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Antibacterial phototoxic effects of synthetic asymmetric and glycosylated curcuminoids in aqueous formulations Studies on curcumin and curcuminoids. LIV

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ABSTRACT

The aim of this study was to evaluate the *in vitro* phototoxic potential of synthetic asymmetric and glycosylated curcuminoids on planktonic model bacteria by counting the colony forming units. The Gram-positive *Enterococcus faecalis* and the Gram-negative *Escherichia coli* were exposed to aqueous solutions of the curcuminoids ($\leqslant$2.5 μM) in the presence or absence of selected pharmaceutical excipients (Pluronic® F127, PEG 400 and HPγCD) in combination with a low irradiation dose (5 J/cm^2; λ_{max}: 450 nm) of constant irradiance and time. All the asymmetric curcuminoids, but only one of the glycosylated curcuminoids demonstrated substantial phototoxic effect on *E. faecalis* ($\geqslant$4.7 log reduction). Only two of the asymmetric curcuminoids showed a moderate to low phototoxic effect on the more persistent *E. coli*. This study emphasized that aromatic hydroxyl substituents in the *para*-position are important to maintain the phototoxic potential of curcuminoids independent of molecular symmetry. Glycosylation of the aromatic substituents resulted in a substantial loss in phototoxicity towards planktonic bacteria, an apparent change in the non-radiative S_1-decay process and a weaker interaction with Pluronic® F127 compared to the non-glycosylated curcuminoids. The selected excipients Pluronic® F127, PEG 400 and HPγCD strongly influenced the phototoxic potential of the unsymmetrical, non-glycosylated compounds.

1. Introduction

It has previously been demonstrated that symmetrical curcuminoids including the naturally occurring curcumin and bisdemethoxycurcumin, show a potential as photosensitizers in antibacterial photodynamic therapy (aPDT). Haukvik et al. [1–3] demonstrated a significant phototoxic effect of curcumin and symmetric curcuminoids on *Enterococcus faecalis* (*E. faecalis*), *Escherichia coli* (*E. coli*), and *Streptococcus intermedius*. This effect has further been confirmed for curcumin on methicillin-resistant *Staphylococcus aureus* (MRSA) [4,5] and lactobacilli [6]. Curcumin has been reported to possess several biological effects in the ground state such as antibacterial, anticancer, anti-inflammatory, antiviral, and antifungal activities [7,8]. Oral delivery of curcumin is very well tolerated by humans and the compound is approved by the US Food and Drug Administration as a coloring agent in food, cosmetics and drugs [9]. The main obstacle toward exploitation of curcumin as a pharmaceutical active ingredient is its poor water solubility ($<3 \times 10^{-8}$ M) at acidic and neutral pH and its rapid hydrolytic degradation at alkaline conditions [10]. These characteristics address the need of a suitable delivery system to obtain a sufficient concentration and stability for the desired pharmacological effect (*e.g.*, antibacterial phototoxicity). Although the water solubility and stability might be slightly improved for some of the other symmetrical curcuminoids there is a notable instability of their excited state which can limit their use as photosensitizers [11]. Three asymmetric and three glycosylated curcuminoids (Fig. 1) have been synthesized previously in a separate study (Supplementary data [12,19,20]) with the aim to achieve improved physicochemical properties compared to curcumin. The aim of the current study was to evaluate the influence of the structural

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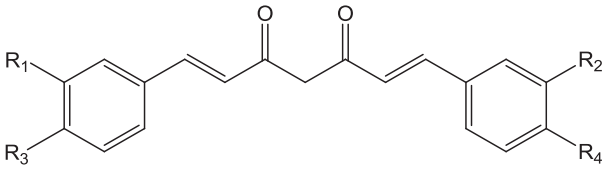

Compound	R_1	R_2	R_3	R_4
Curcumin	-OCH_3	-OCH_3	-OH	-OH
Casym1	-OCH_3	-H	-OH	-OH
Casym2	-OCH_3	-OCH_3	-OH	-OCH_3
Casym3	-OCH_3	-H	-OH	-H
Cglu1	-OH	-OH	-OGlc	-OGlc
Cglu2	-OCH_3	-OCH_3	-OGlc	-OGlc
Cglu3	-H	-H	-OGlc	-OGlc

Fig. 1. Structural formula of the curcuminoids. Curcumin is given as a reference only. The substituent -OGlc refers to glycosylation of the compound (Glc = glucose).

