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Research paper

# Delivery of a hydrophobic drug into the lower gastrointestinal system via an endogenous enzyme-mediated carrier mechanism: An *in vitro* study

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ABSTRACT

Clofazimine (CFZ) is a hydrophobic antibiotic agent which exhibits poor solubility. This poor solubility was overcome herein by the formulation of CFZ with the digestive enzyme pepsin. It is shown that pepsin can actively bind 11 CFZ molecules in the protein's native gastric environment, forming a CFZ-pepsin complex. A dynamic dissolution system, representing both the gastric and intestinal system, was used to analyze this CFZ-pepsin complex, revealing that only CFZ which binds to pepsin in the gastric environment remains in solution in the intestinal environment. The CFZ-pepsin complex displays adequate solution stability for the delivery of CFZ into the lower intestinal system. *In vitro* bioactivity assays against *Clostridium difficile* demonstrated the effectiveness of this CFZ-pepsin complex for the treatment of infectious diseases in the lower intestinal system.

## 1. Introduction

The development of novel antimicrobial drug delivery systems has become an important research concern in recent years [1]. Most disconcerting is the lack of new antibiotic agents in clinical development, and the time constraints associated with clinical trials for such new antibiotics. This lack of innovative antibiotic agents is coinciding with the emergence of increasingly hydrophobic and difficult to formulate drug substances [2]. This trend is usually associated with high throughput screening techniques, which select for increased drug-receptor affinity at the expense of physiochemical properties such as solubility and dissolution rate, factors which are strongly associated with the bioavailability of orally delivered drugs. New and innovative drug delivery platforms are constantly required for the stream of hydrophobic drugs being manufactured. Formulation science has adopted two approaches to overcome hydrophobicity, (i) co-administration of drug substances with solubilising agents and (ii) so-called supersaturating drug delivery systems [2]. These drug delivery strategies are often very susceptible to changes in pH and ionic strength, such as those experienced due to interpatient variability in gastrointestinal fluid. Herein, we report the development of a delivery system, whereby a hydrophobic antimicrobial drug, clofazimine (CFZ), is formulated along with the endogenous digestive enzyme, pepsin, for delivery of the drug into the lower gastrointestinal system.

Gastrointestinal membrane proteins have been known to bind APIs and influence their absorption rate for many years [3–5]. However, the impact of digestive proteins present in the human gastrointestinal system on the solution concentration and absorption rate of orally delivered APIs has not been previously reported. Pepsin is the only proteolytic enzyme found in the human stomach and is known to preferentially cleave peptide bonds between hydrophobic or aromatic amino acids via a large hydrophobic active site cleft [6]. It has recently been shown that porcine pepsin (purified from porcine stomach mucosa, 3450 units/mg protein) can inhibit the crystallization of a hydrochloride salt form of a drug in solutions at low pH and at a pepsin concentration of 3.2 g/L [7]. Similarly, we have previously demonstrated that the solution concentration and stability of CFZ in low pH solutions can be enhanced by increasing the concentration of pepsin in

*Abbreviations:* CDI, *Clostridium difficile* infection; CFZ, clofazimine; CFZB, clofazimine free base; CFZHCl, clofazimine hydrochloride; FAA, Fastidious Anaerobic Agar; m-FaSSGF, modified fasted state simulated gastric fluid; m-FaSSIF, modified fasted state simulated intestinal fluid; KI, inhibitory constant; MRD, Maximum Recovery Diluent; PEP, pepsin; RCM, Reinforced Clostridial Medium

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the system [8]. There are also several studies demonstrating how small hydrophobic molecules can bind with the active-site of pepsin [9–12]. Thus there is a growing body of evidence to suggest that gastrointestinal proteins can influence the solubility of hydrophobic drug substances.

CFZ is an antimicrobial drug currently used to treat *Mycobacterium leprae* [13]. CFZ also exhibits a vast arsenal of antimicrobial potential, showing activity against most Gram-positive pathogens *in vitro* [13]. However, the clinical usefulness of CFZ has historically been hampered by poor solubility [13,14]. Of particular interest is the potential of CFZ in the treatment of *Clostridium difficile* infections (CDI) [15]. *C. difficile* is a Gram-positive, spore-forming, anaerobic bacterium that is present in the lower gut of 1–2% of healthy individuals, where it is not pathogenic. However, when the normal gut microbiota balance of an individual is disrupted, often through antibiotic use, *C. difficile* colonies present in the gut can multiply to opportunistically dominate the local environment, leading to CDIs. In the U.S. alone, it has been recently estimated that *C. difficile* costs the healthcare system in excess of $5 billion annually as well as causing substantial morbidity and mortality [16]. CDIs are notoriously difficult to treat, due to the combination of antibiotic resistance and the spore-forming ability of the pathogen. Being a highly potent and rarely used antibiotic agent, CFZ could relieve some of these associated difficulties, if a solubility enhancing delivery system could be developed.

Historically CFZ has been formulated in its free base form (CFZB), as an oil/wax emulsion capsule, which is marketed as Lamprene® [17,18]. CFZB is insoluble in pure water but does dissolve to some extent in a narrow pH range between 2 and 4 with the highest solution concentration observed at pH 3.75 [8]. Salt forms of CFZ can overcome this poor solubility in pure water, by delivering the drug in the protonated state, but their effectiveness in biorelevant media is limited [8]. The aqueous solubility of CFZ molecules is completely dependent upon the drug molecule being protonated [14]. However, above pH 3.75 the protonated CFZ species ($CFZH^+$) deprotonates and precipitates from solution [14]. Below pH 3.75, $CFZH^+$ ions become susceptible to the common ion effect and subsequently crystallize from solution as CFZ hydrochloride (CFZHCl) [14]. The pH sensitivity of salt forms of poorly water-soluble ionisable drugs in this regard is well established [19]. However, pepsin may provide a solution to this problem [8]. Thus, in this study, we explore, *in vitro*, the application of pepsin as an enzyme-mediated carrier to deliver CFZ into the lower intestinal system.

