Diclofenac-β-cyclodextrin for colonic drug targeting: *in vivo* performance in rats

Amélia C. F. Vieira[1,2,3], Arménio C. Serra[3], Francisco J. Veiga[2], António M. d'A Rocha Gonsalves[3], Abdul W. Basit[1], Sudaxshina Murdan[1*]

[1] Department of Pharmaceutics, UCL School of Pharmacy, London, United Kingdom

[2] Centre for Pharmaceutical Studies, Laboratory of Pharmaceutical Science, Faculty of Pharmacy, University of Coimbra, Portugal

[3] Chymiotechnon and Department of Chemistry, Faculty of Science and Technology, University of Coimbra, Portugal

***Corresponding author:** Sudaxshina Murdan, UCL School of Pharmacy, 29-39 Brunswick Square, London, WC1N 1AX, UK; Tel: +44-207 753 5810; Fax: +44-207 753 5942; Email: s.murdan@ucl.ac.uk

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Abstract

The aim of this *in vivo* study was to assess the ability of the prodrug conjugate diclofenac-β-cyclodextrin to release diclofenac in the colon following oral administration, using sulfapyridine (a metabolite of sulfasalazine) as a marker of colonic absorption. Two groups of rats were used; the test rats received a suspension containing the two prodrugs, diclofenac-β-cyclodextrin and sulfasalazine, while the control rats received a suspension containing the corresponding free drugs, sodium diclofenac and sulfapyridine. The rats were fasted overnight with free access to water before and throughout the first 12 hours of the study. Blood was collected from the tail vein at pre-determined time points and the plasma analyzed for the concentrations of diclofenac and sulfapyridine. Following the oral administration of the two prodrugs, a more extended absorption profile was observed and C_{max} was achieved 10 hours post-dose, in contrast to rapid absorption of the free drugs (t_{max} of diclofenac being 1.3 h, and that of sulfapyridine being 2.1 h) . In addition to a later t_{max}, conjugation of diclofenac to β-cyclodextrin also resulted in a reduced C_{max} and a reduced AUC. The same t_{max} for diclofenac-β-cyclodextrin as for sulfasalazine confirms the colonic metabolism of diclofenac-β-cyclodextrin. This study shows the potential of this new cyclodextrin-based prodrug to target and release diclofenac specifically in the colon following oral administration.

1. Introduction

Colon-specific drug delivery - as a method of targeting drug administration - has gained increasing attraction in recent years for treating both local gastrointestinal, as well as, systemic disorders. A range of approaches ranging from the application of pH changes in the gastrointestinal tract (Ibekwe et al., 2008; Kendall et al., 2009; Liu et al., 2010) to mucoadhesive drug delivery (Varum et al., 2011) and even metabolism by colonic microbiota have been exploited (Basit et al., 2009; Freire et al., 2011; Yadav et al., 2013). Indeed, knowledge of the unique enzymatic activity of the gut flora and the associated mechanisms has enabled the development of marketed prodrugs targeted to this region. For example, azo-bonded prodrugs of 5-aminosalicylic acid (5-ASA), such as sulfasalazine (where 5-ASA is bonded to the carrier sulfapyridine), are used for the management of conditions such as ulcerative colitis (Sousa et al., 2014). In the colon, the azo-bond is cleaved by azoreductases and the drug is released for local action. Due to the fact that azo-bonding of drugs is limited to molecules with an amine functional group, and the fact that the carrier sulfapyridine is toxic, other strategies with different types of linkages and carriers for colon-specific delivery have been explored (Jung and Kim, 2010).

One such strategy is the conjugation of drug molecules to cyclodextrins. The latter are a group of cyclic oligosaccharides derived from starch and consist of glucose units linked by α-1,4 glycosidic bonds in a doughnut-shaped conformation (Szejtli, 1998). They are not absorbed intact in the gastrointestinal tract, but are metabolised by colonic microbiota – in particular by *Bacteroides* spp. - into smaller saccharides (Chourasia and Jain, 2003; Flourie et al., 1993; Park et al., 2000; Sinha and Kumria, 2001). This means that drugs can be covalently bonded to cyclodextrin, forming conjugates and be released in the colon, following cyclodextrin metabolism by colonic bacteria. Thus, natural cyclodextrins have been investigated as carriers for drug delivery to the colon (Minami et al., 1998; Yano et al., 2001).