modifications and of pharmaceutical excipients on the *in vitro* antibacterial phototoxic effect of these asymmetric and glycosylated curcuminoids. Our previous investigation on symmetrical curcuminoids demonstrated that the presence of aromatic OH-groups in the *para (p)* position is important with respect to phototoxic effect, especially towards Gram-negative bacteria [3]. The importance of the *p*-OH substituent could be further evaluated in the non-glycosylated curcuminoids. Glycosylation was performed to increase the aqueous solubility and kinetic stability according to previous reports on curcumin derivatives [12]. Hydroxypropyl-γ-cyclodextrin (HPγCD), polyethylene glycol 400 (PEG 400) and Pluronic® F127 were selected as solubilizers to increase the aqueous solubility even further. Complex formation of curcumin with cyclodextrins and micelles has previously been studied [13–17]. In the presence of cyclodextrins, the solubility of curcumin was shown to increase by a factor of $\geqslant 10^4$ at pH 5 (shake-flask method), and the hydrolytic stability increased 50–500 fold above pH 7. HPγCD was the most efficient solubilizer and stabilizer among the cyclodextrins investigated [14]. The hydrophilic polymer polyethylene glycol 400 (PEG 400) has demonstrated some solubilizing effect on curcumin in aqueous solutions, but did reduce the phototoxic effect of curcumin on *E. coli* as a function of increasing PEG 400 concentration [2]. PEG 400 is however, a useful excipient in many topical preparations and was therefore included in the present work. The nonionic poloxamers (Pluronic®) are also efficient solubilizers of curcumin [1,15]. Pluronic® F127 and P123 have been shown to increase the curcumin solubility by a factor of $\geqslant 10^4$ at pH 5 (shake-flask method). Pluronic® F127 was selected in the present study and applied in a concentration below the critical micellar concentration (CMC). The bacterial phototoxicity of all the curcuminoids were initially screened in PBS. Only the compounds giving a significant reduction in bacterial survival were further screened in the presence of Pluronic® F127. The most promising compounds in this screening (Casym 1 and 3, Fig. 1) were further investigated in formulations containing cyclodextrin or PEG 400. Gram-positive *E. faecalis*, frequently found in root canal-treated teeth [18] and the Gram-negative *E. coli* was used as model planktonic bacteria.

2. Materials and methods

2.1. Materials

The asymmetric compounds (*i.e.,* Casym1–3; Fig. 1) and the glycosylated compounds (*i.e.,* Cglu1–3; Fig. 1) were synthesized and characterized (Supplementary data, [12,19–20]). Phosphate buffered saline (PBS; Lonza, Verviers, Belgium) was adjusted to pH 6.1 to retard hydrolytic degradation by addition of a required volume of 37% hydrochloric acid (HCl) (Merck, Darmstadt, Germany), and aseptically filtered (0.22 μm; Millipore S.A.S, Molsheim, France) prior to use. The distilled water was sterilized by autoclavation before use. Pluronic® F127 and polyethylene glycol 400 (PEG 400) were purchased from Sigma Aldrich (Steinheim, Germany), and hydroxypropyl-gamma-cyclodextrin (HPγCD; Cavasol® W8 HP Pharma) was purchased from Wacker Chemie AG (Burghausen, Germany). All materials were used as received. The water content of HPγCD was determined by use of a Moisture Analyzer MA 30 (Satorius, Goettingen, Germany) prior to use and included in the further calculations.

2.2. Microorganisms

E. faecalis strain FCC120 (ATCC 19433) and *E. coli* (ATCC 25922; Oxoid Ltd., Bassingstoke, UK) were maintained by three times weekly subculture in tryptone soya broth (TSB; Oxoid Ltd.). The bacteria were incubated at 37 °C in the dark.

2.3. Preparation of samples

Stock solutions of the curcuminoids (Fig. 1) were prepared in ethanol (EtOH) at the following concentrations: Casym1 (2 mM), Casym3 (2 mM), and Cglu3 (0.4 mM). Due to low solubility of Casym2 (<0.5 mM) and Cglu1 (<0.4 mM) in EtOH these stock solutions were prepared as saturated solutions in EtOH and filtered (0.22 μm). Cglu2 showed very low solubility in EtOH and a stock solution was therefore freshly prepared as a saturated aqueous solution (<0.4 mM; filtered 0.22 μm).

The stock solutions of Casym1, Casym3, and Cglu3 were further diluted in PBS pH 6.1 to a final concentration of 2.5 μM. A similar dilution of Casym2, Cglu1, and Cglu2 resulted in a final concentration < 2.5 μM. The EtOH concentration was kept constant (0.6%) in the final preparations. Samples of Casym1 and Casym3 were prepared with or without Pluronic® F127, PEG 400, or HPγCD; Casym2 and Cglu3 were prepared with or without Pluronic® F127, while Cglu1 and Cglu2 were prepared only in PBS. The CMC of Pluronic® F127 is reported to be 2.8×10^{-6} M (0.0035% w/v) at pH 7.4, 37 °C [21]. The concentration of Pluronic® F127 was therefore kept at 0.001% w/v (~30% of CMC) in order to keep the solubilizer as unimers rather than micelles in the aqueous preparations [1]. A concentration of 2.5% v/v PEG 400 was used, equal to the concentration applied when the highest phototoxic effect of curcumin was achieved on *E. coli* [2]. HPγCD was used at 5% w/v concentration, and the preparations were equilibrated for 30 min in order to form curcuminoid-cyclodextrin complexes prior to addition of bacteria in the phototoxicity testing (Section 2.7). The final preparations were made immediately before use and kept protected from light throughout the experiments.

2.4. Absorption spectra

The UV–VIS absorption spectra (250–700 nm) of the asymmetric and glycosylated curcuminoids were recorded by a Shimadzu UV-2550 PC UV–VIS spectrophotometer (Shimadzu, Kyoto, Japan). The samples were freshly prepared in PBS pH 6.1 with or without 0.001% Pluronic® F127 (Casym1–3, Cglu3), 2.5% PEG 400 (Casym1, Casym3), or 5% HPγCD (Casym1, Casym3) at a concentration of 2.5 μM curcuminoid if not stated otherwise. Bacteria were not added to the samples to avoid scattering. The accuracy of the wavelength determination was ± 1 nm.