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Lipid components of bile increase the protective effect of conjugated bile salts against antifungal drugs

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ABSTRACT

Fungi and bacteria can persist in the human gall bladder. Previous studies have shown that bile protects *Candida albicans* in this cryptic host niche from antifungals, providing a reservoir for intestinal re-colonization after discontinuation of antifungal therapy. Bile and conjugated bile salts trap antifungals in micelles, thereby reducing their bioavailability and possibly promoting the development of drug resistance. Here we show that the protective effect of bile and conjugated bile salts is not limited to *C. albicans*, but also observed with other fungi. Interestingly, bile, but not conjugated bile salts conferred resistance of *C. albicans* against fluconazole and only bile mediated resistance of *Aspergillus terreus* against voriconazole. To investigate this higher potency of bile we aimed in a step-wise reconstitution of bile from conjugated bile salts. Neither addition of phospholipids nor saturated fatty acids protected from azoles. In contrast, supplementation with polyunsaturated fatty acids increased azole resistance and decreased the critical micelle concentration of conjugated bile salts to the level of bile. Therefore, polyunsaturated fatty acids are vital for mixed micelle formation with high potential to trap antifungals. As biliary infections are difficult to treat, drug efficacy in the biliary system should be tested by using reconstituted synthetic bile.

Introduction

In liver transplant recipients biliary tract infections comprise one of the major complications that increase mortality rates and may inforce the requirement for re-transplantation (Moreno & Berenguer 2006). About 50 % of liver transplant recipients encounter at least one episode of viral, bacterial or fungal infection with *Escherichia coli* and *Klebsiella pneumonia* as the main bacterial infections followed by fungi such as *Candida* or *Aspergillus* species (Chiereghin *et al.* 2017). Furthermore, it has been shown that the bacterium *Listeria monocytogenes* freely replicates in bile implying that the gall

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bladder acts as a source for excretion of these bacteria (Hardy *et al.* 2004). In addition, gall bladder provides a reservoir for *Salmonella enterica* serovar Typhi even after antibiotic therapy causing severe chronic infections frequently accompanied by the development of gall stones (Gonzalez-Escobedo *et al.* 2011).

In a murine model of disseminated candidiasis, we have previously shown that *Candida albicans* uses the gall bladder as a cryptic reservoir under antifungal therapy (Jacobsen *et al.* 2014). Subsequent investigations revealed that bile conferred resistance against commonly used antifungals such as the echinocandin caspofungin and the polyene macrolide amphotericin B (Jacobsen *et al.* 2014). As underlying mechanism of protection we found that antifungals are trapped in micelles that are formed by conjugated bile salts (Hsieh *et al.* 2017). The resulting reduction in the bioavailability of drugs in the biliary system and possibly also in the intestine might thereby not only prevent the clearance of pathogens from these host niches, but might also lead to the development of drug resistant strains. Accordingly, a recent study revealed the emergence of echinocandin resistant *Candida* species in liver transplant recipients after treatment with caspofungin (Prigent *et al.* 2017). Resistant strains were mainly isolated from the digestive system implying that a reduced bioavailability of drugs might have caused this emergence (Prigent *et al.* 2017). Furthermore, case studies indicate that also other fungi and especially *Aspergillus* species are able to cause severe cholangitis (Garcia-Ruiz *et al.* 1998; Erdman *et al.* 2002). Therefore, it appears of high importance to clear pathogenic microorganisms residing in the biliary system. However, this requires the application of drugs that are not trapped in micelles.

Antifungal protection is strictly dependent on a concentration of conjugated bile salts that is above their specific critical micelle concentration (CMC). In this respect, taurocholate displays a higher CMC value at physiological conditions than taurodeoxycholate and, accordingly, higher concentrations of taurocholate are required to confer resistance. However, while both conjugated bile salts effectively protect against caspofungin and amphotericin B, neither of both compounds protected *C. albicans* against the azole fluconazole (Hsieh *et al.* 2017). On the contrary, bile strongly reduced the *in vitro* sensitivity of *C. albicans* against fluconazole and enabled persistence in the gall bladder under fluconazole treatment in a murine model of systemic candidiasis (Jacobsen *et al.* 2014). Since even high concentrations of taurodeoxycholate were not able to confer fluconazole resistance this increased protective effect of bile might be linked to a lower CMC value of bile accompanied by an increased ability to trap antifungals. It has been speculated that the more complex composition of bile including fatty acids and phospholipids leads to mixed micelles that allow a more effective trapping of antifungals (Hsieh *et al.* 2017).

