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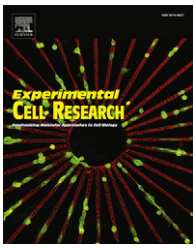

Research Article

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

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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 that 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 h. 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.

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

Abbreviations: BP(s), bile pigment(s); BR, unconjugated bilirubin; BR-DME, Bilirubin dimethyl ester; BR_f, free bilirubin; BRDT, bilirubin ditaurate; BV, biliverdin; BV-DME, biliverdin dimethyl ester; PRO, protoporphyrin; SCGE, single cell gel electrophoresis; TP(s), tetrapyrrole(s); SB, stercobilin; UB, urobilin

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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 as potentially anti-carcinogenic activities 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 antio idant mechanisms [9,11–13], and emphasize a protective physiological role for unconjugated BR in protecting against gastrointestinal and colorectal cancers [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/accumulated including the liver, gall bladder and intestine [17] in addition to the urinary tract and the circulatory system [16].

Thus far, the scientific focus has mainly been directed toward *in vitro* anti-genotoxic properties of BR and BV [5,18], with little attention focused on structurally related metabolites. Unconjugated BR induces cell cycle arrest, apoptosis and cytostasis *in vitro* in multiple cell lines [19]. Besides, BR may be defending against cancer by interfering with pro-carcinogenic signaling pathways, and therefore potently inhibit tumor cell proliferation *in vivo* [19]. Furthermore, BR induces mitochondrial depolarization in colon cancer cells, as it diffuses across the outer mitochondrial membrane, and thereby activates the intrinsic apoptotic pathway [17]. 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, 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.

Materials and methods:

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.

Single cell gel 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 ethidium bromide staining (20 μl of 20 μg/ml/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, GB) 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 1×10^6 cells/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).

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).

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%