetes anti-oxidatives Potential in

den durchgeführten Tests zur anti-oxidativen Kapazität der Testsubstanzen, und es zeigte sich im Comet assay DNA-Schädigung in Krebszellen, was ein toxisches Verhalten von Gallenpigmenten in entarteten Zellen im *in vitro* Modell nahelegt. Diese Resultate bestätigen zum Einen bereits bestehende Ergebnisse bezüglich eines anti-mutagenen/anti-oxidativen Potentials von Tetrapyrrolen, und stellen zum Anderen durch die Erweiterung der Testbatterie um bislang ungetestete Tetrapyrrole und die Anwendung ergänzender Testverfahren, komplett neue Ergebnisse aus dem Bereich der Mutationsforschung dar.

10. LIST OF FURTHER PUBLICATIONS

Wallner M, Blassnigg SM, Marisch K, Pappenheim MT, Müllner E, Mölzer C, Nersesyan A, Marculescu R, Doberer D, Knasmüller S, Bulmer AC, Wagner K-H. **Effects of unconjugated bilirubin on chromosomal damage in individuals with Gilbert's syndrome measured with the micronucleus cytome assay.** Mutagenesis 2012. doi: 10.1093/mutage/ges039

Carvalho-Wells AL, Helmholz K, Nodet C, Mölzer C, Leonard C, McKevith B, Thielecke F, Jackson KG, Tuohy KM. **Determination of the in vivo prebiotic potential of a maizebased whole grain breakfast cereal: a human feeding study.** British Journal of Nutrition 2010; 104 (09): 1353 – 1356.

Conference attendance - oral and poster presentations

The role of bilirubin in human health: implications for oxidative stress-mediated diseases.
Lifestyle, oxidative stress and diabetes mellitus (Modra, Slovakia), November 8 – 9 2012 (oral presentation)

***In vitro* anti-carcinogenic effects of bile pigments and derivatives – evidence from a cell culture study.**
Trieste Yellow Retreat 2012 (Trieste, Italy), June 21 – 22 (oral presentation).

Anti-genotoxic potential of bile pigments – evidence from the Vienna studies
Trieste Yellow Retreat 2011 (Trieste, Italy), June 6 – 7 (oral presentation).

Anti-genotoxic properties of urobilin, stercobilin and protoporphyrin *in vitro.*
Trieste Yellow Retreat 2011 (Trieste, Italy), June 6 – 7 (poster presentation).

Antioxidative and antimutagenic effects of selected bile pigments *in vitro.*
Trieste Yellow Retreat 2010 (Trieste, Italy), March 8 – 9 (oral presentation).

Bacterial bile pigment uptake is concentration dependent.
ASTOX/ANTIOX Symposium 2011 (Vienna, Austria), November 24 (abstract and poster presentation)

Protoporphyrin induces cancer cell damage in the SCGE assay.
ASTOX/ANTIOX Symposium 2011 (Vienna, Austria), November 24 (abstract and poster presentation)

Anti-genotoxic properties of urobilin, stercobilin and protoporphyrin *in vitro.*
ASTOX/ANTIOX Symposium 2011 (Vienna, Austria), November 24 (abstract and poster presentation)

The physiological potential of Bilirubin: A possible antigenotoxic effect?
ISFE Symposium 2011 (Vienna, Austria), December 1 – 2 (poster presentation)

CURRICULUM VITAE

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Date of Birth:	Jan 28th, 1983 in Vienna
Citizenship:	Austria

Current position:

Since April 2009, scientific employee (since May 2012 holding prae-doc position) at the Dept. of Nutritional Sciences, Faculty of Life Sciences, University of Vienna, co-worker within the FWF-Project (No. P21162*) "The physiological relevance of bile pigments: In vitro to in vivo evidence of anti-oxidant, anti-mutagenic, anti-carcinogenic potential and their mechanisms of action",* PhD-student of Nutritional Sciences, tutor and lecturer at the Dept. of Nutritional Sciences.

Education:

July 2007	High School Diploma
Oct 2002 – July 2007	Study of Nutritional Sciences
Feb 2003 – Sept 2008	Study of Sports Sciences Faculty of Life Sciences, University of Vienna Title of master thesis: *"Anti-/Mutagenic and Anti-/Oxidative potential of heating products based on alanin, dextrin 10, glucose and maltose"* Supervisor: Univ.-Prof. Dr. Karl-Heinz Wagner
July 2007	Graduated as Mag. rer. nat. (Nutritional Sciences)
Sept 2008	Graduated as Bakk. rer. nat. (Sports Sciences)
Since April 2009	PhD student at the Dept. of Nutritional Sciences Title of dissertation: *"Anti-mutagenic and anti-oxidative effects of novel bile pigments in in vitro assays"*

Research activities abroad:

Oct 2007 – Dec 2007	Research visit, Dept. of Food Biosciences, University of Reading (UK)
May 2008 – Oct 2008	Research visit, Dept. of Food Biosciences, University of Reading (UK)
Oct 2011 – Dec 2011	Visiting staff, School of Medical Sciences, Griffith University (AUS)

Work Experience:

Since April 2009	Scientific Assistant at the Dept. of Nutritional Sciences

Since Feb 2009	Tutor at the Dept. of Nutritional Sciences
Since Nov 2010	Lecturer at the Dept. of Nutritional Sciences

Professional Qualifications:

- Analytical skills: Cell cultures, bacterial (batch) cultures, Salmonella reverse mutation assay, Fluorescent *in situ* hybridization technique (FISH), Flow cytometry, SCGE/comet assay, Micronucleus assay, Fluorescent-/Microscopy, Antioxidant capacity assays (TEAC, ORAC, FRAP)
- Designing of studies, experience with ethical commissions, statistics (SPSS), *in vitro* trials
- PC skills: Word, Excel, Power Point, statistics and graphic programs
- Languages: German (first language), English (fluent), French (good knowledge)
- Experience in planning and execution of meetings and conferences
- Supervision and mentoring of master- and diploma students
- **Co-evaluation of diploma- and master theses:**

The anti-mutagenic and antioxidative potential of bilirubin dimethyl ester and biliverdin dimethyl ester in the Ames Salmonella assay (Gesa Ziesel)

The antimutagenic and antioxidant potential of stercobilin and urobilin in the Ames Salmonella test (Andrea Steyrer)

Antimutagenic and antioxidative effects of specific bile pigments in the Salmonella Ames Assay (Hedwig Huber)

Untersuchung der Effekte von Gallenpigmenten auf Caco 2 Zellen mittels SCGE Assay (Barbara Pfleger)

Die Wirkung von Tetrapyrrolen auf HepG2 Zellen im Comet Assay (Antonia Rossmann)

Investigation of porphyrin effects in Caco2 cells (SCGE assay) (Elisabeth Putz)

The effects of tetrapyrroles in HepG2 cells using comet Assay (Ursula Schwarz)

Die anti-/mutagene Wirkung von Safranextrakten im Salmonella reverse mutation assay (Roland Holler)

Personal interests:

- Endurance sports (running, cycling, mountainbiking)
- Hiking, mountaineering, climbing
- Travelling
- Licensed massage-therapist (since May 2011)

Appendix

PUBLICATIONS AND MANUSCRIPTS

Bilirubin and related tetrapyrroles inhibit food-borne mutagenesis *in vitro* – implications for pathogenetic processes

Christine Mölzer[a,*], *Hedwig Huber*[a], *Andrea Steyrer*[a], *Gesa Ziesel*[a], *Marlies Wallner*[a], *Andrew C. Bulmer*[b], *Karl-Heinz Wagner*[a, b]

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Key words: *Salmonella typhimurium*, protoporphyrin, urobilin, stercobilin, PhIP, AfB1

Abstract

Bilirubin exerts physiologically important antioxidant effects and is therefore hypothesised to protect against degenerative diseases. Recent *in vitro* studies also indicate its anti-mutagenic action, which is shared by its structurally related precursor, biliverdin. Additional tetrapyrroles, concentrated within the gut, were therefore tested for their anti-genotoxic effects in the *Salmonella* reverse mutation assay, in the presence of the food-borne mutagens aflatoxin B1 (AfB1) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Bilirubin- and biliverdin dimethyl esters, naturally abundant un-/conjugated bilirubin, biliverdin, protoporphyrin, urobilin and stercobilin were tested within physiologically relevant concentrations (0.01 – 2 μmol/plate; 3.5 – 714 μM). In the presence of metabolic activation (S9), the test compounds and mutagens were added to *Salmonella typhimurium* strains *TA102* and *TA98*. Most tetrapyrroles were clearly anti-mutagenic against both mutagens, with protoporphyrin being the most effective against AfB1 in strain *TA102* and against PhIP in *TA98*, followed by the bilirubinoids. Within the *TA98*-AfB1 condition, urobilin was the most effective compound. In all other experiments, the intestinal compounds urobilin and stercobilin were moderately active. In strain *TA98* against AfB1, bilirubin and biliverdin exerted pro-mutagenic responses, which were likely due to their competition with reactive mutagens for glucuronidation and detoxification. This study reports that intestinally abundant tetrapyrroles prevent genotoxicity induced by poly-/heterocyclic amines, which could be specifically important for (gut) health. Furthermore, the data suggest unconjugated bilirubin and biliverdin could increase the risk for frame-shift mutation in the presence of AfB1, by competing for phase-II detoxification.

AfB1: aflatoxin B1; **AfM1:** aflatoxin M1; **AfQ1:** aflatoxin Q1; **2-AF:** 2-aminofluorene; **BP(s):** bile pigment(s); **BR:** unconjugated bilirubin; **BR-DME:** bilirubin dimethyl ester; **BRDT:** bilirubin ditaurate (disodium); **BV:** biliverdin hydrochloride; **BV-DME:** biliverdin dimethyl ester; **BVR:** biliverdin reductase; **CYP(s):** cytochrome P450; **DMSO:** dimethyl sulfoxide; **HCA(s):** heterocyclic amine(s); **HMOX-1:** haem oxygenase 1; **p-/HCA(s):** poly- and heterocyclic amine(s); **PhIP:** 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; **PRO:** protoporphyrin IX; **SB:** stercobilin hydrochloride; ***S. typhimurium:*** *Salmonella typhimurium;* **TA98/102:** *Salmonella typhimurium* strains TA98 and TA102; **TP(s):** tetrapyrrole(s); **UB:** urobilin hydrochloride.

1. Introduction:

Aflatoxin B1 (AfB1) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) represent food contaminating mutagens derived from nuts, cereals and cooked meat [1, 2]. Upon absorption and transport to the liver, the molecules undergo oxidation and glucuronidation by cytochrome systems (CYPs) and UDP-glucuronosyltransferases (UGTs) [3], prior to excretion in a process commonly referred to as phase-I and -II detoxification. Esterification of bioactivated intermediates in the liver represents a key event, limiting the concentration and time that the short lived reactive oxygenated molecules can potentially react with DNA [4]. Such interaction leads to DNA strand breaks [5], mutation [6, 7] and adduct formation [8, 9], all of which are important precursors to malignant transformation, particularly in the liver, biliary and gastrointestinal tract [10-14].

Aflatoxin B1 is metabolized involving CYP1A1, CYP1A2 (formation of exo-/endo-epoxide and AfM1) and CYP3A4 (formation of AfQ1 and exo-epoxide [15, 16], which can be found in the urine as biomarkers of exposure [17]). Overexpression of CYP1A1/-1A2 after AfB1 exposure is documented in cultured human hepatocytes [18]. In the human liver however, previously mentioned CYP3A4 predominates and differences in enzyme expression (e.g. CYP3A5) due to genetic polymorphisms influence inter-individual AfB1 susceptibility [19, 20].

The main cytochrome system involved in PhIP activation is CYP1A2 which transforms the compound to its 2-hydroxy-amino intermediate. Subsequent conjugation/esterification by sulfotransferase and/or acetyltransferase generates o-sulfonyl or o-acetyl esters [21]. However, in humans PhIP metabolism is dominated by glucuronidation (UGT-glucuronosyltransferase isoenzymes; UGT1) [22]. The UGT1 complex (especially UGT1A and UGT1B) contributes substantially to the biotransformation of PhIP to its main metabolites 2-hydroxy-PhIP and 4'-hydroxy-PhIP [23, 24].

Importantly, the liver also plays an essential role the metabolism of relevant, potentially protective molecules with porphyrin structure [25-28]. For example unconjugated BR [29-32] and its derivatives are endogenous haem catabolites formed within the liver and spleen. Haem oxygenase (HMOX-1/-2), and NADPH/biliverdin reductase (BLVRA) generate BV and subsequently BR, which is glucuronidated in the liver by the UGT1A family [33]. Interestingly, deficiency in UGT1A1 activity, as seen in Gilbert's Syndrome, results in a mildly elevated unconjugated hyperbilirubinemia [25-28, 34] and may interfere with xenobiotic metabolism. After excretion into the bile, conjugated BR is further metabolized in the gut, forming urobilin (UB) and stercobilin (SB), which are re-absorbed or eliminated via the urogenital- (UB) and the intestinal tract (SB). Due to their accumulation in the gut, UB and SB could provide protection

from carcinogenesis by physically interacting with food-borne contaminants [35, 36] hindering their absorption, or by reacting with oxygenated mutagen intermediates in the liver [28], preventing oxidative DNA damage/adduct formation.

Current evidence indicates that elevated blood BR concentrations are strongly associated with chemo prevention [37, 38] cardio protection [39, 40], improved glucose tolerance [41], beneficial lipid profile, reduced body mass [42], and with a better prognosis in neurodegenerative disease [43]. Data mostly on BR and BV support strong multimodal anti-mutagenic [25, 26] and antioxidant activities of bile pigments (BPs) [44-46] in vitro and in vivo [30-32, 46, 47]. Furthermore in vitro anti-carcinogenic properties including pro-apoptotic and anti-proliferative protoporphyrin (PRO) effects have been reported [48-50]. Despite these findings, the ability of tetrapyrroles (TPs) to inhibit DNA-damage caused by dietary-derived mutagens remains unexplored. Revealing inhibitory effects against these agents would represent very important contributions to understanding the chemo-preventative effects of BR and related TPs in epidemiological studies. Furthermore, by exploring the effects of structurally related compounds including BR's and BV's dimethyl esters (BR-/BV-DME) would assist in understanding the importance of molecular interaction between TPs and mutagens, and the role of TP glucuronidation in the bioactivation of pro-mutagens/-carcinogens. Furthermore, with significant amounts of UB, SB and PRO within the gut, revealing novel effects of these compounds could lead to the development of additional therapeutics to prevent cancer in at-risk-populations [51].

A few studies have explored the effects of structurally related compounds including chlorophyllin, and suggest anti-mutagenic activity through test compound/mutagen complex formation [51-54]. The present study aimed to 1) reveal the effects of endogenous TPs on AfB1- and PhIP- (dietary-derived) induced mutation, and 2) demonstrate the importance of BP physico-chemical structure/competition for phase-II glucuronidation on DNA mutation.

2. Materials and methods

2.1. Salmonella reverse mutation assay:

The Salmonella reverse mutation assay is a well-accepted screening test to evaluate the mutagenic potential of chemicals in vitro [55]. Mutagenic substances revert mutated bacteria to their wild type variant, which allows them to grow. Genotoxicity (or vice versa anti-genotoxic

effects) of compounds can therefore be assessed by quantifying bacterial growth. The conducted experiments followed the method of Maron and Ames [56] and included 25 min of pre-incubation. S9 liver homogenate (S9 microsomal fraction from Aroclor 1254 pre-treated rats) was used as an enzymatic activation system in all of the assays.

2.1.1. Chemicals:

Bilirubin IXα (BR) [CAS# 635-65-4], bilirubin conjugate (ditaurate) disodium (BRDT) [CAS# 635-65-4], biliverdin IXα (BV) [CAS# 55482-27-4], bilirubin dimethyl ester (BR-DME) [CAS# 19792-68-8], biliverdin dimethyl ester (BV-DME) [CAS# 10035-62-8], protoporphyrin IX (PRO) [CAS# 553-12-8] as well as urobilin (UB) [CAS# 28925-89-5] and stercobilin (SB) [CAS# 34217-90-8] were purchased from Frontier Scientific, UK. Compound purity (> 98 %) and solubility were assessed using high pressure liquid chromatography (HPLC) and spectrophotometry. The S9 liver homogenate was from MP Biomedicals, Illkirch (France). All other reagents were from Sigma Aldrich Austria (unless otherwise noted), were of the highest analytical grade available and were stored and used according to instructions. 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine was purchased from Toronto Research Chemicals, Canada). Test compounds *(supplementary material 1)* were solubilised in DMSO (CAS# 67-68-5) were protected from light using foil, and used immediately. Composition and preparation of all necessary reagents and solutions can be found elsewhere [26].

2.1.2. Bacterial strains:

S. typhimurium strains were obtained from Dr. Bruce N. Ames (University of California, Berkley, USA). Frozen permanents were stored at -80 °C until use. Prior to the tests being conducted, the strains were attested to their genetic integrity and spontaneous mutation rate [55]. Two distinct strains were adopted to consider multiple mechanisms of protection from mutation: the DNA repair proficient strain *TA102 (hisG428 mutation),* detecting A/T base pair damage and small deletions provoked by cross-linking agents, can be reverted by mutagens causing oxidative damage, and *TA98 (hisD3052 mutation)* primarily detecting G/C base pair and frame-shift mutations. The *Salmonella* strains used in the experiments have different mutations in various genes in the histidine operon; each of these mutations is designed to be responsive to mutagens that act via different mechanisms [55].

2.1.3. Sample preparation:

Following preceding experiments [26] and a series of solubility assays, six doses of BRDT, BV and previously untested BR-DME, BV-DME, UB, SB and PRO were investigated at 0.01, 0.05, 0.1, 0.5, 1 and 2 µmol/plate (3.5 µM – 714 µM). Bilirubin was tested over a range of five doses including: 0.01, 0.05, 0.1, 0.5 and 0.75 µmol/plate (3.5 µM – 268 µM). The respective maximum sample doses had been ascertained by *1)* testing the maximum amount of DMSO that did not result in bacterial cytotoxicity (350 µl/plate, approx.. 12 % v/v) and *2)* by the respective maximum solubility of each compound (supernatant analysis via spectrophotometry: BR, BRDT, BR-DME: 455 nm; PRO: 410 nm; UB, SB: 400 nm; BV, BV-DME: 380 nm; read on a Perkin Elmer Lambda 2 UV/VIS spectrophotometer after high-speed centrifugation; HPLC analysis was performed as previously published [57, 58]).

2.1.4. Positive and negative controls:

Table 1. Positive controls in use. DMSO served as a negative control.

Compound	Occurrence	Class	Solvent	Strain(s)	Conc. [mol/plate]	S9^
Aflatoxin B1 (AFB1)	Food borne (agricultural produce, nuts, cereals) [19]	Planar polycyclic amine; mycotoxin; mutagen	DMSO	*TA98*	0.8×10^{-7}	+
				TA102	0.24×10^{-6}	+
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)	Food borne; high temperature cooking of meat [40, 41]. Alternative source: tobacco smoke [72]	Planar heterocyclic amine (HCA), 2-aminoimidazole structure [73]; mutagen	DMSO	*TA98*	0.1×10^{-7}	+

^S9: „+“ indicates metabolic activation was used.

2.2. Experimental design:

The assays were performed in a dimly lit laboratory under sterile working conditions (Laminar airflow cabinet Safemate 1.8; Bioair Euroclone, Italy). Samples were protected from light throughout the experiments and were freshly prepared in amber vials before each test. 500 µl of PBS buffer or S9-mix (19.75 ml $_dH_2O$, 25 ml PBS buffer, 500 µl $MgCl_2$ (0.85 M), 0.5 ml KCl (1.65 M) and 2 ml NADP (90.8 mM), 250 µl glucose-*6*-phosphate (1.08 M) and 2 ml of S9.), 100 µl of overnight culture, 200 µl of TP solution (in DMSO) were merged in sterile test tubes. For anti-mutagenic testing 100 µl of mutagen (in DMSO or DMSO alone for controls) were added to each tube *(Table 1)*. After 25 mins of pre-incubation (37 °C, on rotary-shaker) 2 ml of molten top agar were added to every tube. The mixtures were poured onto minimum glucose agar plates which were incubated at 37 °C for 48 hrs. His^+ revertants were counted manually after having routinely checked the background lawn under a microscope (40 x magnification; Olympus CH-2). DMSO final plate concentrations did not exceed 10 % v/v.

Each sample was tested in triplicate and all of the assays were independently repeated again. Every test included three "positive control plates" (mutagen only) and six "negative control plates" (no mutagen, no pigment) as well as three "no treatment plates" (no mutagen, no pigment, no DMSO).

For a substance to be classified genotoxic in the *Salmonella* reverse mutation assay, the number of revertant colonies on the test compound plates had to exceed twice the number of colonies grown on the solvent control plates (negative control) [55].

In addition to investigating the anti-mutagenic effects of TPs, the compounds' spontaneous mutagenic activities were tested; for each sample the respective highest and lowest concentrations were applied without mutagen addition. None of the tested samples caused mutagenesis in either of the bacterial strains ($p \geq 0.05$).

2.3. Statistical analysis:

Data were analyzed using IBM SPSS 17.0 for Windows. A *p*-value ≤ 0.05 was considered significant. All data are presented as mean ± *SD*. Data were tested for normal distribution (Kolmogorov-Smirnov test). Parametric statistical analysis (one-way analysis of variance, and the Scheffé *post hoc* test) were performed on normally distributed data and corresponding non-parametric tests (Kruskal-Wallis test, Dunnet T3 *post hoc* test) on not normally distributed data. To assess the order of effectiveness of the tested compounds, data were analyzed using $IP_{0.5}$ values (percentage positive control inhibition at 0.5 µmol/plate test compound)

3. Results

3.1. AfB1-induced mutagenesis in strain TA102

Bilirubin, BRDT and BR-DME all significantly lowered AfB1-induced mutagenic effects *($p < 0.01$)*. Within this compound group BRDT was most effective *(Fig 1A)*.

As presented in *Fig 1B*, both BV and its methyl ester derivative significantly lowered revertant counts versus positive control *($p < 0.001$),* with BV being most effective.

PRO was the most effective of all tested compounds in *TA102 (Fig 1C)*. In a dose-dependent manner it significantly lowered revertant counts versus positive control *($p < 0.001$)*. Urobilin attenuated AfB1-induced mutation in a dose-dependent manner, with all tested concentrations indicating significant anti-mutagenic effects *($p < 0.05$)*. For SB, an anti-mutagenic tendency without dose-dependency was observed. At 0.01, 0.05, 0.1, 0.5 and 1 μmol/plate, SB reduced the number of revertants significantly versus positive control *($p < 0.05$)*. The overall order of effectiveness based on $IP_{0.5}$ values was calculated as follows (see *Table 2*): PRO > BR = BRDT > BV > BR-DME > SB > UB > BV-DME.

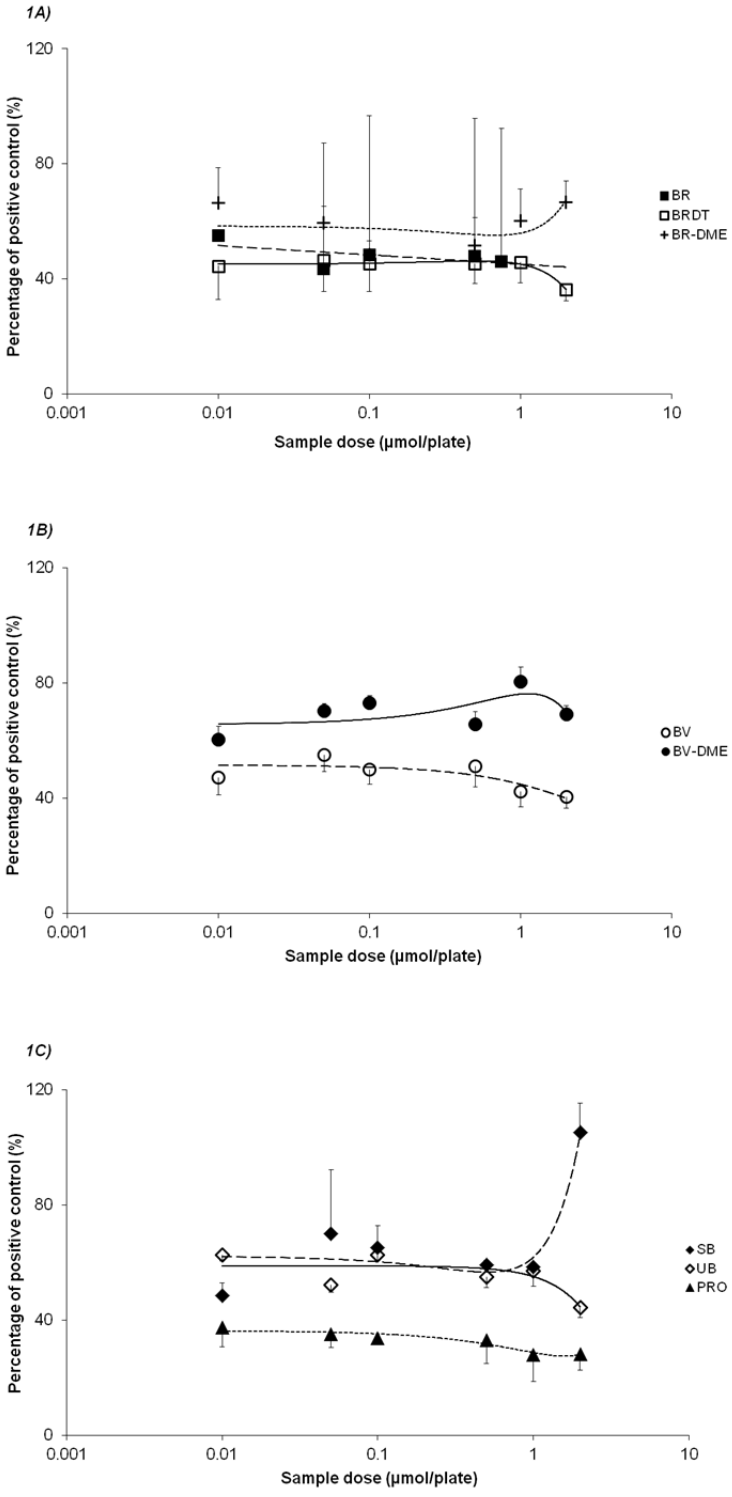

Figure 1A–C. Anti-mutagenic effects of A) BR, BRDT, BR-DME; B) BV, BV-DME and C) UB, SB, PRO against metabolically activated AfB1-induced mutagenesis in *Salmonella typhimurium* strain *TA102*.