We have reported the development of a new cyclodextrin based colonic prodrug – diclofenac-β-cyclodextrin (Figure 1). In contrast to previously synthetized cyclodextrin conjugates produced by conventional methods (Uekama et al., 1997; Yano et al., 2001), our

conjugate was synthetized using microwave irradiation, which allows the synthesis of the conjugate in a short period of time. *In vitro* and *ex vivo* studies showed that this conjugate has the potential to act as a prodrug of diclofenac for colonic delivery (Vieira et al., 2014; Vieira et al., 2013). The non-steroidal anti-inflammatory drug, diclofenac, is an important component of many treatment regimens for inflammatory disorders. Its colonic delivery offers a number of advantages, such as prevention of its adverse gastric effects, targeting to its site of action, for example if the drug is developed for its preventive action in colon cancer (Jasmeet and Nath, 2010; Kaur Saini et al., 2009), and the possibility of delayed drug release and absorption post-dosing, which would enable chronotherapy (i.e. the drug is released at the correct time, i.e. when required), which is widely recognized as a suitable tactic for the management of arthritis (Lin and Kawashima, 2012). It is also known that diclofenac is well-absorbed from the colon, and colonic bioavailability has been shown to be the same as oral bioavailability (Gleiter et al., 1985).

Following our *in vitro* and *ex vivo* studies (Vieira et al., 2014; Vieira et al., 2013) the aim of the work described in this paper, was to establish that diclofenac-β-cyclodextrin can indeed be metabolized *in vivo* in the colon and to release the active diclofenac, following oral administration. To confirm that diclofenac release occurred specifically in the colon, sulfasalazine was administered concomitantly. Sulfasalazine (Figure 1) is a well-known and widely marketed colonic prodrug which, following oral administration undergoes cleavage to 5-aminosalicylic acid (mesalazine) and sulfapyridine upon reaching the colon. Thus, the sulfasalazine metabolites, in particular sulfapyridine which is well absorbed, can be used as an indicator of colonic targeting, providing the transit time through the gastrointestinal tract (Lee et al., 2012; Sjödin et al., 2011). In order to investigate the *in vivo* fate of diclofenac-β-cyclodextrin, experimental animals were dosed with a suspension containing both diclofenac-β-cyclodextrin and sulfasalazine, and the appearance of the expected metabolites, diclofenac and sulfapyridine, in their blood was monitored. A control group of animals was administered a suspension containing the expected metabolites themselves, i.e. diclofenac and sulfapyridine.

Rats were used in our *in vivo* investigations, as this animal model is considered particularly appropriate for the determination of pharmacokinetic parameters following oral administration of powder or liquid formulations (Kararli, 1995a).The total gastrointestinal transit time of rats is similar to that in human) and the rat has been used for *in vivo* studies with diclofenac (León-Reyes et al., 2009; Reyes-Gordillo et al., 2007), sulfasalazine (Chungi et al., 1989 ; Lee et al., 2012) and a number of colon-targeted prodrugs, including those that use cyclodextrins as a carrier (Kamada et al., 2002; Kunhiro Minami et al., 1998).

2. Materials and methods

2.1 Materials

Diclofenac-β-cyclodextrin (MW=1411 g/mol) was synthetized according to the method described by Vieira *et al.* (Vieira et al., 2013). Briefly, diclofenac-β-cyclodextrin was synthesized via a 2-step process involving tosylation of β-cyclodextrin (step 1) followed by nucleophylic substitution of the tosylated β-cyclodextrin by sodium diclofenac under microwaves (step 2). Diclofenac Sodium, sulfasalazine, sulfapyridine and trifluoroacetic acid (TFA) were purchased from Sigma (UK). Sodium chloride, Microtainer tubes containing K_2EDTA, HPLC grades acetonitrile, methanol and water were purchased from Fisher Scientifics UK Limited. Phosphate buffer pH 7.4 was prepared according to the USPXXIV.

2.2. Animals

Healthy adult male Wistar rats (8 weeks old, 240-250 g) were purchased from Harlan Olac Ltd. (Oxfordshire, UK). All the procedures were conducted under license and in accordance with the Home Office standards under the Animals (Scientific Procedures) Act, 1986. The animals were housed in rooms with controlled conditions of 20 ^{o}C, 40-60% humidity and 15-20 air changes per hour. The animals underwent a period of acclimatization, with free access to standard rat chow and water for 7 days prior to the experiment. Twelve hours before the beginning of each experiment, the animals were housed individually in separate metabolic cages. The latter were perforated at the bottom, which allowed the separate collection of rats' urine and faeces and also stopped the animals eating their own faeces. Water was available *ad libitum* through the experiment.

