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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 $p \leqslant 0.05$.