BR: unconjugated bilirubin, BRDT: bilirubin ditaurate (conjugated bilirubin), BR-DME: bilirubin dimethyl ester, BV: biliverdin, BV: biliverdin dimethyl ester, UB: urobilin, SB: stercobilin, PRO: protoporphyrin; AfB1: aflatoxin B1 (0.24×10^{-6} mol/plate).

3.2. AfB1-induced mutagenesis in strain TA98

In contrast to *TA102* results, neither BR nor BRDT acted in an anti-mutagenic manner in strain *TA98*, and increased revertant formation significantly *($p < 0.001$).* However, a significant anti-mutagenic behavior was found over the entire range of BR-DME concentrations *($p < 0.001$; Fig 2A).*

As presented in *Fig 2B,* a similar observation indicated that BV significantly increased revertant formation, whereas its esterified counterpart was significantly anti-mutagenic versus positive control *($p < 0.001$),* at all concentrations.

Urobilin was the most anti-mutagenic of all tested compounds *(Fig 2C).* At all concentrations, UB significantly lowered AfB1-induced mutagenesis by up to 75 % *($p < 0.05$)*. At 1, 2 and 0.5 µmol/plate PRO also lowered revertant counts compared to positive control values. As for SB, significant anti-mutagenic effects at all concentrations (except for 2 µmol/plate) were reported *($p < 0.05$)*. However, the observed effects were not dose-dependent. The overall order of effectiveness based on $IP_{0.5}$ values was calculated as follows *(see Table 2)*: UB > BV-DME > BR-DME > SB > PRO. Bilirubin ditaurate, BR and BV did not attenuate AfB1-induced mutagenesis in this condition.

3.3. PhIP-induced mutagenesis in TA98

Bilirubin, BRDT and BR-DME *(Fig 3A)* were highly effective against PhIP-induced mutagenesis when compared to positive control values *($p < 0.001$)*. All samples attenuated PhIP-induced mutagenesis by over 60 % and appeared equally effective.

A strong dose-dependent effect was noted for BV but not for BV-DME *(Fig 3B).* However, both compounds significantly lowered revertant counts over the entire concentration range versus positive control *($p < 0.001$).*

Both, SB and UB were significantly effective at all tested doses *($p < 0.05$)* and showed strong anti-mutagenic effects towards PhIP-induced genotoxicity. Protoporphyrin significantly lowered PhIP-induced effects by more than 90 %, resulting in a nearly complete PhIP detoxification *($p < 0.001$).* In addition, strong concentration-dependent effects were seen with all of the tested compounds *(Fig 3C).* The overall order of effectiveness was calculated as follows (see *Table 2*): PRO > BR > BV > BR-DME > BRDT > SB > BV-DME > UB.

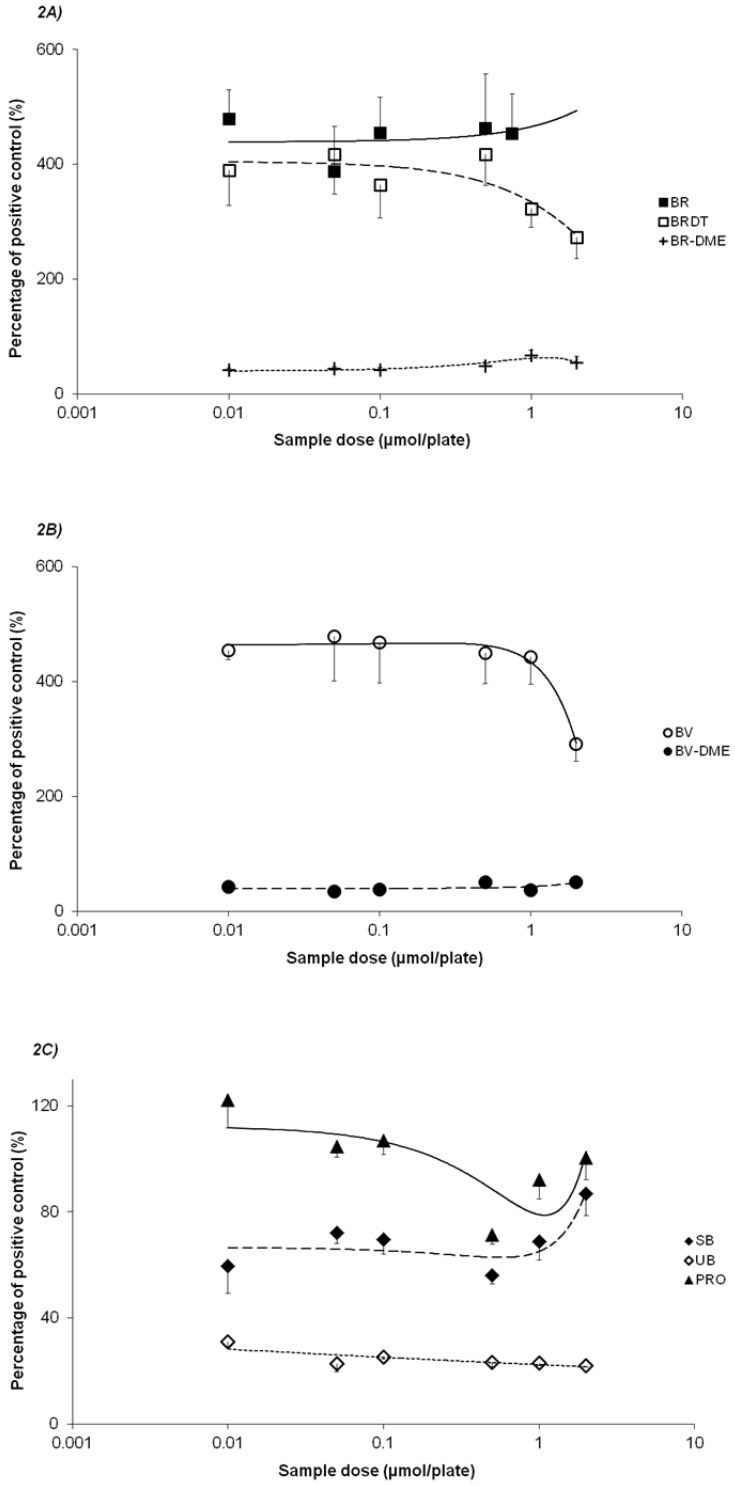

Figure 2A–C. Anti-mutagenic effects of A) BR, BRDT, BR-DME; B) BV, BV-DME and C) UB, SB, PRO against metabolically activated AfB1-induced mutagenesis in *Salmonella* strain *TA98.*
BR: unconjugated bilirubin, BRDT: bilirubin ditaurate (conjugated bilirubin), BR-DME: bilirubin dimethyl ester, BV: biliverdin, BV: biliverdin dimethyl ester, UB: urobilin, SB:

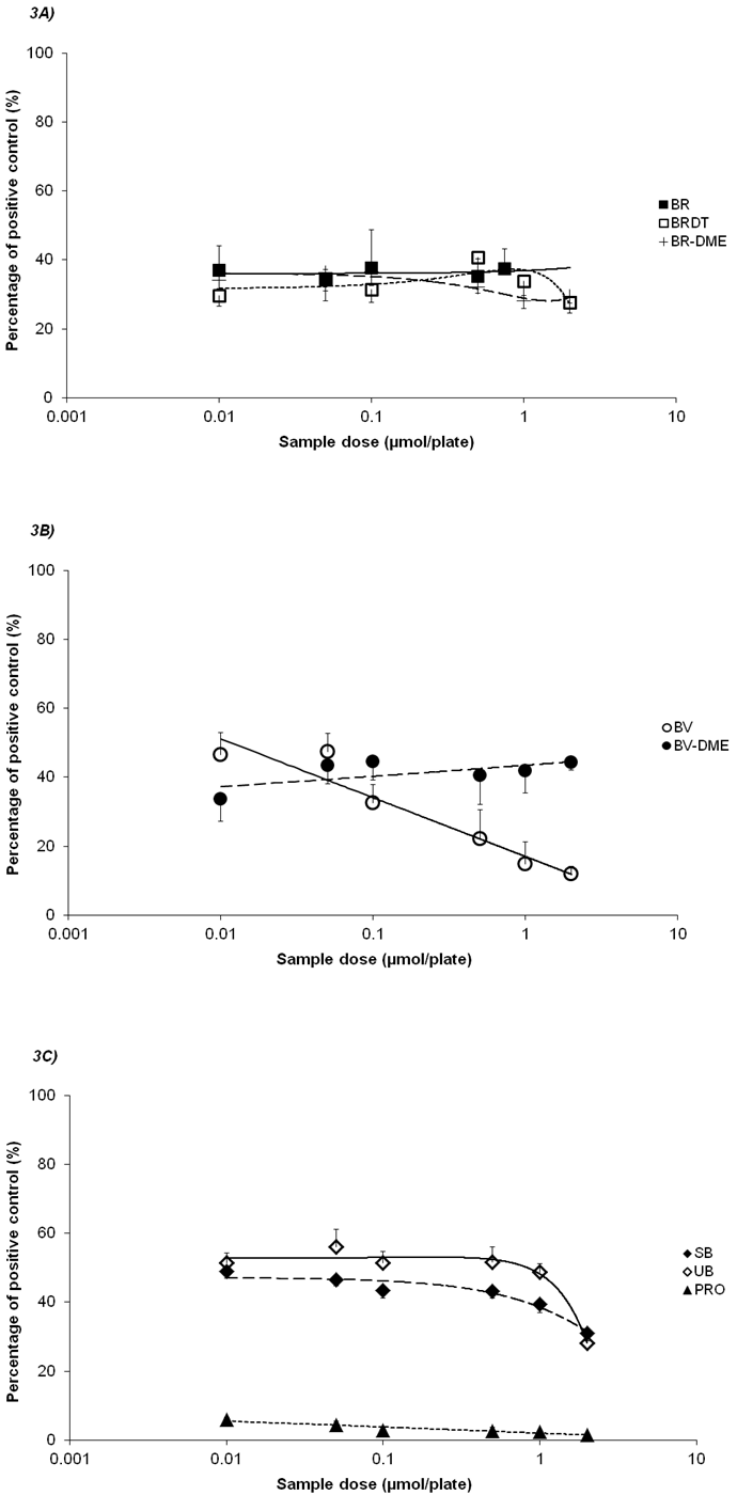

Figure 3A–C. Anti-mutagenic effects of A) BR, BRDT, BR-DME; B) BV, BV-DME and C) UB, SB, PRO against metabolically activated PhIP-induced mutagenesis in *Salmonella* strain *TA98*.
BR: unconjugated bilirubin, BRDT: bilirubin ditaurate (conjugated bilirubin), BR-DME: bilirubin dimethyl ester, BV: biliverdin, BV: biliverdin dimethyl ester, UB: urobilin, SB: stercobilin, PRO: protoporphyrin; PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

Table 2. Anti-mutagenic behaviour of tetrapyrroles against metabolically activated AfB1 and PhIP in *Salmonella typhimurium* strains *TA102* and *TA98.*

Strain	Mutagen [mol/plate]	S9	Compound	$IP_{0.5}$ [pos control inhibition, %]°	Sign. different from ($p \leq 0.05$)#	His^+_{pos} ± SD^
TA102	AFB_1 0.24 x 10^{-6}	+	BR	- 54	BV-DME, UB, SB, PRO	1142 ± 112
			BRDT	- 54	BV-DME, UB, SB, PRO	
			BR-DME	- 45	BR, BRDT, BV, BV-DME, UB, PRO	
			BV	- 52	BR-DME, BV-DME, UB, SB, PRO	
			BV-DME	- 27	BR, BRDT, BR-DME, BV, BV-DME, UB, SB, PRO	
			UB	- 42	BR, BRDT, BR-DME, BV, BV-DME, PRO	
			SB	- 43	BR, BRDT, BV, BV-DME, PRO	
			PRO	- 68	BR, BRDT, BR-DME, BV, BV-DME, UB, SB, PRO	
TA98	AFB_1 0.8 x 10^{-7}	+	BR	+ 352	BRDT, BR-DME, BV-DME, UB, SB, PRO	318 ± 26
			BRDT	+ 268	BR, BR-DME, BV, BV-DME, UB, SB, PRO	
			BR-DME	- 45	BR, BRDT, BV, BV-DME, UB, SB, PRO	
			BV	+ 363	BRDT, BR-DME, BV-DME, UB, SB, PRO	
			BV-DME	- 59	BR, BRDT, BR-DME, BV, UB, SB, PRO	
			UB	- 79	BR, BRDT, BR-DME, BV, BV-DME, SB, PRO	
			SB	- 37	BR, BRDT, BR-DME, BV, BV-DME, UB, PRO	
			PRO	- 11	BR, BRDT, BR-DME, BV, BV-DME, UB, SB	
TA98	PhIP 0.1 x 10^{-7}	+	BR	- 82	BRDT, BR-DME, BV-DME, UB, SB, PRO	789 ± 77
			BRDT	- 64	BR, BR-DME, BV, BV-DME, UB, SB, PRO	
			BR-DME	- 68	BR, BRDT, BV, BV-DME, UB, SB, PRO	
			BV	- 78	BR, BRDT, BR-DME, BV-DME, UB, SB, PRO	
			BV-DME	- 55	BR, BRDT, BR-DME, BV, UB, PRO	
			UB	- 48	BR, BRDT, BR-DME, BV, BV-DME, SB, PRO	
			SB	- 57	BR, BRDT, BR-DME, BV, UB, PRO	
			PRO	- 97	BR, BRDT, BR-DME, BV, BV-DME, UB, SB	

BR: unconjugated bilirubin, BRDT: bilirubin ditaurate, BR-DME: bilirubin dimethyl ester, BV: biliverdin, BV-DME: biliverdin dimethyl ester, UB: urobilin, SB: stercobilin, PRO: protoporphyrin. AfB1: aflatoxin B1, PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. °$IP_{0.5}$: Percentage positive control inhibition at 0.5 µmol/plate (highest common sample dose per plate): "-" indicates mutagen attenuating effect, "+"amplifying effect; ^His^+_{pos}: average positive control revertant counts ± SD. #based on $IP_{0.5}$.

4. Discussion

The current study demonstrates BR and structurally related TPs exert anti-mutagenic/anti-oxidant effects, preventing mutation caused by the food-derived mutagens AFB1 and PhIP. Protoporphyrin was highly effective against AfB1- and PhIP-induced mutagenesis in both *Salmonella* strains, followed (in effectiveness) by TPs containing rubinoid structure. The calculated orders of effectiveness based on $IP_{0.5}$ values were: PRO > BR = BRDT > BV > BR-DME > SB > UB > BV-DME for AFB1 in strain *TA102*, UB > BV-DME > BR-DME > SB > PRO for AfB1 in strain *TA98*, and PRO > BR > BV > BR-DME > BRDT > SB > BV-DME > UB for PhIP in the *TA98* strain.

To date the possible mechanisms of TP protection from mutation are not fully understood. Furthermore, the ability of TPs to inhibit DNA-damage caused by dietary-derived mutagens, remains entirely unexplored. In consideration of the different bacterial strains and the mutagens that were used, multiple anti-mutagenic mechanisms must be considered. *Salmonella* strain *TA102* reverts upon exposure to oxidation, whereas the *TA98* strain mutates upon interaction with mutagens.

AfB1-induced mutagenesis:

So far, physico-chemical studies suggest that both aromatic and planar AfB1 characteristics allow for TP complex formation, inhibiting covalent AfB1 interaction with DNA. *In vitro* studies investigating the anti-mutagenic effects of chlorophyllin and related porphyrins show strong non-covalent complex formation with toxins that bear planar and aromatic structures [63-65]. Therefore, complex formation is routinely used as a method to detect polycyclic compounds in foodstuffs [54]. Similarly, *in vivo* data imply complex formation between chlorophyllins and food-borne mutagens in the gut followed by electrophile scavenging and complexing in the target organ [66, 67]. Thus, chlorophyllin and structurally related pigments including the compounds tested here, are expected to inhibit carcinogen-DNA binding *in vivo,* and consequently prevent DNA damage [52, 53], particularly in the intestinal tract, the primary location of food-derived mutagen accumulation. Dashwood *et al.* (1995) proposed the exocyclic amine group of poly- and heterocyclic mutagens consistently bind to acidic groups in chlorophyllin [51]. If that structural anti-mutagenic hypothesis also applies to other porphyrins, it is likely that BR, BV, SB and UB would also effectively inhibit these mutagens, due to their free carboxylic acid groups. This conclusion was in part confirmed in the strain *TA102* test condition *(Figure 1A – C)*, in which the compounds carrying free carboxylic groups were among the most effective against AfB1. However, test compound behaviour was somewhat reversed in the *TA98*-AfB1 experiments. In this test condition, BR and BV incubation

unexpectedly showed elevated revertant counts (associated with a pro-mutagenic potential). Clearly, free carboxylic acid groups were instrumental to this response, because their methyl ester derivatives acted in a reliable anti-genotoxic manner in both tester strains.

A mechanistic explanation for the observed contradictory results could include AfB1-adduct formation at guanine bases in bacterial DNA [68], which the *TA98* strain is particularly prone to. This would result in pronounced AfB1-induced mutagenesis specifically in this strain. Furthermore this strongly supports a role for BR-/BV-DME in anti-mutagenesis, which were clearly effective in *TA98.* This was very much in contrast to their non-methylated free acidic BR and BV counterparts, as can be seen from *Figure 2A - C*. The esterified compounds retained their anti-mutagenic effectiveness also in strain *TA102*. The most likely explanation for BR's and BV's strong pro-mutagenic effects probably implicates their phase-II metabolism which results in their glucuronidation [69]. Reactive AfB1 intermediates could potentially compete with the same metabolic pathway, increasing the concentration of reactive epoxide, prior to any detoxification/glucuronidation. In contrast, BR-/BV-DME could not be conjugated, and thus would not represent UGT1A substrates, allowing for secondary AfB1 metabolism to stable glucuronides [36]. These data *(Figures 2A and 2B)* strongly indicate that carboxylic acid groups on BR and BV molecules are associated with an increase in mutation, which might compete with AfB1 for phase-II detoxification.

Followed by BR, PRO was the most effective agent against AfB1 in strain *TA102*, which confirms the compounds' potent antioxidant activity, seen previously against *tert*-BOOH-induced mutagenesis. The same study suggested PRO as a potent reducing agent due to its extended π-electron system, which enabled considerable electron donation [28], allowing for substantial covalent binding capacity with biomolecules [70, 71]. This strongly supports a role for PRO in inhibiting AfB1-induced mutagenesis by direct interaction with the mutagen, consequently preventing oxidative damage. Protoporphyrin was moderately effective against AfB1 in strain *TA98,* further supporting the possibility of physico-chemical interaction between them.

PhIP-induced mutagenesis:

The possibility of PhIP and PRO interaction, leading the inhibition of mutation was also reported in the present study. As previously observed in the presence of AfB1 (especially in strain *TA102*), PRO was the most effective anti-mutagen also within the *TA98*-PhIP experiment, which under certain test conditions indicates that PRO is a potent inhibitor of frame-shift mutations at concentrations that exist within the intestine (0.2 – 14 μM [72]). Frame-shift mutations are an important underlying mechanistic event of carcinogenesis in gastric- and colon

cancer [73, 74]. Therefore, these results could be of great importance in explaining a possible role for intestinal PRO in preventing gastro-intestinal carcinogenesis. This is supported by a preceding study (in which PRO was not considered) [27]: BR and BV bacterial absorption strongly protected against frame-shift mutation in the *TA98* strain. It was speculated that the affinity of BPs to protect against frame-shift mutation, might partly explain the protective relationship between serum BR levels and colorectal cancer *in vivo* [38].

5. Conclusion

In summary, these data report that intestinally abundant TPs (UB, SB, PRO) prevent genotoxicity induced by dietary-derived mutagens, which could be specifically important for gut health. The TPs' mechanisms of anti-genotoxic action might include either direct interaction with mutagens (relevant to strain *TA98* data) or *via* their antioxidant effects (in strain *TA102*). Furthermore, the data implicate elevated levels of unconjugated BR and BV could increase the risk for frame-shift mutation in the presence of AfB1, by possibly competing for phase-II detoxification.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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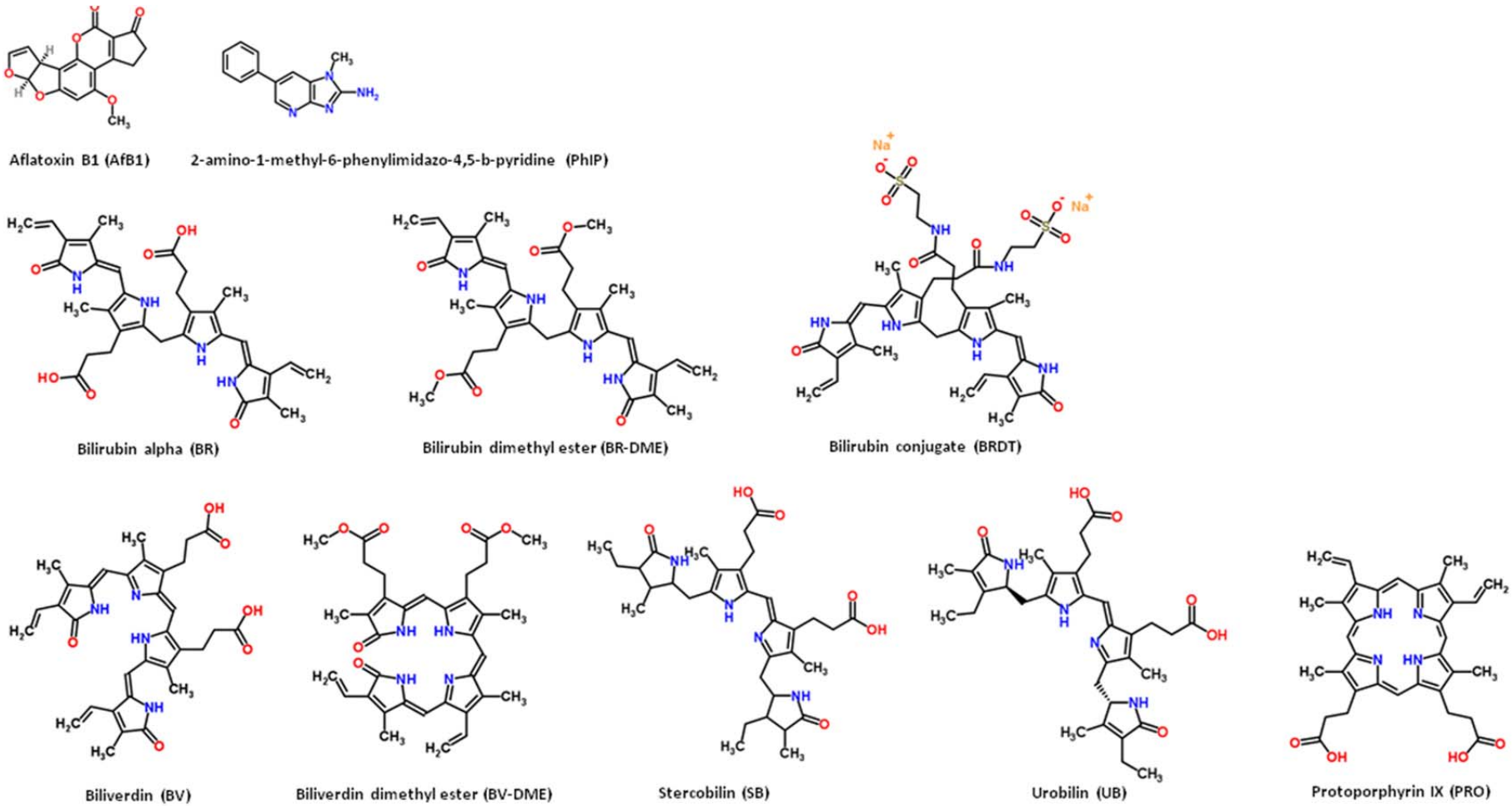

Supplementary material 1. Chemical structures of the test compounds.

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ORIGINAL ARTICLE

In vitro antioxidant capacity and antigenotoxic properties of protoporphyrin and structurally related tetrapyrroles

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Abstract

The antioxidant properties of protoporphyrin IX and related tetrapyrroles are poorly characterized. Therefore, eight tetrapyrroles, five of which are produced *in vivo,* were tested to assess their antioxidant capacities in the *Salmonella* reverse mutation, TEAC, FRAP and ORAC assays. Tertiary-butyl hydroperoxide (*tert*-BOOH) in the presence or absence of metabolic activation (±S9) was added to *Salmonella* strain *TA102* together with the test compounds. In the absence of metabolic activation, the order of effectiveness was protoporphyrin > biliverdin > bilirubin ditaurate > bilirubin > biliverdin dimethyl ester > stercobilin > bilirubin dimethyl ester > urobilin. In the presence of S9, the effectiveness was reversed, with urobilin > biliverdin dimethyl ester > bilirubin dimethyl ester > stercobilin > biliverdin > bilirubin > bilirubin ditaurate > protoporphyrin. In the antioxidant capacity assays FRAP, TEAC and ORAC, mainly bilirubin, bilirubin ditaurate, biliverdin and protoporphyrin showed antioxidant activity. This study reports that previously untested tetrapyrroles of related structure prevent oxidatively induced genotoxicity, and for some, novel underlying mechanisms of antioxidant action were revealed. These results support the physiological importance and biological relevance of tetrapyrroles including protoporphyrin that might act as antioxidants, protecting from oxidatively induced DNA damage, particularly in the tissues/organs where they accumulate.

Keywords: bilirubin, urobilin, stercobilin, *Salmonella*, *tert*-BOOH

Introduction

Bile pigments (BPs) are endogenously formed tetrapyrrolic dicarboxylic acids with porphyrin structure derived from the enzymatic heme degradation [1], which generates both pro- and antioxidant compounds [2], and liberates biliverdin (BV), carbon monoxide and free iron [3]. Subsequently, biliverdin reductase (BLVRA) reduces BV to unconjugated bilirubin (BR) which is glucuronidated and excreted from the body in the bile. The BR metabolites, urobilinogen and urobilinoids, are exclusively found in the intestinal- (urobilin, UB and stercobilin, SB) and the urinary tract (urobilin) [4], where they could contribute to health promotion. So far, the scientific focus has mostly been directed towards the antigenotoxic properties of BV and BR *in vitro* [5–7], their *in vivo* and *ex vivo* antioxidant effects [8–10] and chemopreventive potential [11]. However, the effects of structurally related tetrapyrroles (TPs) have rarely been investigated. Therefore, the antioxidant/antigenotoxic potential of many of the compounds studied here (*e. g.* protoporphyrin (PRO), UB and SB) remain unknown. Furthermore, possible effects of structural modifications to BR and BV on antimutagenicity have not yet been investigated, which might provide mechanistic insight regarding their protective properties. Reduction of oxidative stress through TP antioxidant action could play a key role in lowering the risk for certain diseases *in vivo*. Therefore, testing TPs in terms of their antioxidant capacity *in vitro* would provide essential information on their potential to reduce oxidation processes and subsequent damage to cells.

