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A novel antibiotic-delivery system by using ovotransferrin as targeting molecule

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ABSTRACT

Synthetic antibiotics and antimicrobial agents, such as sulfonamide and triclosan (TCS), have provided new avenues in the treatment of bacterial infections, as they target lethal intracellular pathways. Sulfonamide antibiotics block synthesis of folic acid by inhibiting dihydrofolate reductase (DHFR) while TCS block fatty acid synthesis through inhibition of enoyl-ACP reductase (FabI). They are water-insoluble agents and high doses are toxic, limiting their therapeutic efficiency. In this study, an antibiotic drug-targeting strategy based on utilizing ovotransferrin (OTf) as a carrier to allow specific targeting of the drug to microbial or mammalian cells via the transferrin receptor (TfR) is explored, with potential to alleviate insolubility and toxicity problems. Complexation, through non-covalent interaction, with OTf turned sulfa antibiotics or TCS into completely soluble in aqueous solution. OTf complexes showed superior bactericidal activity against several bacterial strains compared to the activity of free agents. Strikingly, a multi-drug resistant *Salmonella* strain become susceptible to antibiotics–OTf complexes while a *tol*C-knockout mutant strain become susceptible to OTf and more sensitive to the complexes. The antibiotic bound to OTf was, thus exported through the multi-drug efflux pump TolC in *Salmonella* wild-type strain. Further, antibiotics–OTf complexes were able to efficiently kill intracellular pathogens after infecting human colon carcinoma cells (HCT-116). The results demonstrate, for the first time, that the TfR mediated endocytosis of OTf can be utilized to specifically target drugs directly to pathogens or intracellularly infected cells and highlights the potency of the antibiotic–OTf complex for the treatment of infectious diseases.

1. Introduction

The development of novel antibiotics that can be used effectively for a growing number of bacteria that have become resistant to existing antibiotics has become extremely challenging. When bacteria are drug resistant, it does not mean they stop responding, but only at higher doses and this could not be clinically achieved because antibiotics are toxic, water insoluble or do not stay at the site of infection. To break the vicious cycle of drug resistance, it is necessary to develop novel antimicrobial strategies with a unique mechanism of action against the dreadful pathogens. Over the years, several potentially valuable lipophilic antibiotics, targeting the essential type II fatty acid synthesis FASII, such as triclosan (Panagakos et al., 2005; Villalain et al., 2001; Yazdankhah et al., 2006), or folate synthesis, such as sulfonamides (Jenkins et al., 2014; Maury et al., 1987; Pierce et al., 2014), pathways in bacterial cells, have been developed. However, they still have considerable drawback as they are basically insoluble in water, chemically unstable, and are kept out of the bacterial cell by the efflux pump present at the surface of most pathogens or by virtue of membrane exclusion.

Sulfa antibiotics target the enzyme dihydropteroate synthase (DHPS). Most pathogens need DHPS to make the molecule folate, which is required for the production of DNA and some amino acids. These drugs are still widely used against emerging infectious diseases and to prevent infections in patients with weakened immune systems, including patients undergoing cancer chemotherapy. The solubility of sulfa drugs is very low and at high doses they can

Abbreviations: OTf, ovotransferrin; cOTf, control ovotransferrin; SMZ–OTf$_{(n)}$, Sulfamethoxazole–ovotransferrin complex (mole ratio); SMB–OTf$_{(n)}$, sulfabenzamide–ovotransferrin complex (mole ratio); T–OTf$_{(n)}$, triclosan–ovotransferrin complex (mole ratio); WT, wild-type *Salmonella* Enteritidis; ΔTolC, *tol*C-deleted mutant *Salmonella* Enteritidis; DTAF, 5-(4,6-dichlorotriazinyl)aminofluorescein (5-DTAF); NADH, β-Nicotinamide adenine dinucleotide disodium salt reduced form.

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crystallize in the kidneys, constituting a very painful experience. Sulfa drugs are receiving renewed interest, as a possible source of new therapeutic target, for the treatment of infections caused by bacteria resistant to other antibiotics (Jenkins et al., 2014; Nozad et al., 2009). On the other hand, triclosan (TCS) is attractive as antimicrobial agent owing to its specific mechanism of action of inhibiting an essential enzyme, enoyl-ACP reductase (or FabI) that uses NADH to reduce a double bond during the cycle of bacterial fatty acid elongation (Heath et al., 2002). Toxicological and pharmacokinetic studies as well as clinical investigations have shown that TCS generally has no toxic potential in humans (Bhargava and Leonard, 1996). The Food and Drug Administration (FDA) has approved it as oral care drug products, as it is recognized as an over-the-counter or prescription drug based on formulation and application. It has been employed in various cosmetic products and as surgical scrubs (Segundo et al., 2005). The antiplaque and antigingivitis efficacy of TCS-containing dentifrices is well established (Gunsolley, 2006). However, several unfavorable biopharmaceutical properties such as poor solubility in aqueous solvents, rapid photodegradation (Lores et al., 2005), and exclusion at the negatively charged bacterial surface (Yazdankhah et al., 2006) limit TCS therapeutic applications.