To address this question, we studied the *in vitro* resistance of several fungal species in the presence and absence of conjugated bile salts to confirm a general mechanism of bile mediated antifungal protection. Subsequently, we analysed the effect of different fatty acid and lipid components either alone or in combination with conjugated bile salts on antifungal protection with a focus on azole resistance.

Material and methods

Strains and culture conditions

Candida albicans (SC5314), *Saccharomyces cerevisiae* (ATCC 9763) and *Cryptococcus neoformans* (H99 and 1841) were precultivated overnight in 20 ml YPD media (per litre: 10 g yeast extract, 20 g peptone, and 20 g glucose) at 30 °C and yeast cells were harvested by centrifugation at 4000× g. After washing cells twice in phosphate-buffered saline (PBS) cells were suspended and adjusted to selected cell densities in either YPD or MOPS-buffered RPMI 1640 medium (Sigma) containing 2 % glucose (per litre: 10.4 g RPMI 1640, 34.53 g MOPS, 20 g glucose; pH 6.8; subsequently defined as RPMI medium). To obtain conidia suspension of *Aspergillus fumigatus* (CBS144-89) and *Aspergillus terreus* (SBUG844), conidia were plated on 50 mM glucose *Aspergillus* minimal media with nitrate as nitrogen source and 2 % agar (Gressler *et al.* 2011). Plates were incubated at 37 °C for 3–4 d (*A. fumigatus*) or 5–6 d (*A. terreus*). Conidia were harvested in 10 ml sterile PBS and filtered through a 40 μm cell-strainer. After a washing step in PBS, conidia were diluted to defined concentrations in RPMI medium and used for drug resistance analyses.

Preparation of antifungals for sensitivity analyses

Stock solutions of caspofungin (5 mg ml^{-1}; Cancidas, Merck, Germany) and flucytosine (10 mg ml^{-1}) were prepared in PBS and filter sterilized. Stock solutions of amphotericin B (4 mg ml^{-1}) and voriconazole (16 mg ml^{-1}) were prepared in DMSO. Fluconazole at 2 mg ml^{-1} was purchased from B. Braun, Germany. All drugs were diluted in the respective media used for resistance analyses. Controls were prepared according to the solvents used for stock solutions of the respective antifungals.

Preparation of bile solution and lipid solubilisation in conjugated bile salt

Crude porcine bile extract (Sigma, B8631) was solved in either YPD or RPMI medium to give a final concentration of 12.5 % (w/v). Insoluble components were removed by centrifugation at 12 000 × g. The supernatant was filter sterilised and stored at 4 °C in the dark. Sodium taurodeoxycholate hydrate (Sigma, T0875) and taurocholic acid sodium salt hydrate (Sigma, T4409) were dissolved at 100 mg ml^{-1} in either YPD or RPMI medium, filtered sterilised and used as stock solutions. The following saturated fatty acids were used for preparing mixtures with conjugated bile salt: C3 (propionic acid sodium salts, Applichem, A1931), C4 (sodium butyrate, Aldrich, 303410), C8 (sodium caprylate, Fluka, 71339), C10 (sodium decanoate Fluka, 21490), C14 (myristic acid sodium salt, Fluka, 70140) and C16 (sodium palmitate, Fluka, 76165). Media containing 10 mg ml^{-1} taurocholate were supplemented with the indicated amounts of fatty acids. To solubilise C14 and C16, mixtures were heated to 65 °C for 30–60 min. As a source of phospholipids a soy refined lecithin (Mp Biomedicals, LLC) was added at indicated concentrations. As sources of polyunsaturated fatty acids arachidonic acid (Sigma, 10931) and