When examining redox processes within human cell systems, reactive oxygen and nitrogen species (ROS, RONS) possess both potentially beneficial and detrimental effects. When produced in excess, radical species can damage DNA, lipids and proteins, and may thereby contribute to the pathogenesis of cancer and heart diseases [10]. Unconjugated BR represents an important defense against oxidative stress *in vivo* [12]. *In vitro* studies demonstrate that BR scavenges singlet oxygen [13] and serves as a reducing agent for certain (hydro-)peroxides [14]. Also, a number of other RO(N)S can be scavenged by BPs [15], suggesting that BR and BV act similar to vitamin C and scavenge both $1e^-$ and $2e^-$ oxidants [16]. Similar to many physiological antioxidants, BR and BV protect membrane lipids from oxidation by acting in synergy with membrane incorporated α-tocopherol [17]. These effects are enhanced when BR is albumin-bound [18], which fixes the compound in an out-of-plane conformation, beneficial for H-abstraction by radical species [19]. Despite already proven antigenotoxic effects of BR, BV and BRDT against multiple classes of mutagens [5–7], comparatively little is known about the antigenotoxic capacity of structurally related physiological TPs. For example, the antioxidant

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capacity of urobilinoids is only described by Nakamura and colleagues [20], who compared *i*-urobilinogen in terms of its 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity to other known antioxidants such as BR, *α*-tocopherol and *β*-carotene. Among those compounds tested, *i*-urobilinogen most potently inhibited free radical-induced lipid oxidation [20]. The potential of UB and SB remain unexplored.

The antioxidant effects of BR and BV dimethyl esters (BR-/BV-DME), were exclusively investigated by McLean [21], Chepelev [22] and colleagues, who concluded that hydrogen atom transfer, proton-coupled electron transfer and single electron transfer are possible mechanisms of antioxidant action. Despite these findings, it is unknown whether these compounds retain the antigenotoxic capacity of BV and unconjugated BR.

From the little available data on PRO, *in vitro* evidence suggests that the compound exerts antioxidant capacity in the absence of light; however, its relative activity compared to other TPs, remains unknown [23]. Testing these effects is important because heme is catabolized to a number of molecules containing a conjugated system of double bonds *in vivo*, including PRO, BV, BR, UB and SB. To understand the possible physiological importance of these molecules, it is first essential to describe their *in vitro* antioxidant activities, using assays that test different mechanisms of radical reduction.

In summary, the antioxidant/antigenotoxic activity of a small selection of heme catabolites has been investigated so far. Therefore, this study aimed to test the antigenotoxic potential of five novel TPs and discuss mechanisms behind their antioxidant activity by applying multiple *in vitro* assays. These data will assist in understanding their potential beneficial activities in terms of disease prevention.

Materials and methods

Salmonella reverse mutation assay

The *Salmonella* reverse mutation assay is a screening test to evaluate the anti/mutagenic potential of chemicals *in vitro*, and is based on mutagen-induced bacterial wild type reversion. Data were generated as previously published [24]. S9 liver homogenate (S9 microsomal fraction from Aroclor-treated rats) was applied as an enzymatic activation system in specified experiments.

Chemicals. Bilirubin IX*α* (BR) [CAS# 635-65-4], bilirubin conjugate (ditaurate) disodium (BRDT) [CAS# 635-65-4], biliverdin IX*α* (BV) [CAS# 55482-27-4], bilirubin dimethyl ester (BR-DME) [CAS# 19792-68-8], biliverdin dimethyl ester (BV-DME) [CAS# 10035-62-8], protoporphyrin IX (PRO) [CAS# 553-12-8] as well as urobilin (UB) [CAS# 28925-89-5] and stercobilin (SB) [CAS# 34217-90-8] were purchased from Frontier Scientific, UK. Chemical structures of the test compounds can be found in the supplementary section (Supplementary material 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2012.715371). The S9 liver homogenate was purchased from MP Biomedicals, France. All other reagents and mutagens were obtained from Sigma Aldrich, Austria (unless otherwise noted), were of the highest analytical grade available and stored according to instructions. Throughout the test procedures all test compound solutions (DMSO) were protected from light using foil. Composition and preparation of all necessary solutions can be found elsewhere [6].

Bacterial strain. Salmonella typhimurium (S. typhimurium) strain *TA102* was kindly provided by Dr. Bruce N. Ames and stored as frozen permanents at −80°C until use. Prior to the tests being conducted, the strain was attested to its genetic integrity and spontaneous mutation rate [25] in our laboratory. To test for the pigments' ability to prevent oxidative DNA damage, the DNA repair proficient strain *TA102* was selected. This strain detects A/T base pair damage and deletions provoked by cross-linking agents and can be reverted by mutagens that cause oxidative damage, for example through the production of free radicals [25].

Tetrapyrrole sample preparation. Based upon preceding experiments [6], six doses of BRDT, BV and PRO (0.01, 0.05, 0.1, 0.5, 1 and 2 µmol/plate) were applied in the present study to encompass their physiologically relevant concentration range. Bilirubin and previously untested UB, SB and BR-/BV- dimethyl esters (BR-DME, BV-DME) were tested up to 0.75 µmol/plate (due to solubility limitations). Maximum doses had been ascertained by (1) testing the maximum amount of DMSO that did not result in bacterial toxicity and (2) by the respective maximum solubility of each compound (supernatant analysis conducted photometrically at 380 and 450 nm; HPLC analysis after high-speed centrifugation). DMSO toxicity was monitored by screening the background lawn present on minimal glucose agar plates after addition of increasing amounts of DMSO (0–500 µl). The applied positive control/mutagen was synthetic, organic tertiary-butyl hydroperoxide (*tert*-BOOH; Merck, Austria), diluted in dH_2O and used at a concentration of 0.75×10^{-6} mol/plate. DMSO served as negative control.

Experimental design. All assays were performed under light exclusion and sterile working conditions (Laminar airflow cabinet Safemate 1.8; Bioair Euroclone, Italy). Before each test, all stock TP solutions and diluted samples were freshly prepared. 500 µl of 1 × PBS buffer or S9-mix, respectively (19.75 ml dH_2O, 25 ml PBS buffer, 0.5 ml $MgCl_2$ (0.85 M), 0.5 ml KCl (1.65 M) and 2 ml NADP (90.8 mM), 250 µL glucose-*6*-phosphate (1.08 M) and 2 ml of S9), 100 µl of overnight culture, 200 µl of BP solution (in DMSO) were mixed in sterile glass tubes. For antimutagenic testing 100 µl of mutagen (in DMSO or DMSO alone for controls) was added to each tube. After 25 minutes of pre-incubation (37°C, on rotary-shaker), 2 ml of molten top-agar was added to every tube. The

mixtures were poured onto minimum glucose plates, followed by 48 hrs of incubation at 37°C. Bacterial colonies (His^+ revertants) were counted manually after having routinely checked the background lawn under a microscope (Olympus CH-2; 40x magnification).

As recommended in the literature, each sample concentration was tested in triplicate, with positive and negative controls tested six times, with all assays performed twice [6,24–26]. For a substance to be considered genotoxic in the *Salmonella* reverse mutation test, the number of revertant colonies on the test compound plates has to exceed twice the number of colonies produced on the solvent control plates (negative control) [25].

Besides investigating antimutagenic effects of TPs, spontaneous mutagenic effects were also tested for each compound. The respective highest and lowest doses of each compound were applied without mutagen addition.

Measures of antioxidant capacity

To assess the antioxidant/reducing powers of the test compounds, the trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC) and the ferric reducing antioxidant potential assays (FRAP) were conducted as the most popular methods published in the international literature [27–29]. Tetrapyrrole stock solutions were prepared in DMSO at concentrations of 2.5, 0.75, and 0.25 mM for BV and BRDT, and 0.75, 0.5 and 0.25 mM for the remaining compounds, due to solubility limitations, which had been assessed spectrophotometrically (refer to paragraph 2.1.3.).

In the TEAC assay, oxidation of 2,2′-azino-bis(3-ethylbenzoathiazoline-6-sulfonic acid), ABTS is initiated by persulphate, forming the ABTS radical. The ability of the test compound to reduce this radical is measured spectrophotometrically as published previously [30].

The ORAC assay is based on the inhibition of the peroxyl-radical induced oxidation which is triggered by thermal decomposition of the azo-compounds 2,2′-azo-bis(2-amidino-propane dihydrochloride) AAPH. Over time, radicals generated from this decomposition, quench the fluorescein signal. Subsequent antioxidant addition stabilizes the fluorescence signal, which is expressed relative to trolox equivalents. Measurements were performed as previously published [31].

In the FRAP assay, reduction of the ferric tripyridyltriazine complex (Fe^{3+}) to the ferrous complex (Fe^{2+}) in the Fe^{3+}/Fe^{2+} TPTZ redox system, is measured at pH 3.6. Change in absorbance is directly related to the nonthiol reducing power of antioxidants present in the sample. Absorbance was measured as previously published [27].

Statistical analysis

All data were analyzed using SPSS 17.0. Data were tested for normal distribution by applying the Kolmogorov-Smirnov test and histograms. Parametric statistical analyses (ANOVA), followed by the *post hoc* Scheffé or Dunnet T3 tests, were performed on normally distributed data and corresponding nonparametric tests (Kruskal-Wallis H-test) were used for skewed data. A p-value ≤ 0.05 was considered significant. $IP_{0.5}$ values (percentage of positive control inhibition at 0.5 µmol TP plate concentration) were used to assess the orders of effectiveness in *S. typhimurium* tests, and were determined using Derive 6 (Texas Instruments Inc.). A variety of curve algorithms were fitted to the data using Micrsoft Excel 2007 (linear, logarithmic, polynomial, exponential) and in every instance the algorithm with the highest R^2 value was chosen [6]. $IP_{0.5}$ values were interpolated from these algorithms using Derive 6 to determine the orders of TP effectiveness in the reverse mutation assays and antioxidant capacity assays. Besides, IC_{75} values were calculated to assess test compound concentrations (µmol/plate) needed to attenuate positive control effects by 25%.

Results

Bilirubin, BV, BRDT, UB, SB, and BR-/BV-DME were tested in *S. typhimurium* strain *TA102* to assess their potential to modulate spontaneous or induced oxidative DNA damage. In the absence of *tert*-BOOH none of the tested compounds caused toxicity or mutagenesis ($p > 0.05$; data not shown).

Prevention of oxidative stress-induced mutagenesis

tert-BOOH-induced oxidation (−S9). Protoporphyrin was the most active compound of those tested, and inhibited 35% of *tert*-BOOH-induced oxidative damage (Figure 1A). All PRO doses yielded significant dose dependent reductions in revertant counts compared to positive control values ($p < 0.05$). In contrast, UB and SB failed to significantly reduce the number of revertant colonies and UB showed no dose-dependent effect (Figure 1A). Also BV from 0.5 to 2 µmol/plate significantly and dose-dependently lowered revertant colony growth compared to positive control values ($p < 0.05$, Figure 1B), similar to BV-DME at 1 and 0.1 µmol/plate ($p < 0.05$, Figure 1B).

Unconjugated bilirubin at 0.75 µmol/plate tended to lower *tert*-BOOH-provoked oxidative stress, and inhibited 20% of the induced damage. Bilirubin dimethyl ester significantly lowered revertant counts at 1 and 0.5 µmol/plate compared to positive control values ($p < 0.05$). All concentrations of BRDT, except for 0.01 µmol/plate significantly decreased colony formation in a dose-dependent manner, compared to positive control ($p < 0.01$, Figure 1C).

For comparison, the percentage positive control inhibition of each pigment at 0.5 µmol/plate ($IP_{0.5}$) was calculated (Table I). From these percentages, the order of TP effectiveness (based on $IP_{0.5}$) in the absence of S9 was determined as follows: PRO > BV > BRDT > BR > BV-DME > SB > BR-DME > UB.

tert-BOOH-induced oxidation (+ S9). With metabolic activation, results for PRO and UB were reversed: PRO was ineffective whereas UB and SB significantly attenuated

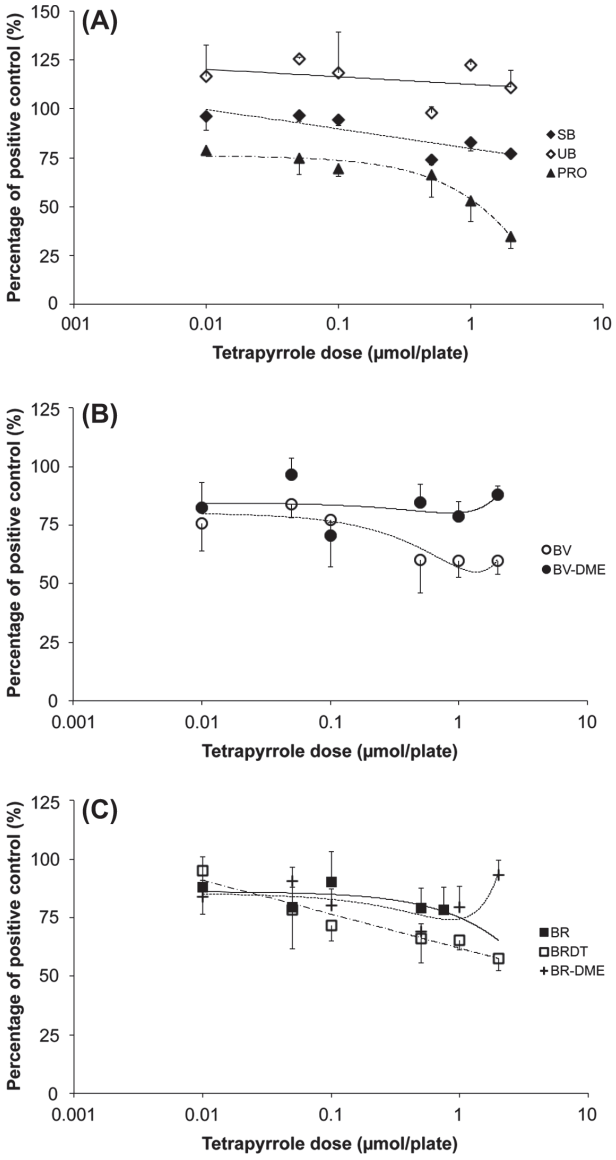

Figure 1. A–C The effects of TPs (A) SB, UB, PRO, (B) BV, BV-DME and (C) BR, BRDT, BR-DME on *tert*-BOOH-induced mutation in *TA102*. BR: unconjugated bilirubin, BRDT: bilirubin ditaurate, BR-DME: bilirubin dimethyl ester, BV: biliverdin, BV-DME: biliverdin dimethyl ester, UB: urobilin, SB: stercobilin, PRO: protoporphyrin.

tert-BOOH-induced DNA damage equally over the entire concentration range ($p < 0.05$). However, no dose-dependency was observed (Figure 2A).

At all concentrations, BV-DME significantly lowered revertant formation ($p < 0.05$), compared to positive control, and was generally more effective than BV. However, also BV significantly lowered *tert*-BOOH-induced oxidation at 0.1, 0.5, 1 and 2 µmol/plate (Figure 2B).

Bilirubin and BRDT exerted a nonsignificant antioxidant effect at or below 0.1 µmol/plate, and BR-DME significantly lowered revertant formation at all doses compared to positive control counts ($p < 0.05$; Figure 2C).

In the presence of S9, the calculated order of antioxidant TP effectiveness at 0.5 µmol/plate was as follows (Table I): UB > BV-DME > BR-DME > SB > BV > BR > BRDT > PRO.

Antioxidant capacity of tetrapyrroles

Considering the test compound-specific solubility limits, stock concentrations of 2.5, 0.5, and 0.25 mM for BV and BRDT and of 0.75, 0.5 and 0.25 mM for the remaining TPs were tested in the TEAC, ORAC and FRAP assays. Results are presented in Figure 3A–C, and orders of effectiveness according to linear regression calculations are summarized in Table II.

The data (Figure 3A–C) attest to dose-dependent antioxidant capacities for most of the tested compounds. Protoporphyrin was highly effective in the FRAP assay, and was moderately active in the ORAC and TEAC assays. In contrast, UB and SB were weakly and generally least effective in all of the antioxidant capacity assays by showing no reductive power in the FRAP assay against Fe^{3+}, a moderate activity in the ORAC and TEAC assay versus trolox. The remaining compounds (BR, BRDT, BV, BR-/BV-DME) possessed moderate to high antioxidant activities in the various assay systems (Table II).

Discussion

A series of novel (physiological) TPs were investigated in the *Salmonella* reverse mutation assay and multiple antioxidant capacity assays (TEAC, ORAC, FRAP) and were compared to already tested, biologically relevant BR and BV. In the absence of metabolic activation (-S9) the order of effectiveness in the *Salmonella* reverse mutation assay was PRO > BV > BRDT > BR > BV-DME > SB > BR-DME > UB. In the presence of S9 the antimutagenic effectiveness was reversed, suggesting UB > BV-DME > BR-DME > SB > BV > BR > BRDT > PRO. These data imply that cytochrome P450 activity within the microsomal S9 mixtures probably oxidized PRO, BV and BR leaving only UB and SB to remain active (in the +S9 condition). Furthermore, these data also indicate that methylation of BR's and BV's propionate groups protect them from cytochrome oxidation, allowing them to remain active, in the presence of metabolic activation. In the antioxidant capacity assays most TPs possessed moderate to high antioxidant activities in comparison to trolox and Fe^{2+}. The only exceptions were UB and SB which possessed no FRAP ability, however, retained moderate ORAC and TEAC activity. These data are the first to indicate that UB and SB act as antioxidants by donating hydrogen atoms to incumbent radical species.

It is evident that BR and BV are potent antioxidants *in vitro* [21] as well as under *in vivo* conditions [8–10]. They possess antimutagenic and antioxidant properties against multiple classes of mutagens in the *Salmonella* reverse mutation assay [6,7], both of which are generally attributed to electron/hydrogen donation and to free electron scavenging of peroxyl and superoxide radicals [12,21].

To reveal possible mechanisms of antioxidant action of TPs, pigment solutions were tested in well-established assays to assess their direct (reducing) and indirect (oxidation preventative) antioxidant properties. The ORAC assay

Table I. The modulatory effects of structurally related TPs on genotoxicity in *S. typhimurium* strain *TA102*.

Strain	Mutagen [mol/plate]	S9	Test compound	$IP_{0.5}$ [pos control inhibition, %]#	IC_{75} [inhibitory dose, μmol/plate]°	His^+_{pos}
TA 102	*tert*-BOOH 0.75×10^{-6}	–	BR	−20	1.15	
			BRDT	−34	0.126	
			BR-DME	+2	0.62	
			BV	−35	0.15	1871 ± 150
			BV-DME	−19	n/a^	
			UB	+4	n/a^	
			SB	−17	2.93	
			PRO	−36	0.04	
TA 102	*tert*-BOOH 0.75×10^{-6}	+	BR	−20	11.86	
			BRDT	−9	2.78	
			BR-DME	−39	1.94	
			BV	−22	0.28	1344 ± 145
			BV-DME	−44	n/a^	
			UB	−47	2.69	
			SB	−38	n/a^	
			PRO	+15	1.65	

BR, unconjugated bilirubin; BRDT, bilirubin ditaurate; BR-DME, bilirubin dimethyl ester; BV, biliverdin; BV-DME, biliverdin dimethyl ester; UB, urobilin; SB, stercobilin; PRO, protoporphyrin; #$IP_{0.5}$: percentage positive control inhibition at 0.5 μmol TP plate concentration (highest common stock sample dose): "−"indicates *tert*-BOOH attenuating effect, "+" amplifying effect; $IP_{0.5}$ was selected to assess the order of TP effectiveness. °IC_{75}: test compound concentration (μmol/plate) needed to attenuate positive control effects by 25% (inhibitory dose). His^+_{pos}: average positive controls revertant counts ± SD. ^n/a indicates TPs were devoid of antioxidant activity and/or curve progressions were inappropriate which made IC_{75} calculation impossible/uncertain.

(hydrogen atom transfer; HAT) specifically detects the compounds' antioxidant capacity towards peroxyl radicals generated in the aqueous phase. The FRAP assay (single electron transfer; SET) exclusively measures the compounds' iron reducing capacity, and TEAC detects the samples' antioxidant action towards the ABTS radical and combines both, the SET and HAT reaction types [32].

Bilirubin and BV, together with BRDT proved most effective in the TEAC assay (Figure 3A), which suggests either electron donation or H-transfer as their underlying mechanism of action. Referring to the compounds' reducing capacity, Benzie and Strain found 1 mol of BR reduces 4 mols Fe^{3+} in the FRAP assay [27]. In the present study however, 1 μmol of BR was found to reduce 1.05 μmol Fe^{3+} (Table II). The observed decreased effect and the generally changed orders of compound effectiveness between the *in vitro* systems might have been due to the albumin-free (and DMSO containing) conditions in the antioxidant capacity assays, versus protein containing bacteria. Binding of BR to albumin alters the antioxidant behavior of the compound, and causes a strained conformation that favors electron donation from the C10 methylene bridge [3].

The presented results show differences in the antioxidant potential of the free acids (BR and BV) versus their methyl esterified counterparts. The two methylated conjugates were moderately to weakly active in all of the tests (TEAC, ORAC, FRAP; Table II). Both compounds possess eight double bonds (with BR's containing two additional hydrogens that can be abstracted) and proved almost equally effective in terms of their antioxidant action, although BR-DME was clearly more effective than BV-DME in the TEAC assay. These findings can probably be attributed to the reduced C10 bridge of BR, allowing hydrogen atom donation. Performing standardized kinetic antioxidant assays, MacLean and colleagues showed an antioxidant behavior of both methylated compounds dependent upon free N-H groups for internal H-bonding, the nature of the solvent and on the attacking radical. Their hypothesized mechanisms of action included hydrogen atom transfer, proton-coupled electron transfer and single electron transfer [21] and are supported by the findings presented here.

In the *Salmonella* reverse mutation assay results, neither BR-DME nor BV-DME significantly protected against *tert*-BOOH-induced oxidation in *TA102* (-S9) at the concentration range tested. However, in the presence of S9 both methyl esters exerted significant antigenotoxic effects compared to positive control ($p < 0.05$; Table I). These divergent results indicate that cytochrome P450 redox system-attributed structural modifications of BPs are active possibly including de-esterification of the methyl esters and yielding unconjugated and bioactive compounds [33]. Correspondingly, if unconjugated pigments were added, we propose BP oxidation by cytochrome P450s, including rat CYP1A5, resulted in C10 bridge oxidation and formation of dipyrollic fragments as identified by DeMatteis et al. [34]. Moreover, the specific organic properties of dimethyl ester compounds have a great affinity for lipid membranes, which might have altered their cellular distribution. In contrast, BRDT would remain in solution and unconjugated BR could be bound to lipids and proteins including albumin and glutathione-S-transferases.

Although very little is known about the antioxidant properties of PRO, one report shows that it can effectively inhibit lipid peroxidation: when Williams et al. tested PRO, lipid peroxidation was dose-dependently inhibited under light protection, suggesting antioxidant activity based upon peroxyl radical scavenging [23]. Another study reports that PRO is a potent antioxidant,

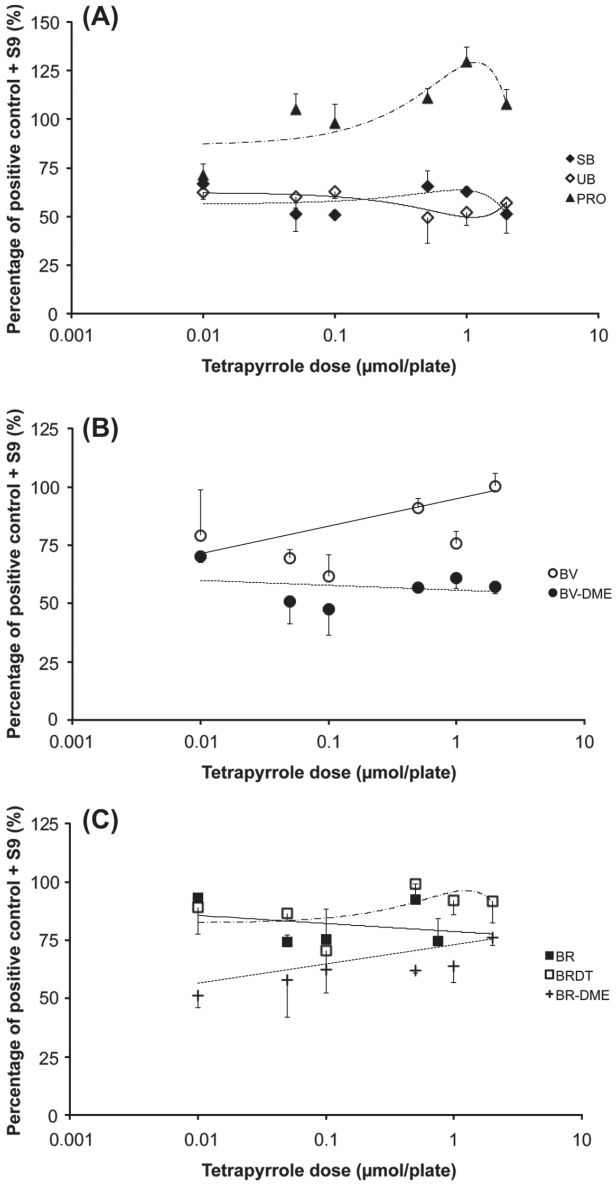

Figure 2. A–C The effects of TPs (A) SB, UB, PRO, (B) BV, BV-DME and (C) BR, BRDT, BR-DME on *tert*-BOOH-induced mutation (+ S9) in *TA102*. BR: unconjugated bilirubin, BRDT: bilirubin ditaurate, BR-DME: bilirubin dimethyl ester, BV: biliverdin, BV-DME: biliverdin dimethyl ester, UB: urobilin, SB: stercobilin, PRO: protoporphyrin.