In particular, many intracellular infections remain difficult to treat because of the high hydrophobicity and the poor cellular transport properties of lipophilic drugs. Their toxicity to healthy tissues also poses significant limitations to the use of lipophilic antibiotics. Thus, a renewed commitment to harness the potent lipophilic antibiotics is the development of drug-targeting strategies that enable delivery of the drug to the bacterial cells in a cell-specific targeted manner. Cell-targeted drug delivery requires a carrier molecule that can recognize and bind the target cells then internalized by this cell type. All pathogenic bacterial cells contain different receptors and transporters that are primarily involved in the uptake of small molecules, such as glucose, peptides, minerals, and also xenobiotic drugs (Koepsell et al., 2007). These receptors with internalizing transporters are able to recognize specifically certain proteins and then internalize them or their bound small molecules (Krewulak and Vogel, 2008). Since these receptors are solely localized on the surface of the bacterial cells, targeting of antibiotic drugs to pathogens via carriers that bind these receptors is the most straightforward strategy to obtain efficient uptake of the drug that can treat microbial infections.

It is established that a continued supply of iron is essential for growth of most pathogens and the correlation of bacterial iron-acquisition systems with infectious disease has been well established (Krewulak and Vogel, 2008; Mietzner, 1998). Most bacterial pathogens possess surface receptors that bind transferrins (Moraes et al., 2009; Ekins et al., 2004), which mediate iron acquisition from these proteins (Schryvers and Morris, 1988; Anaya-Bergman et al., 2014; Boradia et al., 2014; Ciuraszkiewicz et al., 2014). Specific antibiotic drug delivery into bacterial cells or intracellularly infected cells via transferrin receptor-mediated endocytosis therefore seems a promising approach.

Ovotransferrin a member of the transferrin family [serm transferrin (Tf), milk lactoferrin (Lf) and avian ovotransferrin (OTf)], monomeric glycoproteins of ~78 kDa that can be divided into two homologous domains, N- and C-lobes, each of which contain an iron-binding site (Giansanti et al., 2012; Gaffney and Valentine, 2012). In early work, we have reported that OTf is bactericidal whether loaded or not with iron and succeeded in identifying a surface exposed bactericidal domain, OTAP-92, located at the lip of N-lobe of OTf (Giansanti et al., 2012; Ibrahim et al., 2000, 1998). The OTAP-92 (10 kDa peptide) contains three disulfide bridges with two of them forming bilooped structural domain, known as, kringle motif, which is conserved in both lobes of all transferrin members. These kringles show significant sequence and structural similarity with kringles of other proteins involved in receptor-mediated cellular event trigger (Ikeo et al., 1995). The OTAP-92 (including kringle domain) was found able to specifically interact with and permeabilize bacterial membranes of Gram-positive and Gram-negative bacteria (Ibrahim et al., 2000). Interestingly, we recently found that reduction with thioredoxin triggered self-cleavage of OTf, leading to specific libration of the two kringle domains (Ibrahim et al., 2006). More strikingly, the librated peptides by self-cleavage of OTf exhibited superoxide dismutase (SOD) activity (Ibrahim et al., 2007), and have shown to cause specific killing of colon and breast cancer cells mediated through induction of apoptosis and loss of mitochondrial membrane potential (Ibrahim and Kiyono, 2009). Since OTf, like all transferrins, has affinity for the receptor (iron-uptake receptor) at the surface of bacteria (Schryvers and Morris, 1988) and human epithelial cells (Tanaka et al., 2004; Gentili et al., 1994, 1993; Mason et al., 1990; Sorokin and Morgan, 1988; Mason and Brown, 1987), it displays unique features worth using as antibiotic drug-targeting molecules.

These features include the presence of a hydrophobic pocket in each domain made of a cluster of aromatic residues (Trp & Phe), which can accommodate a range of phenolic antibiotics through non-covalent aromatic interaction and is conceivably expected to specifically bind the bacterial and human cell membranes. In addition, the reduction sensitive disulfides will enable the release of the loaded drug upon binding to the redox-active bacterial membrane or in the endosomes. In this study, we explore a prospective drug-targeting strategy in which water-insoluble antibiotics are solubilized, delivered directly to pathogens or to intracellularly infected mammalian cells. The strategy is based on the fact that all pathogens as well as human epithelial cells express transferrin receptor (TfR) and selectively take up transferrin, thus, the antibiotic–OTf complex is targeted to pathogens or to cells in which they reside. For this, two sulfa antibiotics, Sufamethoxazole (SMZ) and sulfabenzamide (SBM) as well as the powerful antimicrobial triclosan (TCS) were complexed with OTf, and the antimicrobial activities and anti-infection potencies of the complexes were examined. The study also involves monitoring the ability of OTf to specifically deliver antibiotics to the intracellular bacteria in the cytosol of infected human colon cells.

2. Materials and methods

2.1. Materials

Ovotransferrin, purchased from Inovatech BioProducts Inc (Abbotsford, Canada), was re-crystallized and chromatographically (Sephadex G-50) purified to over 98% as judged by its extinction coefficient at 280 nm and western blotting. Triclosan was from Ciba Specialty Chemicals (Basel, Swiss). Sulfamethoxazol, sulfabenzamide, 5-(4,6-dichlorotriazinyl) aminofluorescein (5-DTAF), fetal bovine serum (FBS) and trypan blue (TB) were from Sigma (St. Louis, MO). McCoy 5a medium was from ICN (Invitrogen, Japan). CyQuant Direct cell proliferation/toxicity assay kit for animal cells was from Molecular Probes (Invitrogen Japan, Tokyo). Brain heart infusion (BHI), trypticase soya broth (TSB) and nutrient agar were from Nissui (Tokyo, Japan). Unless otherwise specified, all other reagents were of analytical grade.

2.2. Microorganisms and cell lines

Microorganisms for antimicrobial assays, *Staphylococcus aureus* (NBRC 14462), *Salmonella* Enteritidis (IFO 3313) and *Escherichia coli* K-12 (IFO 3301) were obtained from the Institute of Fermentation, Osaka (Japan). *Corynebacterium minutissimum* (NBRC 15361) was