## 2. Materials and methods

### 2.1. Materials

Clofazimine (CAS registry number 2030-63-9) was purchased from Beijing Mesochem Technology Co., Ltd. Ortho-phosphoric acid, sulphuric acid, hydrochloric acid and methanol were obtained from Sigma Aldrich and used without further purification. Ingredients for the gastrointestinal media included porcine pepsin, porcine bile extract, porcine pancreatin, sodium taurocholate hydrate, L- α – Phosphatidylcholine, from egg yolk (lecithin), sodium hydroxide and maleic acid and were received from Sigma Aldrich. Sodium chloride was obtained from Fisher Scientific Ireland. Trifluoroacetic acid (Chromasolv®) and haemoglobin (from bovine blood) used in activity assays of porcine pepsin were also purchased from Sigma-Aldrich. Reinforced Clostridial Medium (RCM) was obtained from Merck Millipore, Maximum Recovery Diluent (MRD) was from Oxoid and Fastidious Anaerobic Agar (FAA) was obtained from LabM Ltd.

### 2.2. Dissolution studies of CFZ in 25 mM HCl solutions of pepsin

These experiments were conducted in 100 mL Duran flasks containing PTFE stir bars and 100 mL of 25 mM HCl. Solutions were stirred at 300 rpm on a submersible stir plate in a water bath at 37 °C. When pepsin was added to this media, it was done 30 mins before the experiment began. Following this, 20 mg of CFZ or an equivalent amount of CFZ salt was added to the media to begin the experiment. Each solid form was ground and passed through particle size sieves (Fisher Scientific) to obtain a particle size distribution of 63–90 μm (PXRD was used to confirm no change in solid form had occurred during grinding). After the addition of the solid forms, the flasks were inverted several times to mix the drug into the solution as CFZ exhibits poor wettability. Samples were then withdrawn at predetermined time intervals using preheated 5 mL syringes and hypodermic needles (40 °C) and then filtered using preheated (40 °C) PTFE syringe filters (0.2 μm, 25 mm diameter, Fisher Scientific). CFZ concentration was determined using a double beam UV–vis spectrophotometer (Shimadzu, UV-1800) at a detection wavelength of 488 nm. For calibration of the instrument standard solutions were prepared in the concentration range of 0.1–20 mg/L and good linearity was observed ($R^2 > 0.999$). Samples which exceeded this range were appropriately diluted with the relevant media.

### 2.3. Pepsin activity assays

The enzyme activity was determined as per the Sigma Aldrich protocol with some modifications [20]. A stock solution of 25 mM HCl (i.e. pH 1.6) was prepared at 37 °C. This was then used to make pepsin solutions of 0.1, 0.2 and 0.4 mg/mL. Upon addition of pepsin, these solutions were stirred for 1.5 h at 400 rpm and 37 °C in a water bath with a submersible stir plate. Similarly, a stock solution of 2% bovine haemoglobin (substrate) was prepared in 25 mM HCl at 37 °C. For the activity assay, experiments were carried out in triplicate as follows; 4 mL of substrate solution was added to a glass vial containing a PTFE coated magnetic stir bar and then stirred at 400 rpm and 37 °C for 10 min, 1 mL of the enzyme solution was then added to each vial containing substrate and this was allowed to stir for a further 10 min, 10 mL of 5% trifluoroacetic acid (TFA) was then added after exactly 10 min and this was allowed to stir for a further 10 min. A blank was also prepared whereby enzyme was added following the addition of TFA and not before (i.e. without the digestion proceeding). 1.5 mL of each solution was then pipetted into 1.5 mL Eppendorf tubes, these were then centrifuged for 15 mins at 12,300 rpm and the supernatant was analysed via UV at 280 nm. When looking at the effects of CFZB or CFZ citrate on activity, 10 mg CFZB or the molar equivalent of CFZ citrate (with a particle size of 63–90 μm) was added to the 4 mL solution of substrate in 25 mM HCl solution to form a suspension prior to the addition of the enzyme solution (1 mL). The activity assay then proceeded as described above.

### 2.4. Preparation of simulated luminal fluid (m-FaSSGF and m-FaSSIF)

Modified fasted state simulated gastric fluid (m-FaSSGF) was prepared at pH 1.6 similar to that proposed by Vertzoni et al. [21,22], with a small modification to chloride concentration [8,14]. Modified fasted state simulated intestinal fluid (m-FaSSIF) was prepared at pH 6.5, based on a recipe previously reported by Marques et al. and Vertzoni et al. [22–24], although sodium taurocholate in the solution was replaced with porcine bile extract (PBE) and porcine pancreatin was added to better mimic the mixture of bile acids and enzymes present in the digestion tract [8,25]. The final composition of both the m-FaSSGF and m-FaSSIF are shown in Table 1.

### 2.5. Dynamic dissolution media procedure

Three solid forms of CFZ were investigated in this dynamic dissolution system, CFZB and two salt forms (CFZ sulphate and CFZ phosphate). These solid forms were first administered alone into the dynamic dissolution system i.e. 50 mg of solid form was administered along with 200 mL of water, to mimic administration of a CFZ along