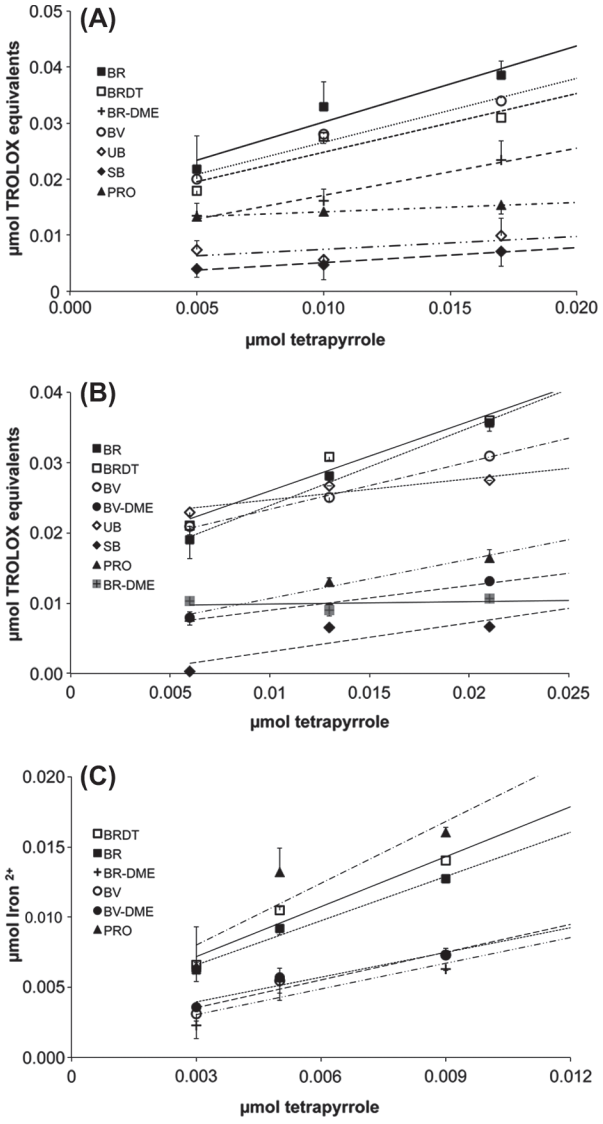

Figure 3. A–C Antioxidant capacities of TPs in the (A) TEAC, (B) ORAC and (C) FRAP assay. BR: unconjugated bilirubin, BRDT: bilirubin ditaurate, BR-DME: bilirubin dimethyl ester, BV: biliverdin, BV-DME: biliverdin dimethyl ester, UB: urobilin, SB: stercobilin, PRO: protoporphyrin.

however, when combined with metals such as cobalt or tin, PRO clearly acted, like heme, in a pro-oxidant manner [35]. These data complement the evidence presented here that shows in the presence of iron and cytochrome activity PRO was pro-oxidant (or lost its antioxidant capacity), whereas it was a potent antioxidant in the absence of S9. Further supporting data indicate that PRO was a weakly to moderately active antioxidant in both TEAC and ORAC assays and was highly effective in the FRAP assay (Table II). This observation suggests clear electron donating capacity of PRO and mechanistically adds to the previously stated hypothesis of Williams et al., who attributed strong peroxyl radical scavenging properties to PRO [23]. Regarding its effects in *Salmonella*, PRO was the most active of all compounds in the -S9 condition, however, it was the least active when metabolic activation was present (Table I). The polarized order of effectiveness is most likely explained by the previously discussed effect of iron-rich liver homogenate application. Furthermore, with eleven double-bonds PRO carries the most extended conjugated double bond system of all tested TPs, allowing it to donate the greatest number of electrons to incumbent radicals. Based on strong π-interactions, its conjugated ring system which is susceptible to redox interactions (*e. g.* with the heme protein cytochrome P450), provides powerful covalent and noncovalent binding capacities towards metals or biomolecules [36,37], supporting a role for the molecule in binding and docking with mutagens. The compound's physic-chemical properties support the conclusion of its strong electron donating capacity/antioxidant activity observed in the aqueous systems of FRAP and the

Table II. Summary of the test compounds' orders of antioxidant effectiveness in TEAC, FRAP and ORAC assays, according to linear regression slope calculations.

TEAC ABTS$^{\cdot}$ reduction; µmol trolox equivalents at 1µmol tetrapyrrole[#]

BR	>BV	>BRDT	>BR-DME	>SB	>UB	>PRO	>BV-DME
1.38µmol	1.16µmol	1.06µmol	0.85µmol	0.27µmol	0.23µmol	0.18µmol	Neg°
	high		**moderate**		**weak**		

ORAC Antioxidant capacity towards peroxyl radicals; µmol trolox equivalents at 1µmol tetrapyrrole[#]

BR	>BRDT	>BV	>PRO	>SB	>BV-DME	>UB	>BR-DME
1.12µmol	1.01µmol	0.69µmol	0.56µmol	0.41µmol	0.36µmol	0.32µmol	0.04µmol

FRAP Fe^{3+} TPTZ reduction to Fe^{2+} TPTZ; µmol Fe^{2+} at 1µmol tetrapyrrole[#]

PRO	>BRDT	>BR	>BV	>BR-DME	>BV-DME	>UB	=SB
1.47µmol	1.19µmol	1.05µmol	0.66µmol	0.61µmol	0.59µmol	Neg°	Neg°

BR, unconjugated bilirubin; BRDT, bilirubin ditaurate; BR-DME, bilirubin dimethyl ester; BV, biliverdin; BV-DME, biliverdin dimethyl ester; UB, urobilin; SB, stercobilin; PRO, protoporphyrin; [#]Trolox/Fe^{2+} values below orders of effectiveness were calculated per 1 µmol compound; °"Neg": after blank subtraction negative values were obtained (therefore compounds were classified as non-active and excluded from respective figures). To classify test compound effectiveness ("high", "moderate", "weak"), tertiles were calculated for each individual assay.

Salmonella reverse mutation assay. Liver homogenates by nature possess high concentrations of free and protein-bound iron that could participate in redox interactions. Consequently PRO could be oxidized or could indeed chelate iron and revert to heme, which is a potent pro-oxidant, abolishing any antioxidant effects. Protoporphyrin occurs in the intestinal tract and is excreted in the feces at a concentration of 0.2–14.3 nmol/g feces [38]. These data suggest that PRO within the intestine could prevent oxidative DNA damage and therefore promote enteral integrity and gut health by an antioxidant mechanism.

Literature reports on the antioxidant effects of UB and SB are rare. Research in this field mostly deals with the compounds' derivatives (*e. g.* urobilinogens, UBG). For UBG, Nakamura et al. reported a slightly superior radical scavenging activity compared to BR. Furthermore, the authors suggest UBG suppressed free-radical-induced lipid oxidation [20], which would consequently argue for a strong antioxidant effect in the lipid phase. Therefore, it was hypothesized that UB would be active in the ORAC assay. Since UBG represents an oxidized form of UB, the antioxidant activity of the latter might be comparatively enhanced. However, results show that UB and SB generally were the least effective of all test compounds (Table II), which also supports the overall outcome from *Salmonella* trials, discussed below. Both compounds were entirely inactive in the FRAP assay with undetectable signals after blank subtraction. However, the compounds were weakly effective in the TEAC and ORAC assays, as hypothesized above. Accordingly, their antioxidant action can be attributed to H transfer within the aqueous phase (also in *Salmonella*). This novel outcome also supports the aforementioned theory of Nakamura et al., which refers to a suppression of free radical-induced lipid oxidation by UBG.

In the *Salmonella* test, UB significantly attenuated *tert*-BOOH effects over the entire range of concentrations, in the presence of metabolic activation. These effects need to be considered relative to the remaining test compounds, most of which did not significantly attenuate *tert*-BOOH effects. Therefore UB and SB were effective relative to the remaining TPs. In the absence of S9 addition, neither SB nor UB showed substantial antigenotoxic potential (Table I). With reference to their greatly reduced number of double bonds within their conjugated system UB and SB represent the most oxidized species tested. Previous studies demonstrate that electron donation from BR occurs via the N-H pyrrole ring [21]. The reductive capacity decreases, if conjugation within the ring is lost, as is the case in UB and SB.

In summary, the obtained results confirm a strong reductive power of PRO, which on its own, was most effective in *Salmonella* (-S9) and the FRAP assay, followed by the rubinoid compounds (UB, BRDT, BR-DME). Upon introduction of S9, antioxidant effectiveness was reversed with UB and SB being amongst the most effective of all tested compounds, followed by the methyl esterified TPs, which are not known to be targets for cytochrome oxidation. The relative potency of UB and SB in preventing oxidative DNA mutation, in the presence of S9, provides very important information as to a novel chemo-preventative role for these compounds in the gastrointestinal and urinary tracts.

Conclusion

This study's findings show evidence of a strong antioxidant behavior of the TPs BR, BV and BRDT in the *Salmonella* reverse mutation assay, and present the first data on an antioxidant activity of BR-/BV-DME, UB, SB and PRO *in vitro.* Stercobilin, UB and PRO all of which are found in the intestinal tract, showed *in vitro* antioxidant activity, which highlights their possible *in vivo* importance in preventing oxidative stress within the gut. Although *Salmonella* reverse mutation assay results cannot be directly transferred to the *in vivo* condition, the data provide preliminary evidence to suggest a possible physiological role of structurally related, partially oxidized TPs, in preventing oxidative DNA damage within the gut. Therefore these compounds could be beneficial if administered enterally for therapeutic means [39].

Declaration of interest

The authors declare that there are no conflicts of interest. This work (grant number P21162-B11) was supported by the Austrian Science Fund (Fonds zur Förderung der wissenschaftlichen Forschung, FWF).

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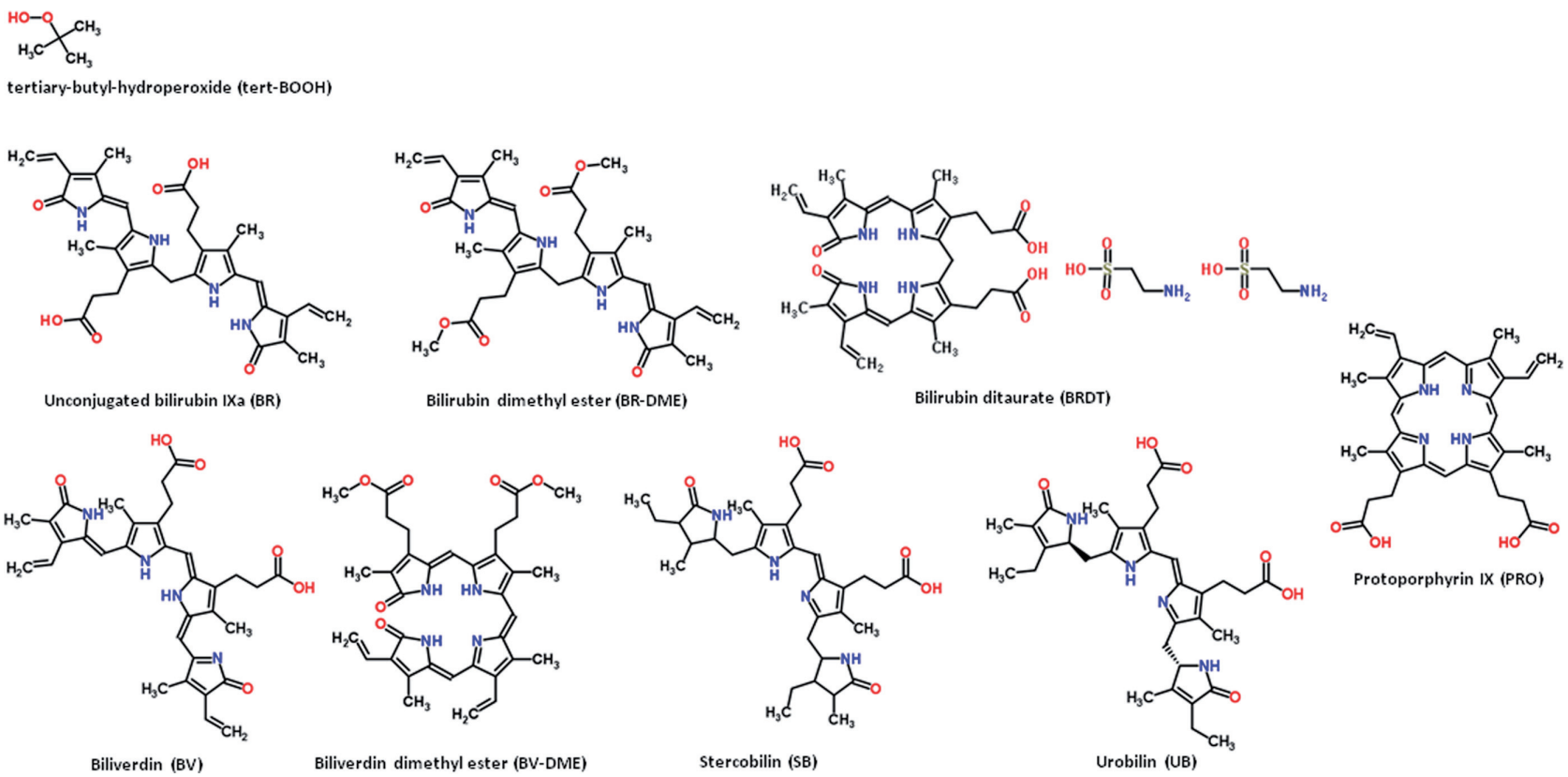
Supplementary material available online

Supplementary material 1. Chemical structures of the test compounds.

Supplementary Material for C. Mölzer *et al. In vitro* antioxidant capacity and antigenotoxic properties of protoporphyrin and structurally related tetrapyrroles. *Free Radical Research* 2012;46(11):1369–1377.



Supplementary material 1. Chemical structures of the test compounds.

In vitro physic-chemical properties of tetrapyrroles against TNFone-induced mutagenesis

Short running tile: Tetrapyrrole/TNFone structural interactions

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Key words: bile pigments, bilirubin, protoporphyrin, *Salmonella,* circular dichroism

Abstract

Bilirubin, the principal and biologically most relevant bile pigment was for long considered to be only a waste product of haem catabolism. However, recent data show its remarkable biological potential, including antioxidant and anti-mutagenic effects. It is now assumed that bile pigments and derivatives exert these effects in multimodal ways, which are likely mediated *via* structure-based mechanisms. Albeit, the major scientific focus so far has been directed towards the compounds' antioxidant action, and specifications on mutagen/tetrapyrrole interactions are lacking. Therefore structurally related bile pigments and derivatives (bilirubin/-ditaurate/-dimethyl ester, biliverdin/-dimethyl ester, urobilin, stercobilin and protoporphyrin) were tested for anti-genotoxicity in the *Salmonella* reverse mutation assay in strains *TA98* and *TA102*, together with the synthetic mutagen and environmental pollutant 2,4,7-trinitro-9H-fluoren-9-one (TNFone). Tetrapyrroles were applied at physiologically relevant concentrations of 0.01 – 2µmol/plate. To explore the physic-chemical background, molecular systems of chlorine e6 porphyrin, bilirubin and biliverdin with TNFone were assayed using circular dichroism techniques, which consistently revealed suprastoichiometric anti-mutagenic effects. Addition of TNFone to chlorine e6 porphyrin, bilirubin/albumin and biliverdin/albumin led to a substantial change in pigment spectral characteristics, providing evidence for tight tetrapyrrole/mutagen interactions. These results are reflected in the bacterial model, in which the majority of compounds significantly attenuated TNFone-induced mutagenesis. Protoporphyrin was the most anti-genotoxic pigment, followed by the rubinoid- (un-/conjugated bilirubin/dimethyl ester) and verdinic- (biliverdin/dimethyl ester) groups of compounds.

1. Introduction

Bile pigments (BPs) are tetrapyrrolic dicarboxylic acids which are endogenously formed within haem catabolism. Haem, to which protoporphyrin (PRO) is an intermediate prefix form, is degraded enzymatically involving haem oxygenase and NADPH/biliverdin reductase (BLVRA). Besides biliverdin (BV) synthesis, carbon monoxide and free iron are also generated. Biliverdin is reduced to bilirubin (BR) *via* BLVRA. Urobilin (UB) and stercobilin (SB) represent further biologically relevant degradation products of BR which are found mainly in the intestinal tract, and are formed by the microflora within the intestinal lumen. An overall estimate of 300mg of BPs is excreted on a daily basis via urine and faeces [Vitek and Ostrow 2009]. It is well established that BR and BV adopt chiral "ridge-tile" [Boiadjiev 1999; McDonagh and Lightner 1985; McDonagh and Lightner 1994; Mugnoli et al. 1983] and helical "lock-washer" [Goncharova and Urbanova 2008] conformations, both of which are stabilized by intramolecular hydrogen bonds. The helical inversion equilibrium between the two enantiomeric M- and P forms [Boiadjiev 1999] is typical for these pigments in solution and causes the zero resultant optical activity of aqueous pigment solutions. However, in the presence of chiral agents, the molecules preferably form one of the diastereoisomers upon complexation with the chiral host [Harmatz and Blauer 1975; Lightner et al. 1986], and become optically active. This enables to follow the subtle structural changes using circular dichroism (CD) spectroscopy [Goncharova and Urbanova 2008; Goncharova and Urbanova 2009]. The vibrational variant of circular dichroism, vibrational circular dichroism (VCD), is a CD technique applied in vibrational spectra. It follows the vibrational transitions that are localized in a molecule and can therefore determine the variation in molecular structure caused by biologically important interaction [Urbanová 2009; Urbanová 2012].

Antioxidant effects have been attributed to BR *in vitro* [Bulmer et al. 2007; Mölzer et al. 2012] as well as it has been found physiologically beneficial [Horsfall et al. 2011; Novotny and Vitek 2003; Vitek et al. 2002; Vitek and Schwertner 2007]. Furthermore, growing evidence suggests that especially BR also acts in a strongly anti-mutagenic manner *in vitro* [Bulmer et al. 2008; Bulmer et al. 2007]. A great number of publications refer to the ability of BPs and derivatives to lessen the genotoxic effects of a range of mutagens other than pro-oxidants in the *Salmonella* reverse mutation assay (Arimoto et al, 1980; De Flora et al, 1994; Arimoto et al, 1995; Tang et al, 1997; Dorner et al, 1997). In that respect, BR, BRDT and BV have recently been found highly effective against 2,4,7-Trinitro-9H-fluoren-9-one (TNFone)-induced mutagenesis [Bulmer et al. 2007]. Observed anti-genotoxic effects were hypothesized to be due to physic-chemical interactions, based on three-dimensional molecule structures [Bulmer et al. 2007]. These findings underline the possible importance of tetrapyrroles (TPs) occurring in biological

systems (organs) where they could protect against different mechanisms of DNA damage induced by various (exogenous) noxae.

To further explore our research hypothesis that TPs inhibit genotoxicity in a structure-dependent manner, the present study aimed to test the anti-genotoxic effects of a variety of yet untested compounds (BR-/BV-dimethyl ester, UB, SB, PRO) against TNFone in the *Salmonella* (*S.)* reverse mutation assay, applying bacterial strains *TA98* and *TA102*. The fluorene derivative TNFone is a synthetic polycyclic nitroamine which was previously found in photocopier toners [Kari 1992]. It is a proven frameshift mutagen [Levin et al. 1979] and was uniformly found mutagenic in a series of genetic toxicity evaluations in *S. typhimurium* strains, with and without metabolic activation, as well as in animal trials [Kari 1992]. Since TNFone, its chemical structure and potential ways of interaction with molecules (*e. g.* DNA bases) have been studied well in the past, it represents an excellent model for testing structure-based anti-mutagenic effects. Tetrapyrrole/mutagen interactions in the sense of co-incubations however, have not been investigated so far, and therefore mechanistic data are entirely lacking. This information however is of high relevance since TNFone represents a common pollutant [Kari 1992] millions of people are exposed to on a daily basis. Furthermore, substantial biliary excretion was found in rats and mice after dermal and oral TNFone-administration [Kari 1992], which underlines the possible importance of a BP-based TNFone-detoxification in the hepato-biliary and gastrointestinal systems.

Thus, we tested *1)* whether a range of mostly untested BPs could modulate TNFone-induced genotoxicity and *2)* how structurally related TPs affect mutagen genotoxicity by exploring possible structure-related mechanisms of action, using circular dichroism spectral studies

2. Materials and Methods

2.1. *Salmonella* reverse mutation assay:

The *S.* reverse mutation assay is a screening method to assess the mutagenic potential of chemicals *in vitro* [Maron and Ames 1983]. Mutagenic substances revert mutated bacteria to their wild type variant which allows them to grow. Based on that, genotoxicity and anti-genotoxic effects of compounds can be assessed by quantifying bacterial growth.

2.2. Chemicals:

Unconjugated bilirubin IXα (BR) [CAS# 635-65-4], bilirubin conjugate (ditaurate) disodium (BRDT) [CAS# 635-65-4], biliverdin IXα (BV) [CAS# 55482-27-4], bilirubin dimethyl ester (BR-DME) [CAS# 19792-68-8], biliverdin dimethyl ester (BV-DME) [B610-9; CAS# 10035-62-8], protoporphyrin IX (PRO) [P562-9; CAS# 553-12-8], chlorine e6 (CL) [CAS# 19660-

77-6] as well as urobilin (UB) [CAS# 28925-89-5] and stercobilin (SB) [CAS# 34217-90-8] were purchased from Frontier Scientific, UK. Compound purity (> 98 %) was assessed using high pressure liquid chromatography (HPLC). All reagents and mutagens were obtained from Sigma Aldrich, Austria (unless otherwise noted), were of the highest analytical grade available and stored according to instructions until use. Sample solutions (prepared in DMSO) were protected from light using cling film and were used immediately. Composition and preparation of all necessary reagents and solutions can be found elsewhere [Bulmer et al. 2007].

2.2.1. Positive/negative controls:

The applied positive control was TNFone (Vitas-M Lab, Russia) dissolved in DMSO which also served as a negative control *(Table I)*.

Table I: TNFone was used as positive control. DMSO served as solvent and negative control.

Compound	Class	Solvent	Strains	Conc. [mol/plate]
2,4,7-TNFone, (> 99%)	Fluorene derivative, non-planar polycyclic nitro-amine	DMSO	*TA98*	0.3×10^{-6}
			TA102	0.2×10^{-7}

2,4,7-TNFone: 2,4,7-trinitro-9H-fluoren-9-one

2.2.2. Bacterial strains:

S. typhimurium strains were kindly provided by Dr. Bruce N. Ames (University of California, Berkley, USA) and stored at -80°C as frozen permanents until use. Following [Mortelmans and Zeiger 2000], the strains were attested to their genetic integrity and spontaneous mutation rate. To consider different mechanisms of mutations two distinct strains were adopted: the DNA repair proficient strain *TA102* detects A/T base pair damage and small deletions provoked by cross-linking agents and can be reverted by mutagens causing oxidative damage; strain *TA98* primarily detects G/C base pair and frameshift mutations [Mortelmans and Zeiger 2000].

2.2.3. Sample preparation:

Based on preceding investigations [Bulmer et al. 2007] and on a series of solubility experiments, six doses of BRDT, BV and newly tested BR-/BV-DME, UB, SB and PRO were assayed at concentrations of 0.01, 0.05, 0.1, 0.5, 1 and 2 µmol/plate. Bilirubin was tested up to 0.75µmol/plate. Respective maximum compound concentrations had been ascertained by *1)* testing the maximum amount of DMSO that did not lead to bacterial cytotoxicity (presence of background lawn on plates) and *2)* by the respective maximum solubility of each compound (supernatant analysis: photometric and HPLC after high speed centrifugation). Bile pigment stock solutions were prepared at 0.025 M and dilutions containing the aforementioned amounts of TPs (in 300 µl) were made in DMSO. For both strains, DMSO toxicity was assessed by screening the background lawn present on minimal glucose agar plates after having added increasing amounts (0 - 500µl) of the diluent.

2.2.4. Experimental design:

The assays were performed in a dimly lit laboratory under sterile working conditions (Laminar airflow cabinet Safemate 1.8; Bioair Euroclone, Italy). Samples were protected from light throughout all experiments and were freshly prepared in amber vials before each test. For the experiments 500 µl of PBS buffer, 100 µl of overnight culture and 200 µl of TP solution (in DMSO) were merged in sterile test tubes. For anti-mutagenic testing 100 µl of mutagen (in DMSO or DMSO alone for controls) was added to each tube. After 25 mins of pre-incubation (37 °C, on rotary-shaker) 2 ml of molten top agar was added to every tube. The mixtures were poured onto minimum glucose plates, followed by 48 hrs of incubation at 37 °C. His^+ phenotype revertants (colonies with reversion of mutations in the histidine operon are able to grow in the absence of histidine [Mortelmans and Zeiger 2000]) were counted manually after having routinely checked the background lawn under a microscope (40 x magnification; Olympus CH-2 light microscope).

Each sample was tested in triplicate and all of the assays were repeated again on a different day. Every test included three "positive control plates" (mutagen only; n = 6), six "negative control plates" (no mutagen, no pigment; n = 6) and three "no treatment plates" (no mutagen, no pigment, no DMSO; n = 3). For a substance to be classified genotoxic in the *S.* reverse mutation assay, the number of revertant colonies on the compound plates had to exceed twice the number of colonies produced on the negative control plates [Mortelmans and Zeiger 2000].
Besides investigating anti-mutagenic effects of TPs also spontaneous mutagenic effects were tested. For each sample, the respective highest and lowest concentrations were applied without mutagen addition.

2.3. Characterization by circular dichroism

The CD spectra of BPs were measured in a quartz cuvette with an optical path length of 1 cm (Starna, USA), using a J-810 spectropolarimeter (Jasco, Japan). The final spectrum was obtained as an average of three accumulations. Spectra were corrected for baseline by subtracting the spectra of the corresponding solvents. Circular dichroism measurements were conducted at room temperature (25 °C). For spectral measurements, BR, BV in the form of sodium salts, and chiral porphyrin CL in double distilled water were at concentrations of 1.2 x 10^{-5} M. The sodium salts of the pigments were prepared by freeze-drying as described before [Goncharova and Urbanová 2007]. Human serum albumin (HSA) was used as a chiral discriminator for BR and BV at the pigment/HSA molar ration of 1/1. The chiral derivative of PRO (CL) was chosen to follow the interaction with TNFone utilizing CD in the absence of a chiral discriminator (chemical structures of compounds can be found in the online *supplementary material 1*). Vibrational CD spectra of CL with TNFone were recorded in DMSO d_6/D_2O (50:50) solution, at a concentration of 0.03 M with a resolution of 8 cm^{-1}, using a Fourier transform infrared spectrometer IFS-66/S (Bruker, Germany), equipped with a VCD/IRRAS module PMA37 (Bruker). A demountable cell with CaF_2 windows and Teflon spacer of a 50 μm pathlength was used. To interpret the VCD and IR spectra of CL with and without TNFone, a possible structure of the CL/TNFone complex was modelled *(Fig. 3)*. Structure optimization was carried out by the HyperChem 8 software package [Hypercube 2012], using the Amber 99 force field [Cornell et al. 1995].

2.4. Statistical analyses:

Values are presented as means ± *SD.* Parametric statistical analyses (ANOVA) and the *post hoc* Scheffé test were performed on normally distributed data, and corresponding non-parametric tests (Kruskal-Wallis H-test, Dunn's post hoc test) on skewed data. Analyses were done using

IBM SPSS 17.0 and Microsoft Excel 2007 for Windows. A p-value ≤ 0.05 was considered significant. To assess differences in revertant growth, data were analyzed using $IP_{0.5}$ values (percentage of positive control inhibition at 0.5µmol of compound per plate) which assisted in standardising the orders of effectiveness in every test. A variety of curve algorithms were fitted to the data using Micrsoft Excel 2007 (linear, logarithmic, polynomial, exponential) and in every instance the algorithm with the highest R^2 value was chosen [Bulmer et al. 2007]. $IP_{0.5}$ values were interpolated from these algorithms using Derive 6 (Texas Instruments) to determine the orders of TP effectiveness in the reverse mutation assay.

3. Results

3.1. Mutagenic testing in S. typhimurium strains TA98 and TA102

Testing TPs for spontaneous mutagenesis revealed no pro-mutagenic effects in neither of the tested compounds and concentrations (*$p > 0.05$; data not shown*).

3.2. Anti-mutagenic testing in S. typhimurium strain TA98

Protoporphyrin attenuated TNFone-induced mutagenesis in a dose-dependent manner, and was the most effective of all tested TPs *($p < 0.01$)* over the entire range of concentrations, in which the highest dose of 2 µmol/plate lowered TNFone effects by 90 % *(Fig. 1A)*.

Also BR and BRDT dose-dependently lowered TNFone-induced mutation. At all tested concentrations the compounds significantly inhibited mutagenesis *($p < 0.05$)*. Bilirubin dimethyl ester was significantly anti-mutagenic from 0.5 µmol/plate *($p < 0.05$; Fig. 1B)*.

Compared to the rubinoid group of compounds, BV and BV-DME, were significantly active over a smaller range of concentrations (0.5 – 2 µmol/plate) compared to positive control *($p < 0.05$, Fig. 1C)*. The overall effectiveness of BV was greater relative to BV-DME, but no dose-dependency was found *(Fig. 1C)*. In contrast, both UB and SB dose-dependently lowered TNFone-induced genotoxicity. Urobilin was significantly anti-mutagenic at all tested concentrations (except for 0.1 µmol), as was SB from 1 µmol/plate *($p < 0.05$, Fig. 1A)*.

The order of effectiveness based on $IP_{0.5}$ values was assumed as follows *(Table II):* PRO > BR > BR-DME > BRDT = BV-DME > UB > SB > BV.

3.3. Antioxidant testing in S. typhimurium strain TA102

As previously seen in strain *TA98,* PRO yielded the most pronounced anti-genotoxic effects of all tested compounds (*$p < 0.01$),* and attenuated TNFone by approximately 75 % at the highest tested concentration. Throughout all concentrations, it reduced TNFone-induced mutagenesis significantly (*$p < 0.01$)* and dose-dependently compared to positive control *(Fig. 2A)*.

Also BR at the highest tested concentration of 0.75 µmol/plate significantly attenuated TNFone-induced mutagenesis *($p < 0.01$)* in comparison to positive control, and a concentration-dependent effect was observed. As already seen in strain *TA98,* also in strain *TA102* BR-DME dose-dependently inhibited TNFone effects *(Fig. 2B)*.

Both BV and BV-DME also significantly lowered TNFone-induced mutagenesis in *TA102* in a dose-dependent manner *($p < 0.05$)*. Somewhat contrasting *TA98* results, the overall anti-mutagenic effects of BV-DME were comparably stronger within these two TPs *(Fig. 2C)*.

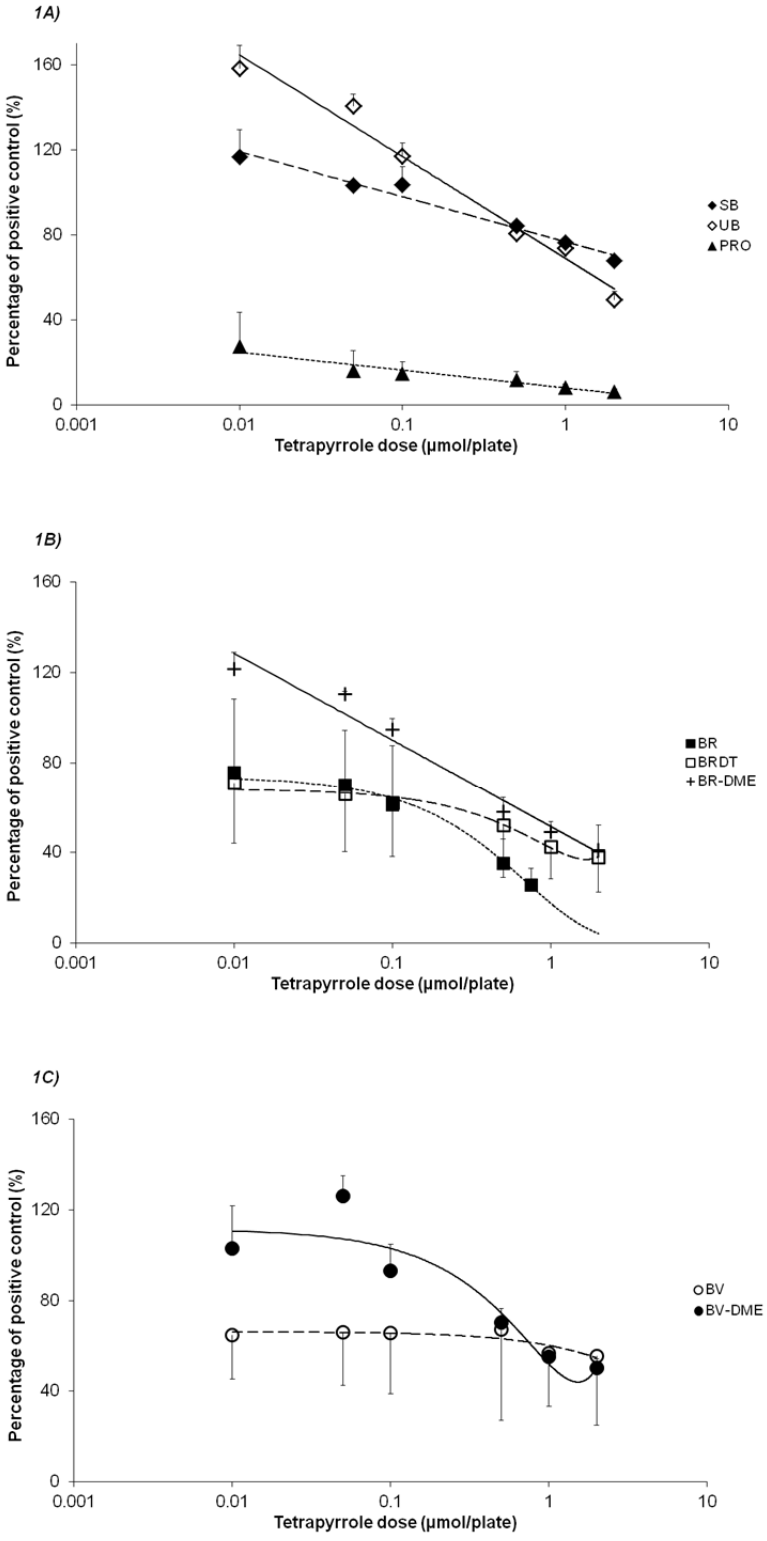
From a concentration of 0.1 µmol/plate also UB and SB significantly and dose-dependently inhibited TNFone-induced genotoxic effects *($p < 0.05$, Fig. 2A)*.

The order of effectiveness based on $IP_{0.5}$ values was assumed as follows *(Table II):* PRO > BR > BRDT > BR-DME = BV > BV-DME > UB = SB.

Table II: The impact of different tetrapyrroles on genotoxicity in *S. typhimurium* strains *TA98* and *TA102*

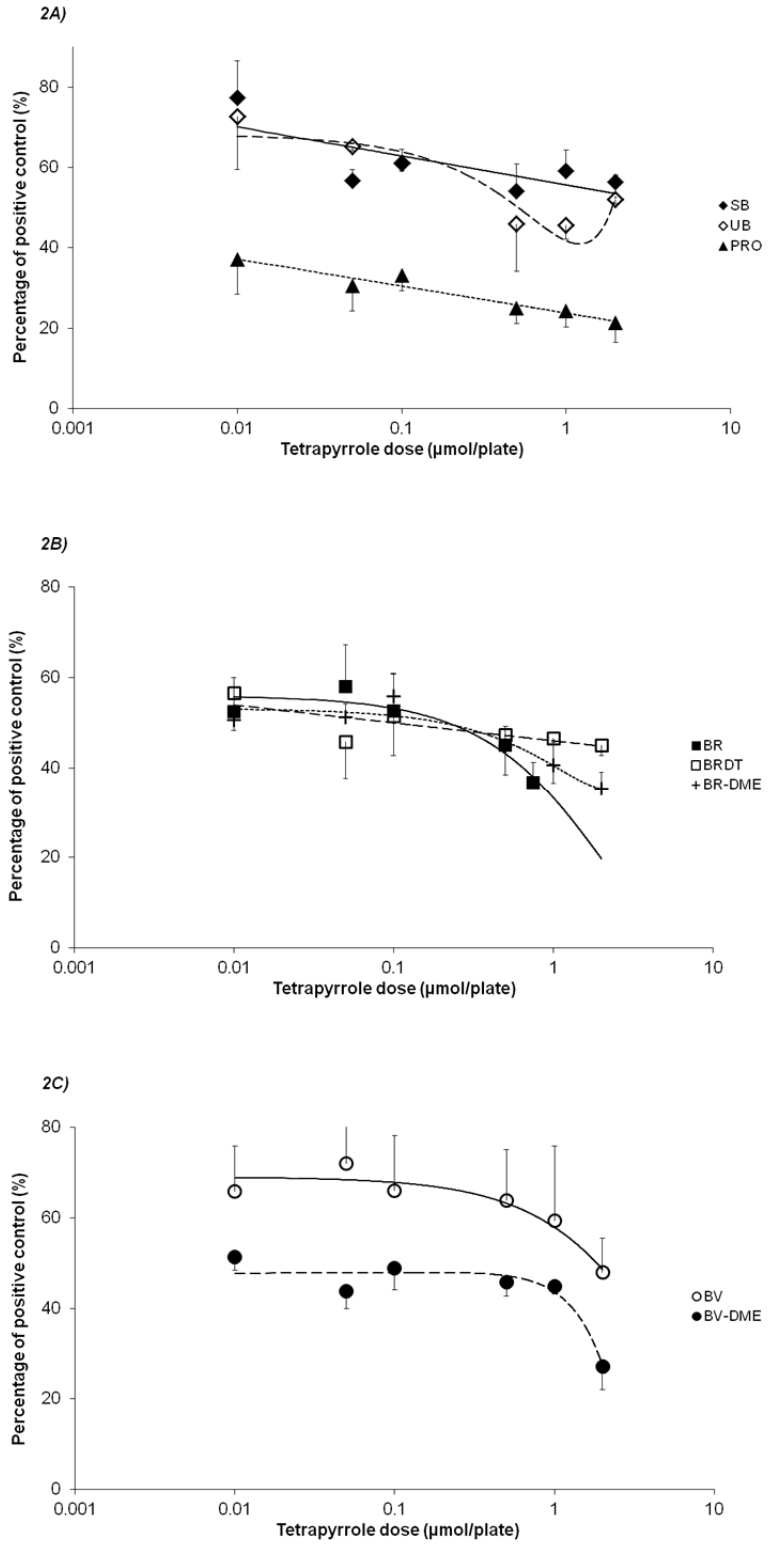
Strain	Mutagen [mol/plate]	Test compound	$IP_{0.5}$ [%]#	His^+_{pos}°
TA 98	TNFone 0.3 x 10^{-6}	BR	-64	
		BRDT	-48	
		BR-DME	-37	
		BV	-37	
		BV-DME	-26	331 ± 22
		UB	-17	
		SB	-17	
		PRO	-90	
TA 102	TNFone 0.2 x 10^{-7}	BR	-57	
		BRDT	-53	
		BR-DME	-54	
		BV	-37	
		BV-DME	-53	1157 ± 148
		UB	-50	
		SB	-42	
		PRO	-74	

BR: unconjugated bilirubin, BRDT: bilirubin ditaurate, BR-DME: bilirubin dimethyl ester, BV: biliverdin, BV-DME: biliverdin dimethyl ester, UB: urobilin, SB: stercobilin, PRO: protoporphyrin. #$IP_{0.5}$: percentage positive control inhibition at 0.5 µmol TP per plate (highest common sample dose), "-" indicates positive control attenuating, "+" enhancing effect; °His^+_{pos}: average positive controls revertant counts ± SD.



BR: unconjugated bilirubin, BRDT: bilirubin ditaurate, BR-DME: bilirubin dimethyl ester, BV: biliverdin, BV-DME: biliverdin dimethyl ester, UB: urobilin, SB: stercobilin, PRO: protoporphyrin.

Fig. 1A – C: Anti-mutagenic effects of A) PRO, UB, SB; B) BR, BRDT, BR-DME and C) BV, BV-DME against TNFone-induced mutagenesis in *S.* strain *TA98.*



BR: unconjugated bilirubin, BRDT: bilirubin ditaurate, BR-DME: bilirubin dimethyl ester, BV: biliverdin, BV-DME: biliverdin dimethyl ester, UB: urobilin, SB: stercobilin, PRO: protoporphyrin.

Fig. 2A – C: Anti-mutagenic effects of A) PRO, UB, SB; B) BR, BRDT, BR-DME and C) BV, BV-DME against TNFone-induced mutagenesis in *S.* strain *TA102.*

3.4. Circular dichroism characterization of pigment/mutagen interactions

The influence of TNFone on CD spectra of the molecular systems composed of pigment and HSA in the case of BR and BV, and without HSA in the case of CL, is demonstrated in *Fig. 3*. Bilirubin and BV showed the typical absorption spectra observed in aqueous solutions and negligible CE signals, whilst a pronounced CE spectrum was observed for CL, which documented its high enantiomeric excess. Addition of HSA (to BR and BV) and TNFone resulted in the appearance of the signal at wavelengths of < 350 nm; the absorption signals in the blue (380 – 480 nm) and red regions (> 600 nm) are characteristic for pigments, whereas BR exerted a pronounced signal only in the blue region. Presence of HSA in BR and BV solutions induced a shift in absorption and strong CD signals as documented earlier [Goncharova and Urbanova 2008]. Chlorine, as a chiral compound without fast racemization in solution, *per se* showed a CD signal without the presence of a chiral agent. The variation of the CD signal intensity of the blue band was chosen as the quantitative parameter, describing the spectral changes upon TNFone addition. Addition of TNFone at the pigment/TNFone ratio of 1 caused 66, 26 and 13 % variation of the blue band CD intensity for CL, BR and BV, respectively. The same spectral variation was observed at the pigment/TNFone ratio from 1 to 3, and about half that variation was observed for pigment/TNFone (= 0.5; *data not shown*). Evidently, TNFone induced pronounced CD spectral changes in the following order: CL > BR > BV.

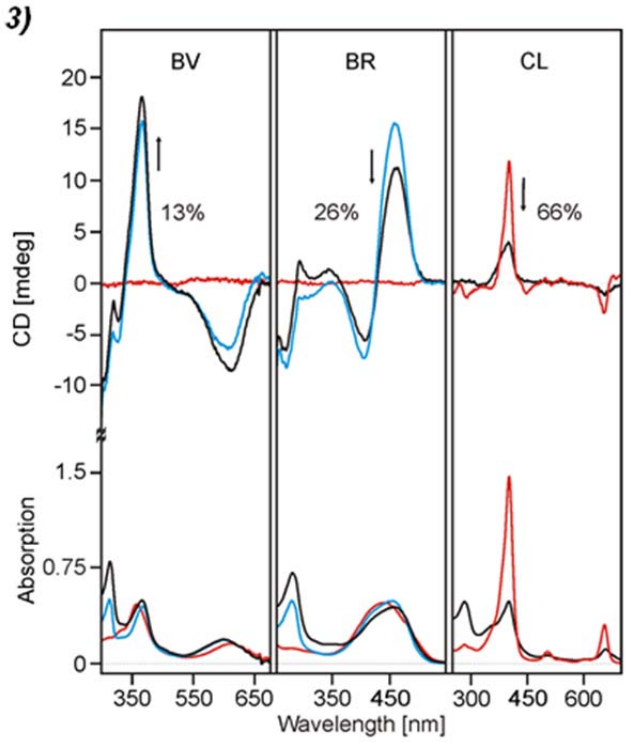


Fig. 3: UV-vis absorption and CD spectra of BV, BR and CL in aqueous solutions (red lines), in the presence of HSA (blue line) and with TNFone (black lines). Concentration of all compounds was 1.2×10^{-5} M.

Figure 4 shows VCD and IR absorption spectra of CL with and without TNFone. Significant VCD spectral changes were observed in the presence of TNFone, which clearly indicated a diastereometric interaction between both compounds. The spectral observations are described in relation to a structure of the complex *(Fig. 5)* which was obtained by the energy minimization using the molecular mechanics. The VCD signals assigned to the symmetric and asymmetric COO^- vibrations at 1590 – 1550 and 1440 – 1380 cm^{-1}, respectively, were observed for CL without TNFone and were assigned to propionate in the 2(S)-propionic acid group *(Fig. 4 and 5, position a)*. Addition of TNFone caused a significant increase in VCD signals at 1580 and 1562 cm^{-1} localized in the region of asymmetric COO- vibrations which became strong, well resolved and of different shape than for CL alone. This can be explained by a chiral spatial fixation of the carboxylic groups in the CL part of the diastereometric complex. Besides the propionate, which is located in close proximity to the chiral center in the CL molecule, especially carboxymethyl and possibly carboxyl groups could be chirally oriented in the complex *(Fig. 5, position b)*.

Upon addition of TNFone, the IR absorption and the negative VCD signal at 1730 cm^{-1} was assigned to the C=O characteristic vibration localized in the flourenone *(Fig. 4)*. In the suggested structure of the complex, the C=O position is appropriate for H-bondings shown in *Fig. 4, position c*. The signals at 1530 and 1340 cm^{-1} were assigned to the asymmetric and symmetric dinitro (NO_2) vibrations, respectively, localized in the TNFone molecule *(Fig. 4 and Fig. 5, positions d)*. A specifically strong VCD signal was observed for symmetric NO_2 vibration at 1340 cm^{-1} *(Fig. 4),* which indicated induced optical activity by possible π/π interaction between the nitro- (N) and dinitro-aromatic rings of TNFone with the CL pyrroles, as assigned in *Fig. 5*. The π/π interaction between the aromatic rings of the two partners in the complex was also confirmed by a strong induced negative VCD signal at 1610 cm^{-1}, characteristic to C=C/C=N vibrations, which were observed upon addition of TNFone. The assignment of the VCD signals was confirmed by the spectra obtained for the half TNFone molar concentration resulting in about half decrease of the observed signals.

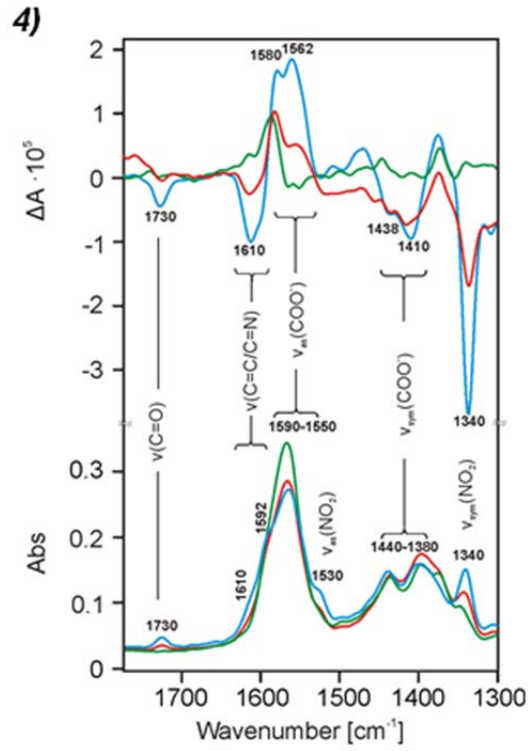


Fig. 4: IR absorption and VCD spectra of CL in DMSO-d_6/D_2O (50:50) (green) and with TNFone for the CL/TNFone molar ratio 1/0.5 (red) and 1/0.25 (blue). Concentration of CL was 0.03 M.

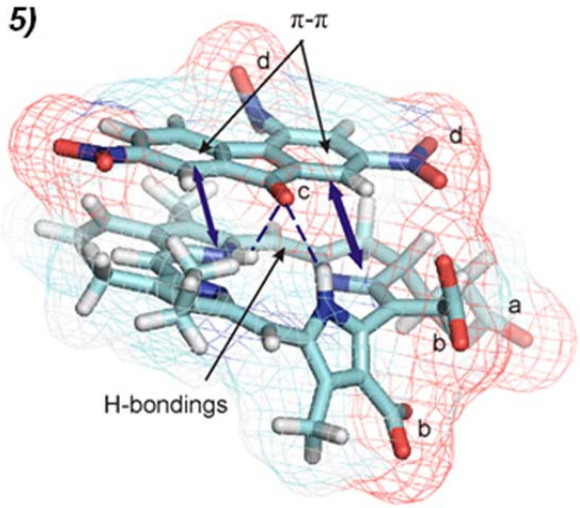


Fig. 5: The structure of the TNFone/CL complex with the molecular orbital mapping.

4. Discussion:

Physiologically relevant concentrations of BR, BRDT, BR-DME, BV, BV-DME, UB, SB and PRO were tested in *S. typhimurium* strains *TA98* and *TA102* to assess their potential to modulate TNFone-induced mechanisms of genotoxicity. In both of the bacterial strains, cyclic PRO was the most effective of those compounds tested. Also linear BR, BRDT and the esterified compounds strain-independently showed high anti-mutagenic activities. Least active in both test conditions were structurally highly inter-related SB and UB.

In a previous study [Bulmer et al. 2007] BR, BRDT and BV dose-dependently inhibited TNFone-induced mutagenesis. These strong anti-mutagenic effects for the above open-chain TPs are supported by our findings and can possibly be discussed with reference to their respective structural characteristics, bringing forward three-dimensional interactions. Both BR and TNFone carry ridge-tile conformation which according to the hypothesis forms the structural basis of their interaction [Bulmer et al. 2007]. Somewhat contrasting this hypothesis, also planar PRO exerted the most potent effects against TNFone-induced genotoxicity in both tester strains. Along with BR, also BR-DME shows a flexed out-of-plane conformation, supporting structural interaction with TNFone, as hypothesized. Urobilin and SB are as well non-planar in shape but do not possess specific flexed conformations, which is reflected in their weak anti-genotoxic activity, as is true for BV and its dimethyl ester derivative.

To prove whether TNFone directly interacts with PRO, chiral CL was used as a model molecule. The variation in absorption induced by the presence of TNFone in CL solutions *(Fig. 3),* were analogical to the previously described absorption spectra [Gladkova et al. 2010], caused by oxidations of CL. Our pronounced CD spectral changes induced by the addition of TNFone showed strong interactions between both compounds. The VCD results confirmed this interaction between CL and TNFone. The induced VCD signal in originally optically inactive TNFone observed in the presence of CL was localized in the region C=O and NO_2. Upon the CL/TNFone interaction, the pronounced variation in the VCD signal was observed for the COO^- vibrations localized in the CL molecule. The observed spectral changes are consistent with the suggested complex structure *(Fig. 5)*. In *Salmonella*, PRO was most active against TNFone, followed by the rubin group of compounds. Comparing this order of effectiveness to CD results, strongly accentuates a direct compound/mutagen interaction, despite the compound's planar shape. In both *Salmonella* strains PRO was clearly the most effective of all compounds tested, and strongly interacted with TNFone. Why planar PRO in contrast to other planar TPs is able to interact with TNFone remains uncertain. Reasons for its strong anti-mutagenic effects other than structural interactions could be its most substantial conjugated double bond system of all tested compounds. This conjugated ring system provides a large number of double bonds and therefore

has the greatest binding capacity of all tested BPs due to strong π-interactions. This theory is also supported by the relatively weak anti-mutagenic effects of the least conjugated compounds UB and SB. Both compounds were equally active in both strains over the entire concentration range, which might have substantial implications for colon carcinogenesis, since these pigments are present at high concentrations within the intestinal lumen.

The changes in CD intensity of the BR/HSA and BV/HSA adducts upon addition of TNFone, document interactions with it. The preserved position of the CD signal suggests minor variation of the chiral structure of the BR and BV part of the associates. This fact supported the idea that the spatial structure of pigments and TNFone fitted well and enabled the effective interactions, whereas BR acted as a more favorite partner for interactions than BV.

In *Salmonella* BV was moderately to weakly anti-mutagenic in both tester strains, which can probably be attributed to its planar shape and comparably smaller π-electron system. Its anti-mutagenic effectiveness was reflected by CD spectral changes, in which BV's interaction with TNFone was the weakest of those compounds tested (PRO > BR > BV). Both BV and BV-DME are planar in shape which possibly explains the weaker protective potential against flexed TNFone, at least in strain *TA102*. Regarding their anti-mutagenic effects in both bacterial strains, both compounds were weaker than BR. The –COOH functional groups of BV carry two $–CH_3$ groups to the C8 and C12 positions of the BV-DME molecule. In the *TA98* condition BV was generally more effective than BV-DME. In *TA102* the effects were inverted. In this experimental setting BV-DME was clearly more effective than BV. Therefore, it seems that aspects of functional supplementation influenced the compounds' anti-mutagenic activities.

As for BR, BRDT and BR-DME in both strains similar orders of compound effectiveness were obtained (BR > BR-DME > BRDT and BR > BRDT > BR-DME). Our results suggest that different functional groups attached to the carboxylic groups of the BR molecule do not seem to play crucial roles in anti-mutagenic action. In other words, free –COOH groups to C8 and C12 of the BR molecule do not seem to be playing key roles in anti-mutagenesis. As previously mentioned, both BR (due to intramolecular hydrogen bonding) and BR-DME possess non-planar structure, and can form BR's preferred ridge-tile conformation [Bulmer et al. 2007; McDonagh et al. 2008] supporting a 3D-interaction. This hypothesis is supported by BR's strong anti-mutagenic effects seen in both *S. typhimurium* strains.

Due to similar anti-genotoxic activities of TPs observed between the different test models, a largely strain-independent anti-mutagenic mechanism can be assumed. Structure-based physic-chemical interactions between test samples and the applied mutagen can be derived for the

majority of TPs, and especially CD spectral changes observed for PRO, BR and BV confirm the compounds' effectiveness against TNFone, and underline a direct structural interaction.

5. Conclusion

This study supports the hypothesis on a strong anti-genotoxic behavior of the tested tetrapyrrolic compounds. In that respect, our novel evidence supports existing data on an anti-mutagenic activity of BR and BV and possible underlying physic-chemical events. Especially PRO continuously showed an enormous anti-mutagenic potential in our trials and strong PRO/TNFone structural interactions were newly revealed. Bilirubin was found to be the most effective among those compounds with known biological relevance. These data form the crucial basis for further interaction studies which might account for known potent biological effects of BPs.

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7. Statement of author contributions

K. H. W. and A. C. B. are grant recipients and contributed to this article by providing constructive expert input and by proof-reading. M. U., I. G. and S. O. did the physic-chemical analyses (CD, VCD), summarised and interpreted the obtained data. L. V. established the important scientific contact and contributed substantially to this manuscript with his biochemical and medical expertise. Together with M. U., I. G. and S. O. he wrote the manuscript parts on structural interactions. H. H., A. S. and G. Z. did parts of the *Salmonella* analyses. M. W. substantially assisted with the statistical analyses and proof-read the manuscript. C. M. planned and designed the experiments and did a major part of it. She also wrote the main body of this article. All co-authors approved the final manuscript and agreed to its submission to Environmental and Molecular Mutagenesis.

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Supplementary material 1. Chemical structures of the test compounds.

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Extracellular and intracellular anti-mutagenic effects of bile pigments in the *Salmonella typhimurium* reverse mutation assay

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ABSTRACT

In vitro anti-genotoxic properties of bile pigments have been explored and confirmed recently. Despite these reports mechanisms to explain DNA protection by endogenous bile pigments remain unclear. Surprisingly, the quantification of cellular pigment absorption which could represent a fundamental prerequisite for intracellular (e.g., anti-mutagenic) effects, has not been explored. Therefore, we aimed to measure the amounts of un-/conjugated bilirubin as well as biliverdin absorbed into colonies of *Salmonella typhimurium*, utilising HPLC analyses, and to observe whether intracellular compound concentrations could predict anti-genotoxic effects. HPLC analyses confirmed that bacterial bile pigment absorption was concentration-dependent. Plate bile pigment concentrations were inversely associated with genotoxicity of all tested mutagens, irrespective of strain and test conditions. However, protection against frame-shift mutation in strain *TA98* most strongly depended on the bacterial absorption of bilirubin and biliverdin, which indicates that bile pigments can protect by intercepting mutations extracellularly and specifically inhibit frame-shift mutations intracellularly.

1. Introduction

Bile pigments (BPs) such as bilirubin (BR) and biliverdin (BV) are tetrapyrrolic, dicarboxylic compounds derived from the enzymatic heme degradation. They are distributed throughout the body and thus could play an essential role in systemic and tissue-specific health promotion. Numerous studies have identified anti-mutagenic and anti-oxidative activity of specific tetrapyrroles (TPs) in vitro (Asad et al., 2001; Bulmer et al., 2007). In vivo data also demonstrate disease prevention through vasoprotection, inhibition of inflammation and anti-oxidant activity (Bulmer et al., 2008b; McCarty, 2007). Multiple underlying mechanisms of anti-genotoxic action have been hypothesised but remain to be confirmed. Surprisingly, no publication has focused on quantifying cellular BP absorption which could form a fundamental basis for intracellular action. Therefore, we aimed to quantify the absorption of BR, BV and conjugated BR (ditaurate; BRDT) into two distinct strains of *Salmonella typhimurium* (*S. typhimurium*) via HPLC analyses. It was hypothesised that BPs would be absorbed in a dose-dependent manner into bacteria, and that extracellular (plate) and intracellular (absorbed) BP concentrations would broadly protect against genotoxicity mediated by various mutagens.

2. Materials and methods

2.1. Salmonella reverse mutation assay

The *Salmonella* reverse mutation assay is an in vitro test assessing the mutagenic potential of chemicals. Bacterial wild-type reversion in the presence of mutagens, allowing growth and colony formation represents its fundamental, technical basis. Experiments were conducted as previously published (Maron and Ames, 1983), and included 48 h of BP incubation at 37 °C. In some assays, S9 liver homogenate (S9 microsomal fraction from Aroclor-treated rats) was used as an enzymatic activation system. Bile pigment concentrations were tested in triplicate, negative/positive controls were tested in each assay ($n = 6$). Experiments were repeated again on a different day and results were then pooled ($n = 6$ minimum).

2.1.1. Bacterial strains

Two strains of *S. typhimurium* were tested: *TA102*, susceptible to oxidative damage, reverts by cross-linking agents, *TA98* detects

Abbreviations: AfB1, aflatoxin B1; BP(s), bile pigment(s); BR, unconjugated bilirubin; BRDT, bilirubin ditaurate; BV, biliverdin; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; *S. typhimurium/Salmonella*, *Salmonella typhimurium*; *t*-BOOH, tertiary butyl hydroperoxide; TNFone, 2,4,7-trinitro-9H-fluoren-9-one.

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Table 1
Correlations between BP plate concentrations and bacterial pigment absorption in *Salmonella* strains *TA98* and *TA102* ("availability-based absorption").

Compound	Strain	Mutagen conc. [mol/plate]	S9	Correlation (r) BP absorption × BP plate conc.
BR	*TA98*	TNFone, 0.3×10^{-6}	–	0.693**
BR	*TA102*	TNFone, 0.2×10^{-7}	–	0.972**
BR	*TA98*	PhIP, 0.1×10^{-7}	+	0.687**
BR	*TA98*	AfB1, 0.8×10^{-7}	+	0.982**
BR	*TA102*	*t*-BOOH, 0.75×10^{-6}	–	0.691**
BV	*TA98*	PhIP, 0.1×10^{-7}	+	0.949**
BV	*TA98*	AfB1, 0.8×10^{-7}	+	0.972**
BV	*TA102*	AfB1, 0.24×10^{-6}	+	0.949**
BV	*TA102*	*t*-BOOH, 0.75×10^{-6}	–	0.949**
BV	*TA102*	*t*-BOOH, 0.75×10^{-6}	+	0.949**
BRDT	*TA98*	PhIP, 0.1×10^{-7}	+	0.885**
BRDT	*TA102*	AfB1, 0.24×10^{-6}	+	0.972**
BRDT	*TA102*	*t*-BOOH, 0.75×10^{-6}	–	0.878*
BRDT	*TA102*	*t*-BOOH, 0.75×10^{-6}	+	0.814**

S9: metabolic activation system (microsomal fraction from Aroclor-treated rats); BP: bile pigment; BR: unconjugated bilirubin; BV: biliverdin; BRDT: bilirubin ditaurate; TNFone: 2,4,7-trinitro-9H-fluoren-9-one; PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; AfB1: aflatoxin B1; *t*-BOOH: *tertiary* butyl hydroperoxide.
** Significant on $p \leqslant 0.01$.
* Significant on $p \leqslant 0.05$.

frame-shift mutations and base-pair deletions (Mortelmans and Zeiger, 2000). Strains were kindly provided by Dr. Bruce N. Ames and were attested to their genetic integrity and spontaneous mutation rate (Mortelmans and Zeiger, 2000) in our laboratory.

2.1.2. Chemicals

Unconjugated bilirubin 1Xα (CAS# 635-65-4), conjugated bilirubin (ditaurate; CAS# 635-65-4) and biliverdin 1Xα (CAS# 55482-27-4) were purchased from Frontier Scientific Europe, UK. Chemical structures can be found online (Supplementary material 1). Pigment purity (>98%) and solubility were measured using HPLC and spectrophotometry. The S9 liver homogenate was obtained from MP Biomedicals, France. All other reagents and mutagens were purchased from Sigma Aldrich, Austria (unless otherwise noted), were of the highest analytical grade available, and stored according to instructions. Bile pigment solutions were prepared in DMSO, protected from light, and used immediately. Composition and preparation of all necessary solutions can be found elsewhere (Bulmer et al., 2007). To assess different possibilities of anti-genotoxic action (e.g., structural interactions, radical scavenging, complex formation), four different mutagens were applied at their respective appropriate concentrations (Table 1): 2,4,7-trinitro-9H-fluoren-9-one (J & K Ltd., China; TNFone), *tertiary*-butyl hydroperoxide (Merck; *t*-BOOH), aflatoxin B1 (AfB1) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (Toronto Research Chemicals, Canada; PhIP).

2.1.3. Bile pigment sample preparation for the Salmonella reverse mutation assay

Based on preceding investigations (Bulmer et al., 2007), BRDT and BV were tested at concentrations of 0.01, 0.05, 0.1, 0.5, 1 and 2 μmol/plate (equals 3.4, 17.2, 34.5, 172.4, 349 and 689.6 μM). Unconjugated BR was tested over a range of five doses including: 0.01, 0.05, 0.1, 0.5 and 0.75 μmol/plate (equals 258.6 μM). Maximum BP plate concentrations had been ascertained by (1) testing the maximum amount of DMSO that did not result in bacterial cytotoxicity (200 μl/plate) and (2) by the respective maximum solubility of each test compound (spectrophotometric supernatant analysis: BR, BRDT: 455 nm; BV: 380 nm), read on a Perkin Elmer Lambda 2 UV/VIS spectrophotometer after high-speed centrifugation.

2.2. Sample preparation for HPLC analyses

Briefly, *S. typhimurium* colonies (⩾1 mm in diameter) were collected from agar plates, and were lysed for 30 min in 40 μl of isocratic mobile phase (950 ml HPLC-grade methanol, 50 ml HPLC-grade water, 24.2 g n-dioctylamine and 6.01 g glacial acetic acid per litre). Supernatants were diluted at 1:4, and injected (50 μl) into a Hitachi HPLC, equipped with a Shimadzu SPD-M20A detector, and a C18 reverse phase column (5micron, 250 × 4.6 m) (Brower et al., 2001; Bulmer et al., 2008b). Oven temperature was set at 35 °C, column pressure at 140 bar. Sixteen BP standards were run, ranging from 500 to 0.01 μM. The method's detection limit (LOD) was calculated at 18 nM. Photographs of bacterial colonies can be found online (Supplementary material 2).

2.3. Measurement of total protein content

As a reference parameter for bacterial BP absorption, the total protein content in each diluted sample was measured photometrically (Bradford, 1976). Bile pigment concentrations were then expressed as nmol/mg total protein.

2.4. Statistical analyses

Data were analysed using SPSS 17.0. A *p*-value ⩽0.05 was considered significant. Data were tested for normal distribution using

Table 2
Correlations between BP plate concentrations or bacterial BP absorption, respectively, and anti-mutagenic effects in *Salmonella* strains *TA98* and *TA102* ("availability-based anti-mutagenic effects" and "absorption-based anti-mutagenic effects").

Compound	Strain	Mutagen conc. [mol/plate]	S9	Correlation (r) BP plate conc. x anti-mut. effects	Correlation (r) BP absorption x anti-mut. effects
BR	*TA98*	TNFone, 0.3×10^{-6}	–	−0.794**	−0.938**
BR	*TA102*	TNFone, 0.2×10^{-7}	–	−0.682**	−0.639**
BV	*TA102*	TNFone, 0.2×10^{-7}	–	−0.493**	−0.124
BV	*TA98*	PhIP, 0.1×10^{-7}	+	−0.927**	−0.917**
BV	*TA98*	AfB1, 0.8×10^{-7}	+	−0.473**	−0.827**
BV	*TA102*	AfB1, 0.24×10^{-6}	+	−0.601**	−0.216
BV	*TA102*	*t*-BOOH, 0.75×10^{-6}	–	−0.607**	−0.570
BRDT	*TA98*	TNFone, 0.3×10^{-6}	–	−0.475**	−0.198
BRDT	*TA102*	TNFone, 0.2×10^{-7}	–	−0.378*	−0.203
BRDT	*TA98*	AfB1, 0.8×10^{-7}	+	−0.548**	−0.665
BRDT	*TA102*	*t*-BOOH, 0.75×10^{-6}	–	−0.754**	−0.481

S9: metabolic activation system (microsomal fraction from Aroclor-treated rats); BP: bile pigment; BR: unconjugated bilirubin; BV: biliverdin; BRDT: bilirubin ditaurate; TNFone: 2,4,7-trinitro-9H-fluoren-9-one; PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; AfB1: aflatoxin B1; *t*-BOOH: *tertiary* butyl hydroperoxide.
** Significant on $p \leqslant 0.01$.
* Significant on $p \leqslant 0.05$.

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the Kolmogorov–Smirnov test. Parametric statistical analysis (one-way ANOVA) and the *post hoc* Scheffé test were performed on normally distributed, and corresponding non-parametric tests (Kruskal–Wallis H-test, Dunn's *post hoc* test) on skewed data. Relationships between (1) BP bacterial absorption and plate concentrations; (2) BP bacterial absorption and anti-genotoxic effects; and (3) between BP plate concentrations and anti-mutagenicity were determined by performing bivariate

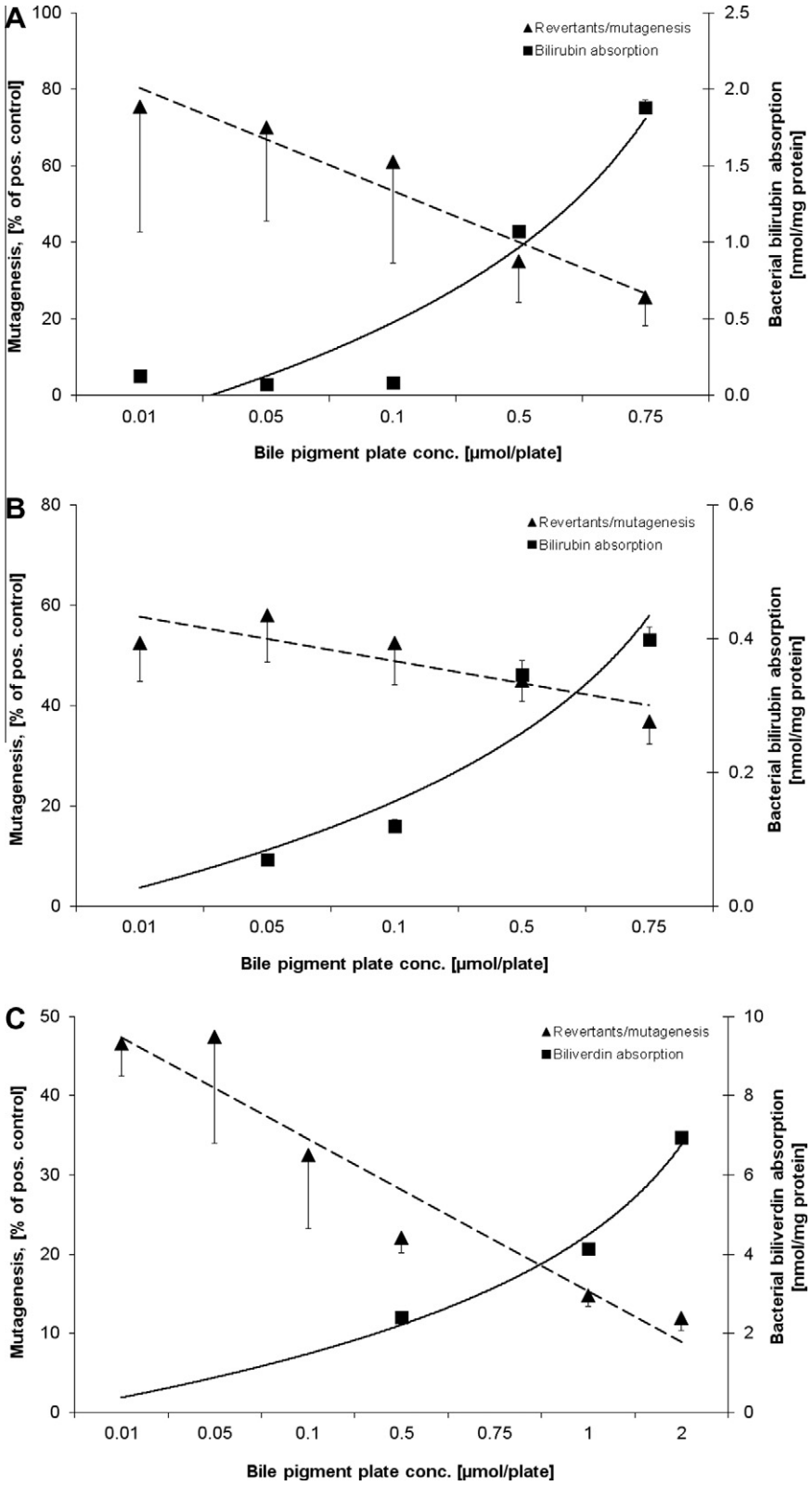

Fig. 1. (A–C) Relationship between bacterial BP absorption and observed anti-mutagenic effects against different mutagens; (A) BR in strain *TA98* with TNFone, (B) BR in strain *TA102* with TNFone, (C) BV in strain *TA98* with PhIP and with S9.

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correlations (Pearson or Spearman for parametric and non-parametric data, respectively).

3. Results

HPLC analyses showed significant concentration-dependent BP absorption from agar plates, which was independent of strain, test condition (± S9) and the applied mutagen (Table 1). Furthermore, anti-mutagenic effects of all BPs against all tested mutagens were observed (Table 2).

The relationships between BP plate concentrations, bacterial BP absorption and observed anti-mutagenic effects are shown in Fig. 1A–C and in Supplementary material 3. Significant inverse relationships were found between BR plate concentrations in strains *TA98* and *TA102* and anti-mutagenic action against TNFone, as well as between BV plate concentration and PhIP mutagenesis in *TA98* (Fig. 1A–C). A typical chromatogram showing BR absorption into strain *TA98* is shown in Fig. 2. Furthermore, significant correlations between pigment plate concentrations and anti-genotoxicity were found for all test conditions (Table 2), indicating a stronger concentration-dependent anti-mutagenic effect based on extracellular (vs. intracellular) BP concentration. Interestingly, the anti-mutagenic effects of BR and BV were most strongly dependent on the bacterial BP absorption exclusively in strain *TA98* (Table 2).

An entirely novel observation was also made in that the obtained HPLC spectra (*not shown*) suggest appearance of BR in plates supplemented with BV, which could imply biliverdin reductase activity in *S. typhimurium*. The ratio of BV to BR (BV:BR) bacterial concentrations calculated from HPLC chromatograms (at 1 μmol/plate BV) approximated 4.4:1 in *TA98* and 9.6:1 in *TA102*.

4. Discussion

This study is the first to report on bacterial BP absorption and its relationship with observed anti-mutagenic effects. When exposed to mutagens, extracellular (plate) BP concentrations negatively correlated with genotoxicity. Furthermore, testing in *TA98* revealed that BV and BR absorption was more strongly related with anti-mutagenesis, when compared to the anti-mutagenic effect relative to plate concentrations.

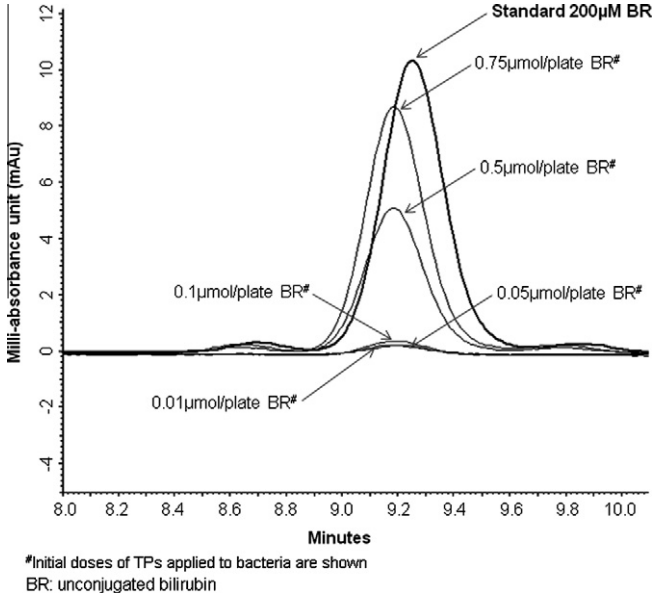

Fig. 2. Chromatogram of BR (with TNFone) in *Salmonella TA98* colonies.

Previous reports refer to the ability of BPs to act in an anti-oxidant and anti-genotoxic manner in vitro (Asad et al., 2001; Bulmer et al., 2007) and in vivo (Boon et al., 2012; Horsfall et al., 2011). Vastly unclear to date however, are the underlying mechanisms of anti-genoxic action. In this context mainly electron scavenging or hydrogen donating capacities (MacLean et al., 2008) and structural interactions between BPs and mutagens (Hayatsu, 1995) are discussed. However, data on cellular compound absorption are lacking and so far only one recent report on enzymatic BRDT reduction in bacteria (Konickova et al., 2012) exists. Therefore, we explored whether bacterial BP absorption was more closely related to anti-mutagenesis compared to extracellular BP concentrations around *S. typhimurium* experiencing genotoxic stress.

In this study, physiologically relevant concentrations of BPs were tested. Un-/conjugated BR is found in the blood, the liver, the intestine (where about 70% are recycled via the enterohepatic cycle), and the urinary tract. In these compartments BR is further metabolised, recycled and/or excreted (Klatskin, 1961). The liver and gut, which are sites of BP accumulation, are at particular risk of genotoxicity due to the absorption, metabolism (Guengerich, 2000; Turesky et al., 2002) and excretion of mutagens. The abundance of BPs within these organs suggests BPs could exert physiological protection against DNA damage specifically at these sites.

Interestingly, BR and BV absorption strongly protected against frame-shift mutation in the *TA98* strain. This mutation represents an important mechanism of pathogenesis in gastric and colorectal cancers (Kim et al., 2010). We speculate that the affinity of BPs to protect against frame-shift mutation, might partly explain the protective relationship between serum BR levels and colorectal cancer in vivo (Zucker et al., 2004). It should be emphasised, however, that BPs do protect against oxidative and frame-shift mutation when present extracellularly, indicating a clear role for BPs in neutralising mutagens before entering cells. Furthermore, it should be noted that BR causes apoptosis in cancer cells in vitro (Keshavan et al., 2004), providing an additional mechanism for chemoprevention. These data further emphasise the importance of therapeutically elevating BR concentrations for the prevention of cardiovascular disease and cancer (McCarty, 2007). Reports to indicate that BV and BRDT are readily absorbed across cultured enterocytes (Bulmer et al., 2008a) support this theory. These data confirm that potential anti-mutagenic BP effects in vivo could be induced by increasing concentrations in the gut lumen (Bulmer et al., 2011) where food-borne mutagens are found, or by increasing blood BP content in vivo to impart protection from DNA damage (Wallner et al., 2012). Although the results of these in vitro experiments cannot be directly extrapolated to in vivo settings, the results suggest BPs in the extracellular milieu (e.g., in the gut lumen/blood) could play a key role in cellular protection, by intercepting mutagens before they arrive at their site of action (e.g., DNA).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2012.08.004.

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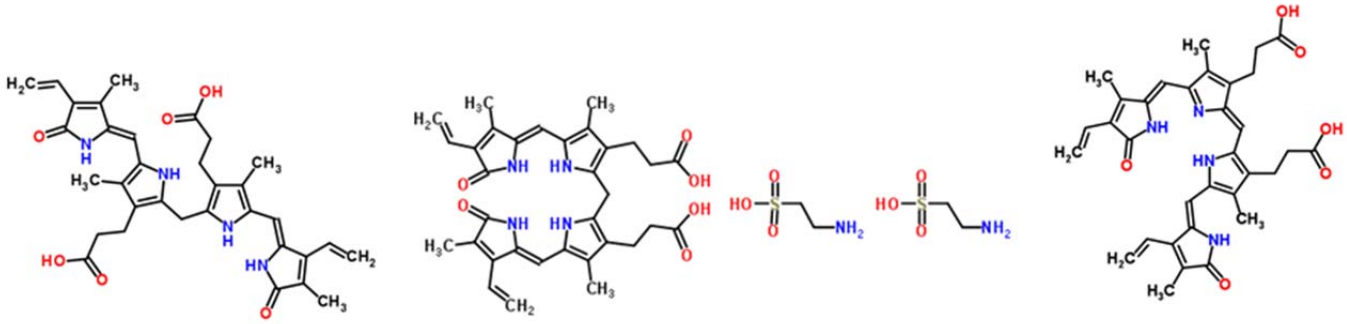
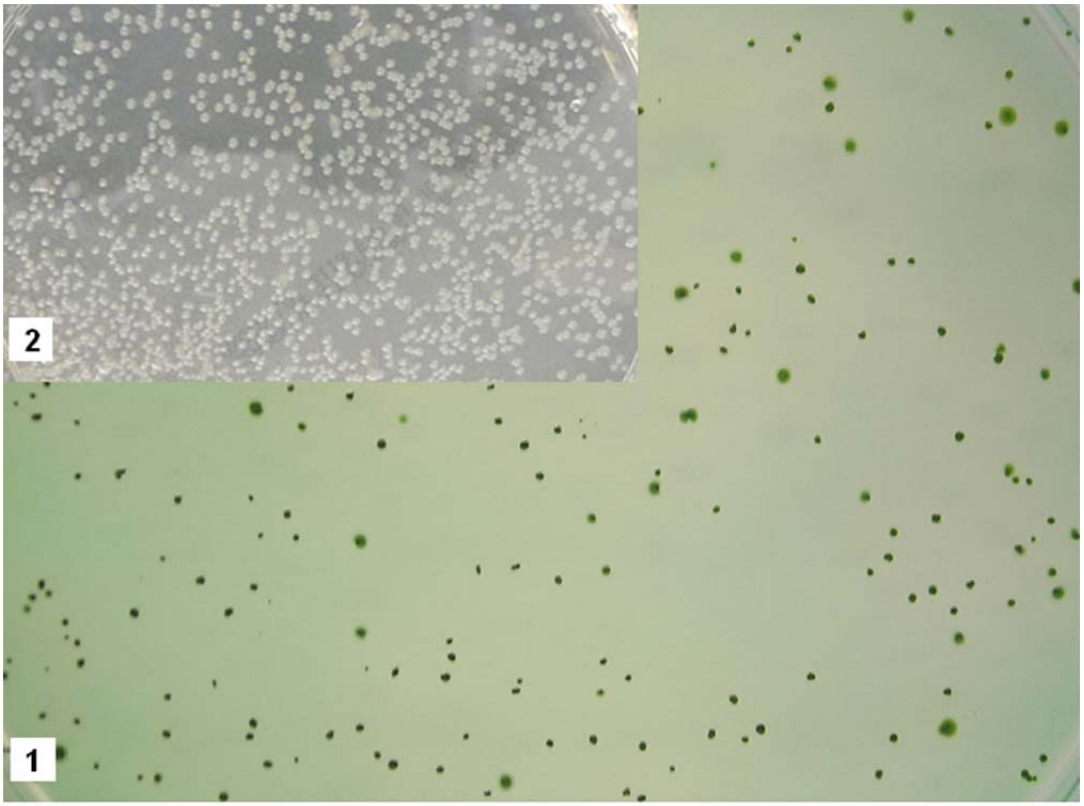
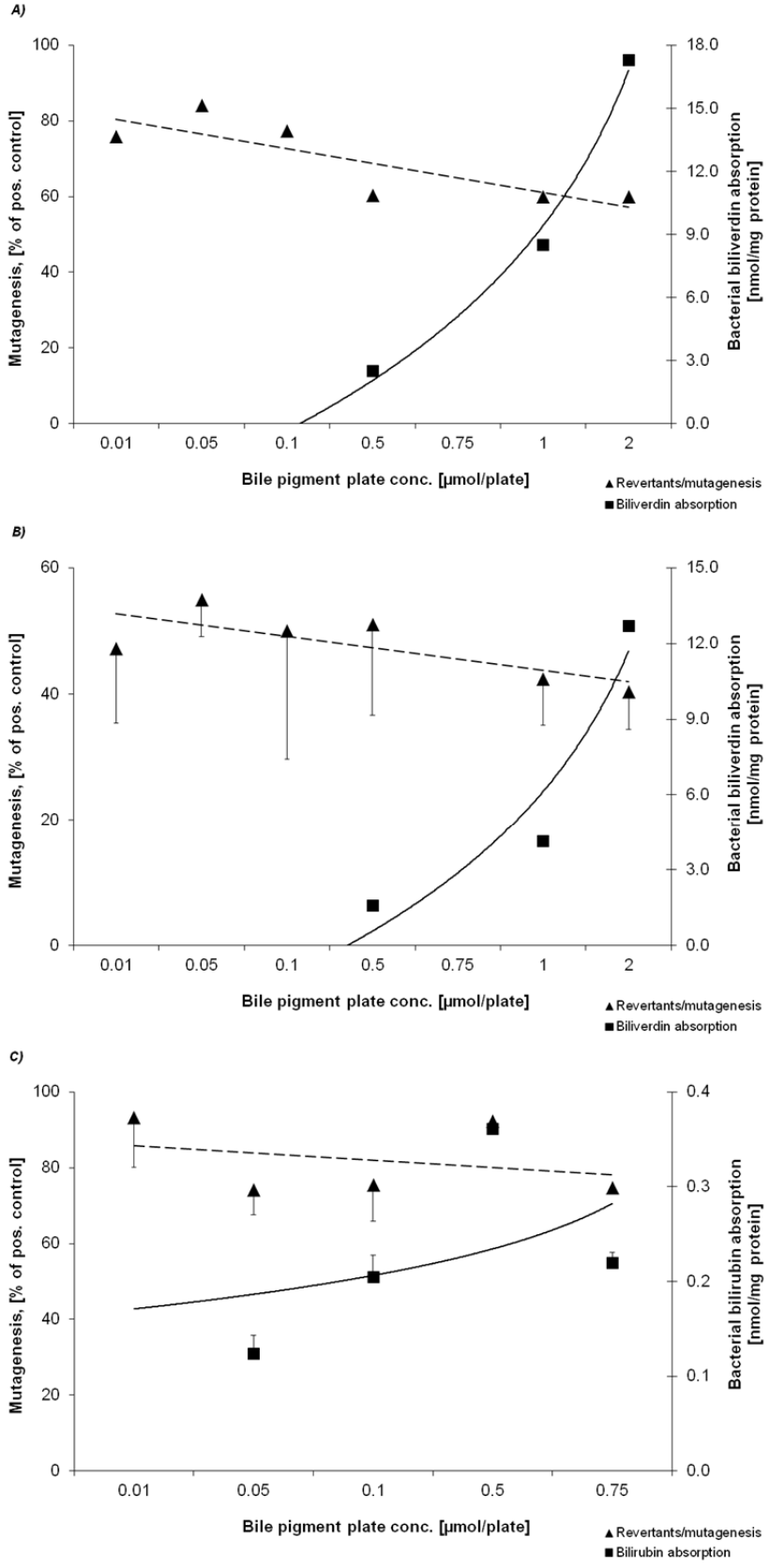


Supplementary material 1. **Chemical structures of the test compounds**



Supplementary material 2. Salmonella typhimurium TA102 colonies with **(1)** and without **(2)** 2µmol/plate BV



Supplementary material 3. Relationship between bacterial BP absorption and observed anti-mutagenic effects against different mutagens; A) BV in strain *TA102* with *tert*-BOOH, B) BV in strain *TA102* with AfB1 + S9, C) BR in strain *TA102* with *tert*-BOOH.
BP: bile pigment, BR: unconjugated bilirubin, BV: biliverdin, *tert*-BOOH: *tertiary*-butyl hydroperoxide, AfB1: aflatoxin B1, S9: rat liver homogenate.

In vitro DNA-damaging effects of intestinal and related tetrapyrroles in human cancer cells

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Key words: stercobilin, urobilin, protoporphyrin, SCGE, comet

Abstract

Epidemiological studies report a negative association between circulating bilirubin concentrations and the risk for cancer and cardiovascular disease. Structurally related tetrapyrroles also possess *in vitro* anti-genotoxic activity and may prevent mutation prior to malignancy. Furthermore, few data suggest tetrapyrroles exert anti-carcinogenic effects *via* induction of cell cycle arrest and apoptosis. To further investigate whether tetrapyrroles provoke DNA-damage in human cancer cells, they were tested in the single cell gel electrophoresis assay (SCGE). Eight tetrapyrroles (unconjugated bilirubin, bilirubin ditaurate, biliverdin, biliverdin-/bilirubin dimethyl ester, urobilin, stercobilin and protoporphyrin) were added to cultured Caco2 and HepG2 cells and their effects on comet formation (% tail DNA) were assessed. Flow cytometric assessment (apoptosis/necrosis, cell cycle, intracellular radical species generation) assisted in revealing underlying mechanisms of intracellular action. Cells were incubated with tetrapyrroles at concentrations of 0.5, 5 and 17 μM for 24 hrs. Addition of 300 μM *tertiary* butyl hydroperoxide to cells served as a positive control. Tetrapyrrole incubation mostly resulted in increased DNA-damage (comet formation) in Caco2 and HepG2 cells. Tetrapyrroles that are concentrated within the intestine, including protoporphyrin, urobilin and stercobilin, led to significant comet formation in both cell lines, implicating the compounds in inducing DNA-damage and apoptosis in cancer cells found within organs of the digestive system.

1. Introduction:

Bile pigments (BPs) such as bilirubin (BR), biliverdin (BV) and structurally related tetrapyrroles (TPs) are formed naturally within the human body. Derived from heme within hemoglobin and heme-containing enzymes, endogenous TPs possess porphyrin structure and carry a conjugated system of double-bonds [1]. Heme catabolism requires the action of heme oxygenase (HMOX-1/-2) and biliverdin reductase (BLVRA), within certain organs including the liver and intestine. Tetrapyrroles derived from this process are found in the bile (mainly conjugated BR), and in the intestinal milieu (mainly stercobilin SB, urobilin UB and protoporphyrin PRO) [2, 3]. The literature reports anti-mutagenic, antioxidant as well potentially anti-carcinogenic activity of BR and BV *in vitro* [4-8]. Further studies suggest an important role for mildly elevated circulating BR in preventing disease in human subjects [9-13] by antioxidant mechanisms [9, 11-13], and emphasize a protective physiological role for unconjugated BR in protecting against gastrointestinal and colorectal cancer [14]. These effects might also be related to BR's intestinal abundance [14], with recent evidence indicating the potential efficacy of intestinal absorption [15, 16]. Consequently, physiologically abundant TPs could play significant health promoting roles in the organs where they are absorbed/accumulate including the liver, gall bladder and intestine in addition to the urinary tract and the circulatory system [16].

Thus far, the scientific focus has mainly been directed towards *in vitro* anti-genotoxic properties of BR and BV [5, 17], with little attention focused on structurally related metabolites. Unconjugated BR induces cell cycle arrest, apoptosis and cytostasis *in vitro* in multiple cell lines [18]. Besides, BR may be defending against cancer by interfering with pro-carcinogenic signaling pathways, and therefore potently inhibit tumor cell proliferation *in vivo* [18]. Furthermore, BR induces mitochondrial depolarization in colon cancer cells [19]. However, data concerning the effects of related tetrapyrrolic compounds on cancer cell biology are entirely lacking, with the only exception being PRO which is successfully applied in the clinic [20]. Despite the use of PRO and derivatives in the photodynamic treatment of skin cancer [21, 22], *in vitro* comet data and evidence on pro-apoptotic, anti-carcinogenic and anti-proliferative effects of PRO in different cell culture models are rare [22-26]. Also the DNA-damaging effects of BR in cancer cells have been investigated only once [27], when applying the single cell gel electrophoresis (SCGE/comet) assay.

Stress stimuli such as DNA-damage provoke cellular responses including oxidative stress, cell cycle arrest and apoptosis, the latter of which is mainly controlled by the action of tumor suppressors [21, 22, 26, 28]. Many chemotherapeutics including purine and pyrimidine analogs

as well as alkylating agents induce DNA-damage within rapidly proliferating cells in an attempt to selectively target malignant cells. To assess whether TPs exert comparable effects on cancer cell lines (*e. g.* induce free radical formation), five essentially untested TPs (BR-/BV dimethyl ester (BR-/BV-DME), UB, SB, PRO) were investigated together with BR, BRDT and BV in human colorectal adenocarcinoma (Caco2) and hepatocellular carcinoma (HepG2) cells. These cell lines represent meaningful models for TP *in vitro* testing, since both the liver and intestine represent central organs for BP metabolism [29]. To elucidate cellular regulatory mechanisms in response to TP exposure, flow cytometry analyses (apoptosis/necrosis, intracellular radical species (ROS), cell cycle) were conducted to reveal underlying mechanisms of TPs action, relevant to cancer cell biology [21, 22, 26, 28, 30], while the comet assay was applied to determine the extent of DNA damage.

2. Materials and methods:

2.1. Chemicals

Unconjugated bilirubin IXα (BR) [CAS# 635-65-4], bilirubin conjugate (ditaurate; disodium; BRDT) [CAS# 635-65-4], biliverdin IXα (BV) [CAS# 55482-27-4], bilirubin dimethyl ester (BR-DME) [CAS# 19792-68-8], biliverdin dimethyl ester (BV-DME) [CAS# 10035-62-8], protoporphyrin IX (PRO) [CAS# 553-12-8] as well as urobilin (UB) [CAS# 28925-89-5] and stercobilin (SB) [CAS# 34217-90-8] were purchased from Frontier Scientific, UK, and were dissolved in DMSO. Solubility was tested spectrophotometrically and (purity) *via* HPLC [15, 31]. DMSO final concentrations in media did not exceed 0.1 %. Test compounds were stored in airtight and lightproof containers at -80 °C until use, and were protected from light throughout all test procedures using foil-covered containers. Other chemicals were purchased from Sigma Aldrich, Austria (unless otherwise noted), were of the highest analytical grade available and were stored and used according to instructions.

2.2. Single cell electrophoresis assay

The comet assay measures DNA single- and double strand breaks in eukaryotic cells embedded in 1 % low melting agarose (LMA; Invitrogen Austria), fixed on agarose pre-coated microscope slides (1 % normal melting agarose, NMA; Invitrogen Austria). After cell lysis at pH 10 and 20 min of DNA unwinding (as well as 300 µM *tertiary*-butyl hydroperoxide (*tert*-BOOH) treatment for positive controls), cells were exposed to a directed electric field (Electrophoresis CSL-10M40, Biozym Austria; pH >13). After ethidiumbromide staining (20 µl of 20 µg/ml per

gel), DNA migration/comet formation was evaluated using a fluorescent microscope (Zeiss Germany) equipped with a camera (Hitachi Austria). Komet 5.5. software (Andor Technology, Germany) assessed comet tail DNA content which was expressed as a percentage of total cellular DNA (% tail DNA). The method of Azqueta *et al.* [32] was used to measure both DNA single- and double strand breaks. Human lymphocytes were replaced by cancer cells, using 1 x 10^6 cells per ml. Eight gels (two per slide; per compound and concentration) were prepared for statistical and quality assurance analysis, six gels of which at minimum were randomly counted (50 cells/gel).

2.2.1. Human cancer cell lines

Cytotoxicity was assessed in two cell lines (Caco2 and HepG2) that were originally derived from primary human tumors as reported by the provider (source as below). Cell viability and cell counts were assessed using a trypan blue assay-based automated cell counter (Countess; Invitrogen, Austria).

2.2.1.1. Cell culture media

Cells were obtained from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute, DSMZ), and were maintained using standard culture techniques. Caco2 cells were cultivated in antibiotic-free Dulbecco's modified essential medium (DMEM with high glucose; PAA Austria), and HepG2 cells in Eagle's minimum essential medium with Earle's salts (MEM; PAA Austria) in sterile 25 cm^2 filter cap flasks (SPL Life Sciences Inc., Austria). Media were supplemented with 10 % (v/v) fetal bovine serum (FBS; PAA Austria), 5ml of non-essential amino acids, 1 ml of Na-pyruvate (HepG2) or 20 % FBS *(v/v)* and 1 ml of 100 mM Na-pyruvate (Caco2). Experiments were conducted between passages 17 – 37 for Caco2, and 34 – 54 for HepG2 cells. Media were changed every second day and on the day before and after cell splitting, which was performed at 70 – 80 % confluence, using 1 ml per flask of Accutase solution (PAA Austria). For TP incubation, 4.5 x 10^5 cells/ml were seeded in sterile 24 well plates (SPL Life Sciences Inc., Austria) and were allowed to adhere for 24 hrs. All cultures were maintained at 37 °C in a humidified atmosphere of 95 % air, 5 % CO_2.

2.3. Flow cytometric analyses

After 24 hrs of TP incubation, cells were harvested from 24 well plates and transferred to 5 ml FACS tubes (BD Biosciences, Austria). After treatment including centrifugation, washing and staining steps, cell suspensions were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, Austria).

2.3.1. Apoptosis/Necrosis assay

The applied detection kit (r-phycoerythrin (PE) annexinV apoptosis detection kit I, BD Pharmingen Austria) was used to measure apoptosis and necrosis *via* flow cytometry. This assay requires the Ca^{2+}-dependent affinity of annexinV to phosphatidyl serine as well as the specificity of 7-aminoactinomycin-d (7-AAD) for guanine-cytosine DNA-base pairs for the assessment of apoptosis and necrosis. Briefly, 1 x 10^6 cells/ml were washed in Ca^{2+}/Mg^{2+}-free 1 x phosphate buffered saline (PBS; PAA Austria) and re-suspended in binding buffer, were doubly labelled with PE-stained annexinV (ex_{max}: 496, em_{max}: 575 nm; FL-2), and 7-AAD (ex_{max}: 546, em_{max}: 647 nm; FL-3) and were incubated for 15 min in the dark prior to cytometric analyses [33, 34].

2.3.2. Intracellular radical species

To detect intracellular reactive oxygen species (ROS), 10 µM final concentration dihydroethidium (DHE) and 25 µM final concentration dihydrofluorescein diacetate (DCFH-DA), both in DMSO, were added indivdually to incubated cells. Dihydroethidium is cell-permeable and reacts with $O_2^{-\cdot}$ to form oxyethidium. This product interacts with nucleic acids and emits red fluorescence, which can be measured cytometrically (ex_{max}: 329, em_{max}: 373 nm; FL-2) [35]. When DCFH-DA enters the cell, it is cleaved by intracellular esterases to form DCFH, which is then non-specifically oxidized by (hydro)peroxides to fluorescent 7'-dichlorodihydrofluorescein (DCF) (ex_{max}: 498, em_{max}: 522 nm; FL-1) [35]. After washing in Ca^{2+}/Mg^{2+}-free 1 x PBS and prior to measurement, 1 x 10^6 cells/ml were stained with DHE (in FACS tubes after harvesting) or with DCFH (in 24 wells prior to harvesting) and were left to incubate for 20 min in the dark [36].

2.3.2. Cell cycle assay

The cell cycle assay measures the percentage number of cells in the cell cycle phases G0/1, S, G2/M, as well as apoptotic cells (sub G0/1), according to fluorescence intensity, based on the number of DNA copies within cells. Propidium iodide (PI; ex_{max}: 535, em_{max}: 617 nm; FL-2) binds to DNA by intercalating between the bases with a stoichiometry of one PI molecule per 4.5 DNA base pairs. After washing, 1 x 10^6 cells/ml were fixed with 75 % ice-cold absolute ethanol and were left to incubate for 30 min on ice. Cells were re-suspended in 500 µl of phosphate buffer (96 ml 0.2 M Na_2HPO_4 + 4 ml 0.1 M $C_6H_8O_7$), and after centrifugation, 50 µM of 100 µg/ml RNase and 200 µl of 50 µg/ml PI (both in $_dH_2O$) were added [37, 38]. Cell suspensions were measured immediately.

2.4. Statistical analyses

To evaluate DNA-damaging effects of TPs in the comet assay, 50 cells per gel were counted. As a measure of DNA-damage, mean percentages of total DNA (% tail DNA) were calculated in Microsoft Excel. Statistical analysis was completed using IBM SPSS 19 for Windows. Normal distribution of the data was assessed using the K-S test. To calculate differences in DNA-damage between groups, ANOVA was performed on parametric data and Kruskal-Wallis H-test on non-parametric data, followed by the Dunnet-T3 *post-hoc* test, assuming non-homogenous variances. Pearson bivariate correlations were performed on parametric, Spearman rho on non-parametric data. A p-value ≤ 0.05 was considered significant.

3. Results:

3.1. DNA-damage/comet formation in Caco2 and HepG2 cell lines:

3.1.1. Protoporphyrin, urobilin, stercobilin

In both cell lines, PRO at the highest and lowest tested concentrations induced significant DNA-damage versus negative control *($p < 0.05$),* as was the case in HepG2 cells, also in comparison to positive control *($p < 0.05$; Figures 1A and 2A)*. Following UB incubation in both cell lines, DNA-damaging effects tended to be or were significantly increased compared to negative control *($p < 0.05$; Figures 1B and 2B).* In HepG2 cells, SB elevated DNA-damage compared to negative and positive control *($p < 0.05$; Figure 2C).*

3.1.2. Bilirubin, bilirubin ditaurate, bilirubin dimethyl ester

In Caco2 cells, the highest tested BR concentration led to significantly reduced comet formation compared to negative control *($p < 0.05$; Table 1).* Somewhat in contrast, conjugated BRDT at

0.5 µM showed significantly elevated DNA-damage versus negative control in Caco2 cells *($p < 0.05$; Table 1)*. In HepG2 cells however, BRDT did not induce DNA-damage compared to negative or positive control *($p < 0.05$; Table 1)*. Bilirubin dimethyl ester incubation with Caco2 and HepG2 cells showed no DNA-damaging effects.

3.1.3. Biliverdin, biliverdin dimethyl ester

In Caco2 cells, BV and BV-DME did not significantly increase DNA-damage versus negative control *($p < 0.05$; Table 1)*. However, BV incubation in HepG2 cells caused significantly elevated DNA-damage at all tested concentrations compared to the negative control *($p < 0.05$; Table 1)*. Also BV-DME at the lowest and highest tested concentration led to significantly elevated DNA-damage compared to negative control *($p < 0.05$; Table 1)*.

3.2. Correlations between DNA-damage/comet formation and flow cytometry parameters:

3.2.1. Protoporphyrin, urobilin, stercobilin

In HepG2 cells, a positive relationship between PRO-induced comet formation and intracellular ROS (superoxide) production was determined *($p < 0.05$; Table 2)*. Superoxide formation after PRO incubation in HepG2 cells was positively correlated to apoptosis *(r 0.529, $p < 0.05$)*.

3.2.2. Bilirubin, bilirubin ditaurate, bilirubin dimethyl ester

For BR, a positive correlation was determined between comet- and intracellular ROS formation (superoxide and hydroperoxide) in both Caco2 and HepG2 cell lines *($p < 0.05$; Table 2)*. ROS (superoxide) formation in HepG2 cells after BR incubation was positively related to apoptosis *(r 0.704, $p < 0.01$)*.

3.2.3. Biliverdin, biliverdin dimethyl ester

Following BV incubation in Caco2 cells a positive correlation was determined between DNA-damaging effects and ROS formation *(Table 2)*. In HepG2 cells BV-DME-based comet formation significantly correlated with apoptosis *($p < 0.05$; Table 2)*. In HepG2 cells ROS formation after BV incubation non-significantly correlated with apoptosis *(r 0.488, p 0.077)*.

Table 1. Mean % tail DNA in Caco2 and HepG2 cells (± SD) after 24 hrs TP incubations, compared to positive and negative controls.

Caco2

Tetrapyrrole	% tail DNA ± SD	% tail DNA pos control ± SD	% tail DNA neg control ± SD
BR 0.5 µM	18.7 ± 3^{p}	36.5 ± 4	21.3 ± 3
BR 5 µM	21.3 ± 3.8^{p}		
BR 17 µM	13.8 ± 1.9^{np}		
BRDT 0.5 µM	10.8 ± 2.6^{np}	29.1 ± 2.7	7.7 ± 2.0
BRDT 5 µM	11.7 ± 2.6^{p}		
BRDT 17 µM	18.2 ± 4.1^{p}		
BR-DME 0.5 µM	8.7 ± 2.7	21.9 ± 3.9	9.9 ± 4.0
BR-DME 5 µM	11.5 ± 2.3^{p}		
BR-DME 17 µM	15.0 ± 6.9^{p}		
BV 0.5 µM	20.9 ± 4.3^{p}	30.1 ± 5.0	16.5 ± 2.6
BV 5 µM	20.8 ± 2.9		
BV 17 µM	18.4 ± 2.7		
BV-DME 0.5 µM	15.6 ± 3.5^{p}	26.6 ± 2.9	15.5 ± 2.5
BV-DME 5 µM	15.3 ± 3.2^{p}		
BV-DME 17 µM	12.9 ± 2.2^{p}		

HepG2

Tetrapyrrole	% tail DNA ± SD	% tail DNA pos control ± SD	% tail DNA neg control ± SD
BR 0.5 µM	10.8 ± 2.8	18.5 ± 3.9	9.3 ± 1.0
BR 5 µM	9.2 ± 2.9		
BR 17 µM	9.7 ± 1.3		
BRDT 0.5 µM	8.2 ± 0.8^{p}	13.3 ± 0.9	7.4 ± 2.0
BRDT 5 µM	8.8 ± 1.4^{p}		
BRDT 17 µM	8.5 ± 1.1^{p}		
BR-DME 0.5 µM	8.8 ± 2.5^{p}	13.4 ± 2.5	6.8 ± 2.3
BR-DME 5 µM	9.3 ± 1.3^{p}		
BR-DME 17 µM	8.7 ± 2.5		
BV 0.5 µM	17.2 ± 1.4^{n}	16.8 ± 0.7	12.3 ± 1.0
BV 5 µM	15.1 ± 0.9^{np}		
BV 17 µM	16.6 ± 1.3^{n}		
BV-DME 0.5 µM	17.0 ± 2.2^{np}	21.6 ± 2.1	12.3 ± 1.6
BV-DME 5 µM	14.6 ± 2.0^{p}		
BV-DME 17 µM	17.8 ± 1.4^{np}		

BR: unconjugated bilirubin, BRDT: bilirubin ditaurate, BV: biliverdin, BV-DME: biliverdin dimethyl ester, TP: tetrapyrrole.
^{p}significantly different to positive control *($p \leq 0.05$)*
^{n}significantly different to negative control *($p \leq 0.05$)*
^{np}significantly different to negative and positive control *($p \leq 0.05$)*

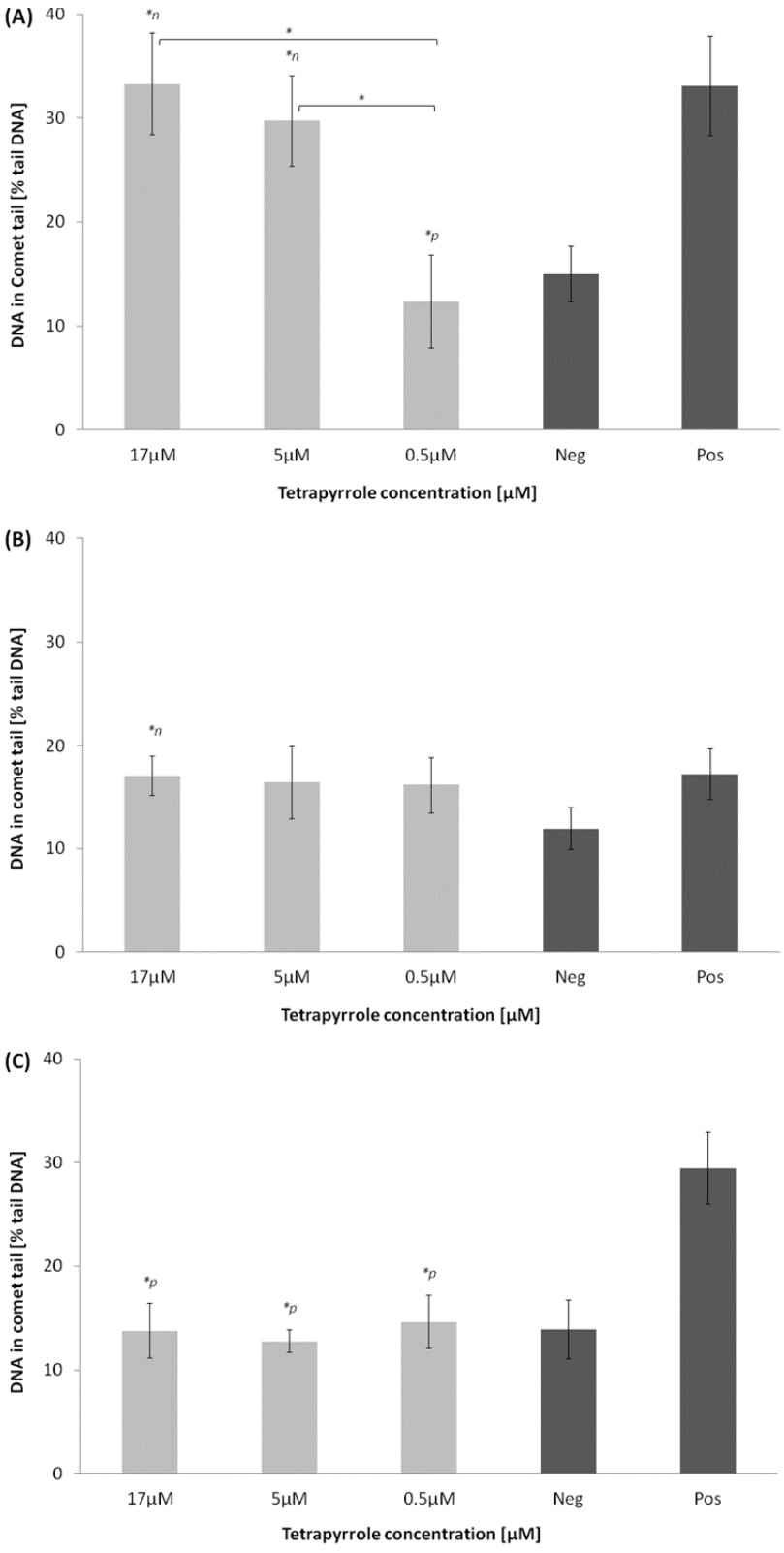

Figure 1 A – C. DNA-damaging effects of (A) protoporphyrin, (B) urobilin and (C) stercobilin in Caco2 cells.
*n: significantly different to negative control *($p \leq 0.05$)*
*p: significantly different to positive control *($p \leq 0.05$)*

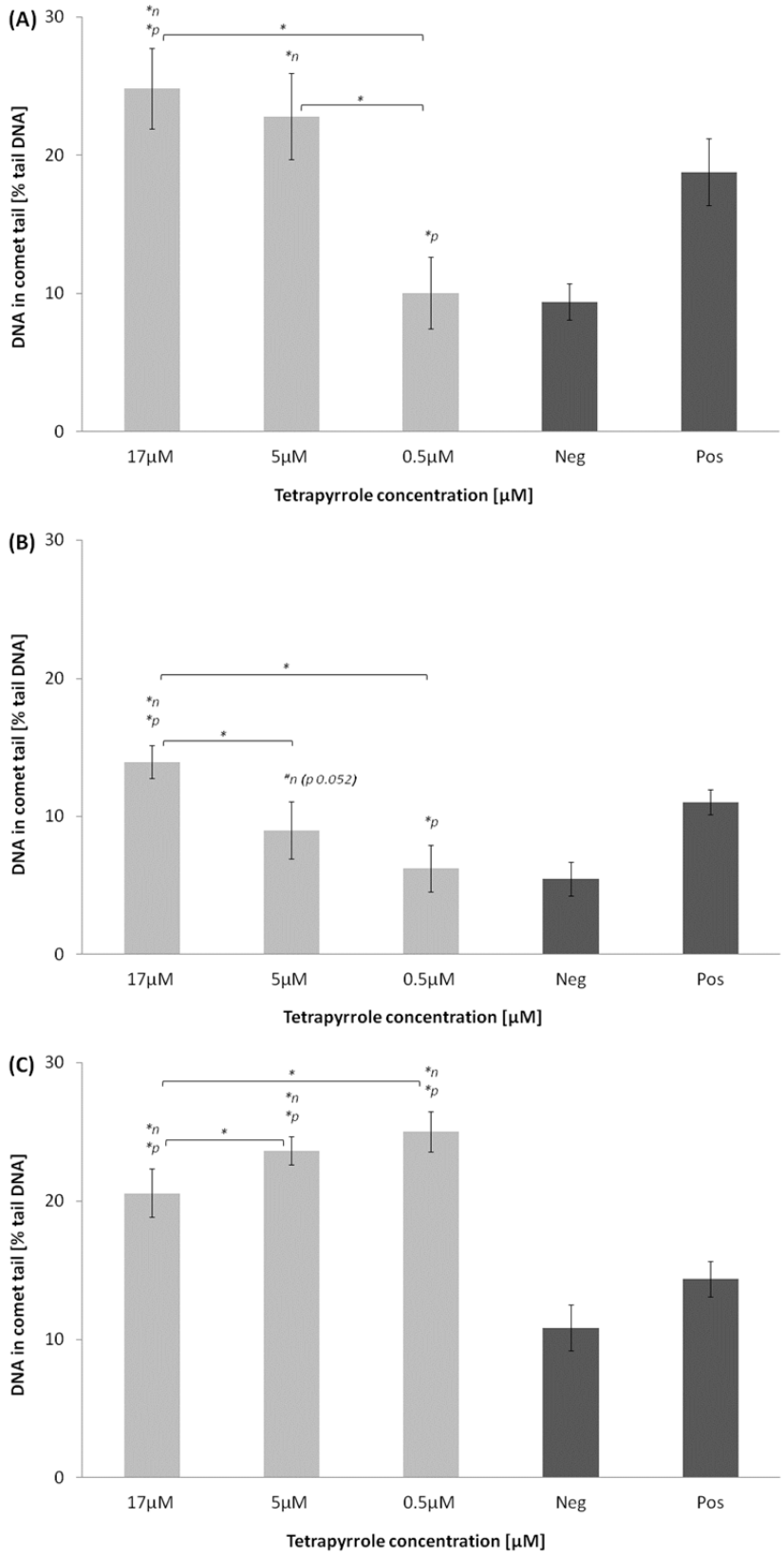

Figure 2 A – C. DNA-damaging effects of A) protoporphyrin, B) urobilin and C) stercobilin in HepG2 cells.
*n: significantly different to negative control
*p: significantly different to positive control
#n: not significantly different to negative control, however strong trend *($p \leq 0.1$)*

Table 2. Correlations (r) of comet tail DNA percentages (% tail DNA) and flow cytometry parameters in Caco2 and HepG2 cells.

	DNA damage/comet formation (% tail DNA)				
Caco2	*BR*	*BRDT*	*BV*	*SB*	*PRO*
Necrosis					-0.726 p=0.011
ROS (Hydroperoxide)	0.727 p=0.011		0.904 p=0.001		

	DNA damage/comet formation (% tail DNA)				
HepG2	*BR*	*BRDT*	*BV-DME*	*UB*	*PRO*
Apoptosis			0.644 p=0.010		
ROS (Superoxide)	0.547 p=0.053[#]				0.583 p=0.036

BR: unconjugated bilirubin, BRDT: bilirubin ditaurate, BR-DME: bilirubin dimethyl ester, BV: biliverdin, BV-DME: biliverdin dimethyl ester, UB: urobilin, SB: stercobilin, PRO: protoporphyrin.
[#]strong correlation, however non-significant on $p \leq 0.05$

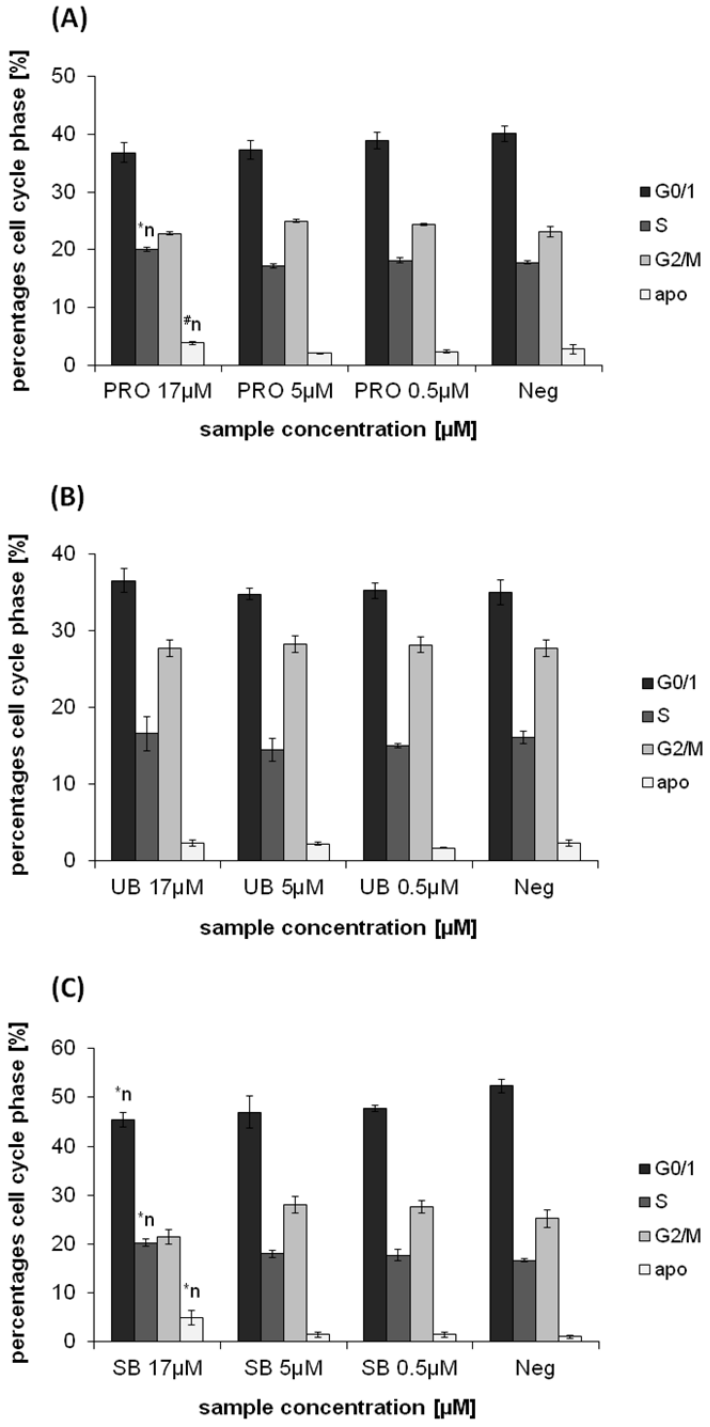

Figure 3 A – C. Effects of 24 hrs PRO (A), UB (B) and SB (C) incubation in Caco2 cells, on cell cycle progression (annexinV-staining).
PRO: protoporphyrin, UB: urobilin, SB: stercobilin; *n: significantly different to negative control *($p \leq 0.05$)*; #n: trends to differ from negative control *($p \leq 0.1$)*.

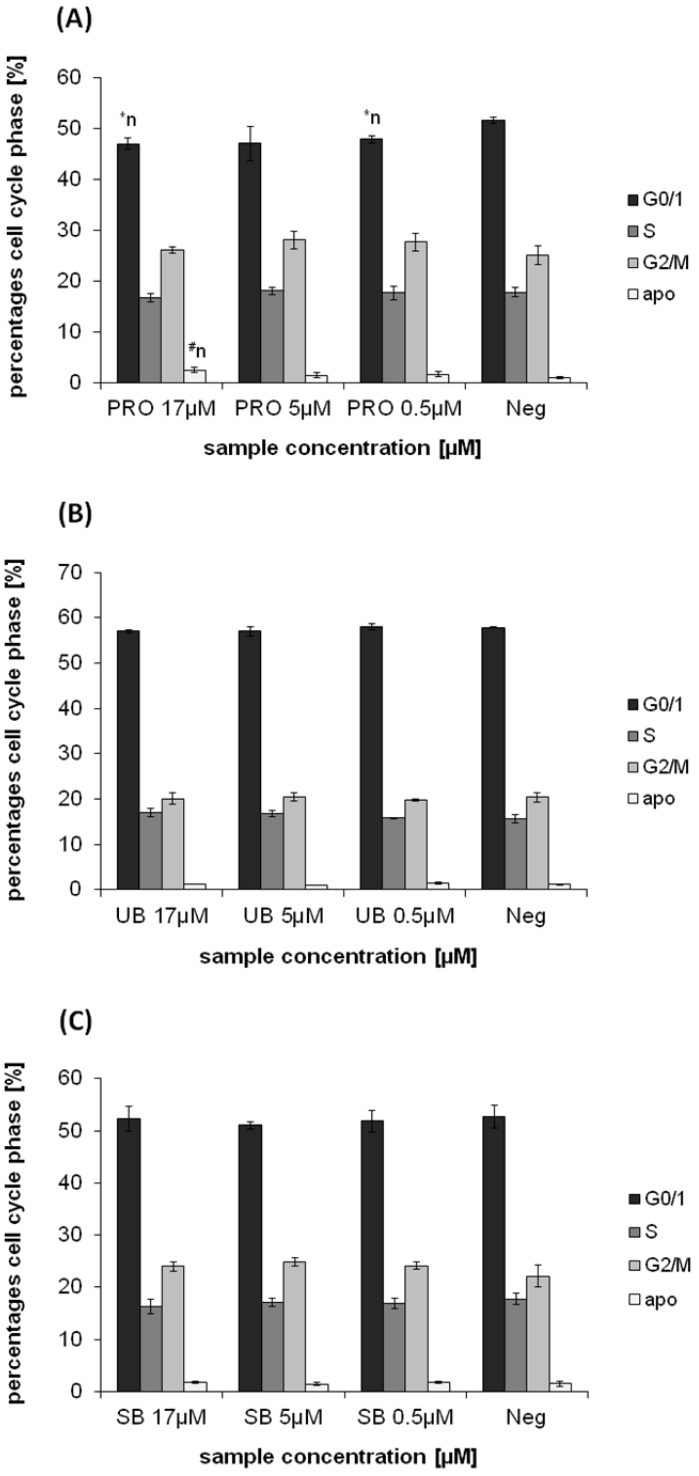

Figure 4 A – C. Effects of 24 hrs PRO (A), UB (B) and SB (C) incubation in HepG2 cells, on cell cycle progression (annexinV-staining).
PRO: protoporphyrin, UB: urobilin, SB: stercobilin; *n: significantly different to negative control *($p \leq 0.05$)*; #n: trends to differ from negative control *($p \leq 0.1$)*.

4. Discussion:

In this study, a range of physiologically abundant TPs (BR, BRDT, BV, UB, SB, PRO) were investigated with non-physiological BR- and BV-DME, to test their effects on ROS accumulation, DNA-damage (comet formation) and apoptosis in HepG2 and Caco2 cell lines. Most of the investigated TPs were found to induce DNA-damage in Caco2 and HepG2 cells, and effects appeared to be most pronounced with intestinal TPs (SB, UB, PRO; *Fig. 1A – C and 2A - C*). Cell viability data *(*for all tests between 86 and 99 % viability; *not shown)* showed no significant differences between the tested TP concentrations and negative controls. Therefore, it can be concluded that comet formation was not due to acute cytotoxic effects, but was predicated by mild TP-induced DNA damage. This was confirmed in both cell lines by elevated levels of apoptosis after BR, PRO and SB incubation *(supplementary material 2; Fig. 3A – C and 4A – C)*, as well as by the lack of positive correlation between DNA-damage and necrosis *(Table 2)*. These data suggest that intestinal BPs induce non-acute toxicity in malignant hepatic and intestinal cancer cells *in vitro.*

Single- and double strand DNA breaks represent critical damage to healthy cells [30]. Whilst mild impairment can yet activate innate repair mechanisms, severe nucleotide breaks can trigger cell death including apoptosis and/or tumorigenesis [39, 40], the latter resulting from genetic amplification or translocation processes leading to oncogene activation [30]. In contrast, radiation [41] and chemotherapeutic treatment can overwhelm cellular repair systems by inducing the production of ROS, which attack and fragment cancer cell DNA, and can cause apoptosis in tumor cells [42]. Cell survival after imperfect repair of DNA-damage on the other hand, can lead to carcinogenesis through mutations, and the formation of many tumors has been associated with the inhibition of apoptosis [43, 44].

Bile pigments (particularly BR) are known for their antioxidant activity at low to moderate concentrations, however, can possess pro-oxidant activity at high concentrations [6, 45]. Furthermore it is important to note that the ratio of BR to albumin represents an important determinant of ‘free’ BR [46]. This concentration influences the degree to which unbound BR can diffuse into cells [47] and interact with the mitochondrial membrane, where it may induce depolarization leading to apoptosis [19]. It is currently unknown whether also related TPs interact with albumin and how they interact with cells within the human organism. Clearly, further pharmacokinetic and bio-distribution studies are required to reveal the *in vivo* fate and consequences of these molecules [15, 16].

In the present study, BR provoked intracellular ROS accumulation *(Table 2)*. This result had been observed previously [27], and BR's anti-cancer behavior had been discussed with reference to a pro-oxidant effect. Particularly with regard to BR, observed toxic or pro-oxidant effects in HepG2 cells seen in the present study *(Table 2; online supplementary material 2)*, can probably be explained by a relatively higher "free" BR concentration in the cell culture media administered to HepG2 cells (10 % FBS addition), compared to the higher albumin binding capacity present in Caco2 cell media (20 % FBS addition). It is likely that the mildly elevated "free" bilirubin concentration resulted in BR absorption [47] into the mitochondrial membrane, leading to mitochondrial dysfunction and increased superoxide and hydrogen peroxide production, by interfering with mitochondrial cytochrome function [48]. Therefore, we hypothesize that BR, despite its known antioxidant potential, cannot neutralize increased production of reactive oxygen species, during mitochondrial depolarization/dysfunction.

As initially stated, DNA-damage is frequently associated with increased ROS production. With relevance to ROS-induced effects, correlations between DNA-damage and ROS formation (superoxide) were found in PRO and BR conditions in HepG2, as well as in BV and BR conditions in Caco2 cells (hydroperoxide). This emphasizes the compounds' potential role in causing ROS-mediated cell death in cancer cells. Elevated oxidative stress after PRO administration in rat hepatocytes had also been reported earlier, in which elevated superoxide formation had been assumed based upon an elevated superoxide dismutase (SOD) activity [49]. Elevated superoxide radical formation has also been detected *in vitro* after PRO administration using spin trapping techniques [50].

With the remaining TPs (BRDT, UB, SB, BR-/BV-DME) no relationships with ROS formation were found in this study. These data suggest that these compounds either induced DNA-damage directly by interfering with DNA or associated molecules, or that radical species other than those measured, account for the observed DNA-damage. In some cases (PRO and BR in HepG2 cells), ROS formation was correlated to apoptosis, which supports a role for the induction of oxidation by certain BPs, leading to cancer cell death *via* activation of apoptotic pathways.

Information regarding the regulation of cell cycle (particularly G0/1; *Fig. 3A - C and 4A - C*) provides further important data for mechanistically explaining cytotoxic results reported in the comet assay. Comet formation in both cell lines, and specifically in HepG2 cells after SB, UB and PRO incubations *(Fig. 1A – C and 2A – C)* are supported by an accumulation of cells in G0/1 phase and an increase in apoptosis, measured using annexinV staining *(Fig. 3A – C and 4A - C)*. This conclusion is particularly evident for SB-induced DNA-damage, which is supported by increased percentage of cells in G0/1 and sub G0/1 (apoptosis) phases. With

reference to these data, SB had the strongest effects on G0/1 cell cycle accumulation and apoptosis in both cell lines. In general, SB and PRO induced more pronounced effects especially in Caco2 cells, whereas UB's effects on apoptosis were comparatively mild *(Fig. 3A - C)*.

In summary, the current results indicate that BPs such as PRO, BR and BV induce radical formation that is related to DNA-damage, which was associated with increased apoptosis (PRO, BR). Since correlations with ROS formation (superoxide and hydroperoxide) were not found in all test conditions, it is also possible that other radical species had been formed such as carbon/nitrogen-centered radicals, which were not detected by the assays utilized in this study. Generally speaking, DNA-damage was induced by TPs leading to elevated ROS production, which agrees with a possible mechanism of DNA damage proposed by Goodsell [42]. In this model, reactive forms of oxygen attack and differently fragment cancer cell DNA involving single- and double stand breaks, and lead to cell death *via* cell cycle arrest and subsequent apoptosis induction.

In normally proliferating lymphocyte cultures, used as a model of healthy human tissue, no increased micronuclei- [13] and comet formation (submitted elsewhere) in the presence of mildly elevated circulating BR have been found recently by our work-group. These data further support the possibility of toxicity and DNA-damaging effects of BPs/derivatives specifically in cancer cells.

5. Conclusions:

Bile pigments with structurally related TPs are reported to possess anti-mutagenic and anti-oxidant effects *in vitro,* and to be of physiological importance by inhibiting oxidative stress *in vivo* and thereby preventing chronic disease [9]. However, data concerning the potential toxic effects of these compounds in cancer cells (beyond BR and PRO) are lacking. This study revealed substantial DNA-damaging effects of tetrapyrrolic compounds in human cancer cells, particularly for PRO, UB and SB in HepG2 and for PRO in Caco2 cell lines. From this evidence, a chemopreventive effect of the compounds might exist within the liver and intestine. Possible underlying mechanisms of DNA-damage (*e. g.* including ROS formation), leading to cell cycle arrest and subsequent apoptosis induction might be responsible for the toxicity observed in malignant cells.

Subsequent future experiments could focus on clarifying the effects of TPs also in non-malignant cells as well as the pigments' DNA-damaging potential in a range of various other cancer cell lines. Furthermore, exploring the effects of TPs also on chromosomal stability (*e. g.* prevention of micronuclei formation) could represent a valuable future scientific approach.

Declaration of interest:

The authors declare that there are no conflicts of interest.

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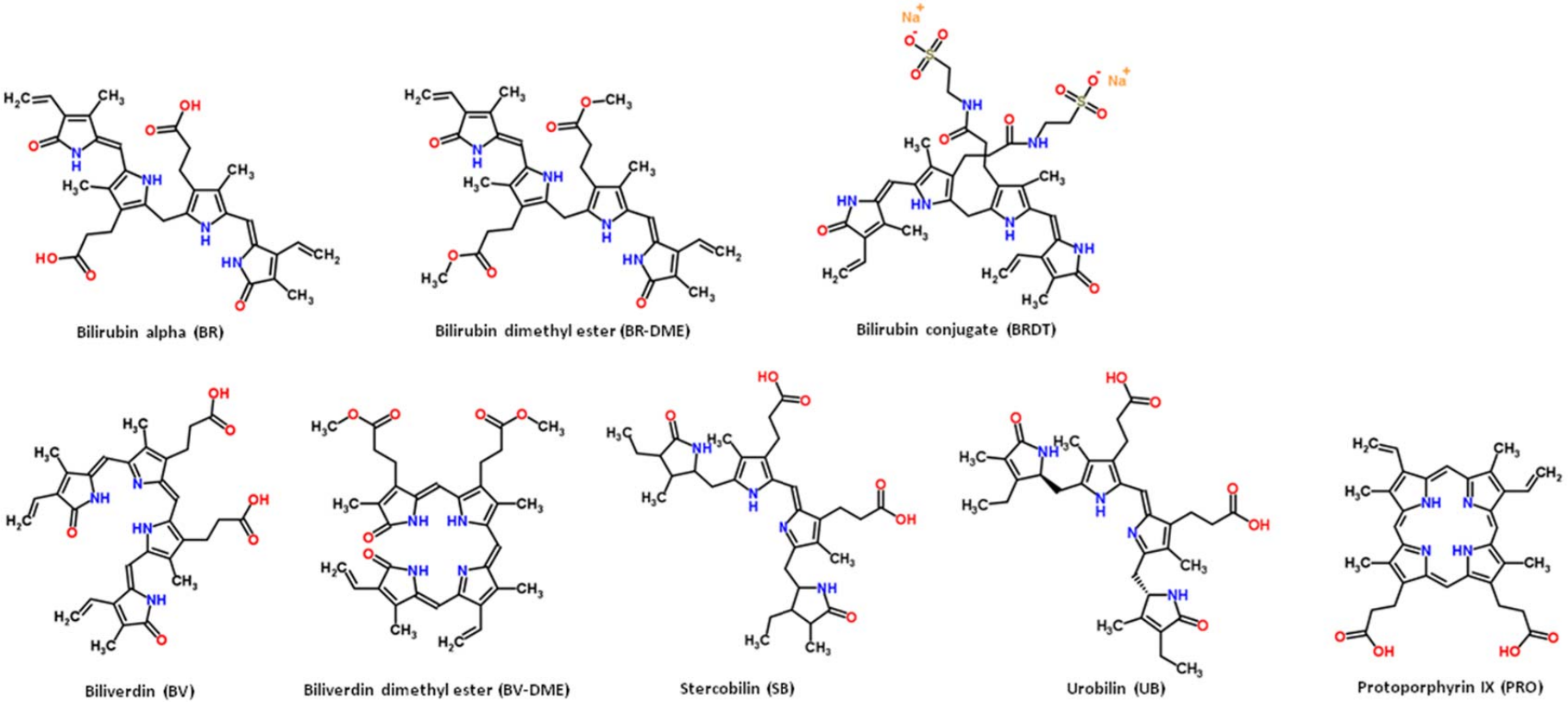
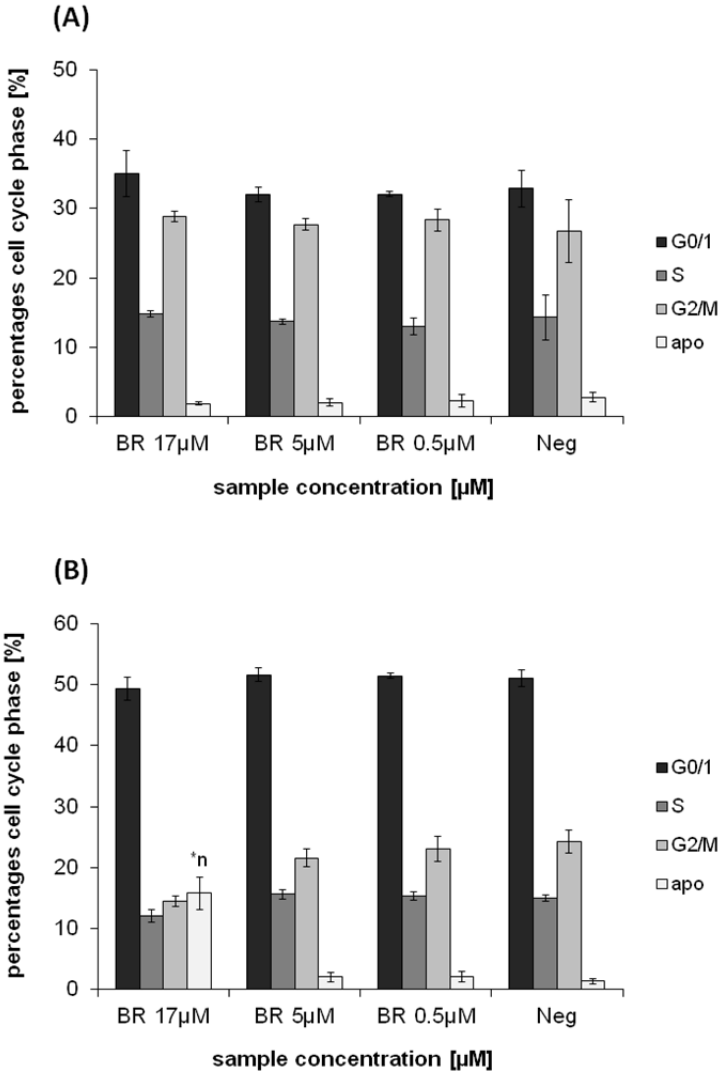


Supplementary material 1. Chemical structures of the test compounds.



Supplementary material 2. Effects of 24 hrs BR incubation in Caco2 (A) and HepG2 (B) cell lines, on cell cycle progression (annexinV-staining).
BR: unconjugated bilirubin; *n: significantly different to negative control ($p < 0.05$).