2.3 *In vivo* investigation

Rats were assigned to two groups of seven: I (test) and II (control). Rats were fasted overnight (12 hours) and again throughout the first 12 hours of the study, but had free access to water. This fasting regimen was chosen based on our previous work which showed faster metabolism of diclofenac-β-cyclodextrin in the colonic contents of fasted rats (Vieira et al., 2014).

Two suspensions – test and control - were freshly prepared in sodium chloride 0.9% w/v. The test suspension contained the two prodrugs, diclofenac-β-cyclodextrin (at 17.7 mg/mL) and sulfasalazine (at 20 mg/mL). The control suspension contained the free drugs at equivalent concentrations i.e. at 4 mg/mL for diclofenac sodium and 12.5 mg/mL for sulfapyridine. Each rat was orally dosed with 1.2 mL of the test or control suspension by gavage. It must be noted that the different rats received the same amount of drug, despite their different masses, which varied from 240g to 250g. Thus, the drug dose/kg of rats varied slightly. This was considered not to influence the results overmuch due to the very small differences among the drug dose/kg for the different rats. The drug doses chosen i.e. sodium diclofenac (~20 mg/kg) and sulfasalazine (~100 mg/kg) were selected based on previous reports (Lee et al., 2012; León-Reyes et al., 2009). These doses were equivalent to ~88.5 mg/kg of diclofenac-β-cyclodextrin and to ~62.5 mg/kg of sulfapyridine, respectively.

Following dosing, the rats were bled via the tail vein at pre-determined time points, which were 30, 90, 120, 180, 240, 360, 480, 720 and 1440 min, for the test rats and 10, 30, 45, 90, 120, 240, 360, 480, 720 and 1440 min for the control rats. The blood samples were centrifuged at 13 000 rpm for 10 min at 4 ºC and the resulting plasma samples were collected and kept at -20 ºC until analysed.

2.4 Simultaneous quantification of diclofenac and sulfapyridine in plasma by HPLC

Frozen plasma samples (100 µl) were allowed to thaw at room temperature. 100 µL of acetonitrile with 2.5 % (v/v) of 0.2 M KOH was added to a plasma sample and the mixture

was vigorously mixed on a vortex mixer for 10 seconds. The samples were then centrifuged for 10 min at 10 000 rpm at 4 ºC. Subsequently, 100 µL of the supernatant was diluted with 20 µL of water and analyzed for the free drugs, diclofenac and sulfapyridine. Analysis was performed by high-performance liquid chromatography (HPLC) using an Agilent 1100 series system equipped with a UV detector. The results were acquired and processed with the Agilent Chemstation Data System Software 7. A XTerra reverse phase C-18 column with 5 µm particle size, 4.6 mm internal diameter and 250 mm length, a sample injection volume of 50 µl, detection wavelength of 273 nm, flow rate of 1 mL/min at 40 ºC and total run time of 24 min were used. A gradient system of 0.1% TFA in water (A) and acetonitrile (B) was followed: 0-15 min, 11-80% (B), 15-22 min 80 – 22% (B), 22-24 min 22-11% (B).
The retention time of sulfapyridine and diclofenac were 5.8 and 16.2 min respectively. A good linearity were established (R^2 = 0.999) for both molecules in the concentration range of 0.25 – 20 µg/mL. The LLOD and LLOQ were 0.05 and 0.25 µg/mL, respectively for both analytes. The percentage recoveries were 98.8%, 81.2% and 74.9% for the diclofenac, and 88.0%, 87.2% and 73.0% for the sulfapyridine at concentrations of 20 µg/mL, 1 µg/mL and 0.25 µg/mL, respectively. The accuracy values ranged from 2.7%-11.8% for diclofenac, and between 0.9-2.2% for sulfapyridine. Intraday precision values ranged from 3.3 - 9.1% for diclofenac, and from 2.8 -8.5% for sulfapyridine, while inter-day precision ranged from 2.5 - 6.0% for diclofenac and from 2.7 - 7.9% for sulfapyridine.

2.5 Pharmacokinetics and statistical analysis

The diclofenac and sulfapyridine concentration in plasma (µg/mL) as a function of time post administration for each rat was plotted, and the maximum diclofenac and sulfapyridine concentration in the plasma (C_{max}) and the corresponding time (t_{max}) for each rat was read from the drug concentration-time profiles, while the area under the drug plasma concentration time curve (AUC0–24 h) was calculated using OriginPro 9.0. Subsequently, the mean tmax, Cmax and AUCs were calculated from the individual rats' data. Statistical analysis was performed using SPSS 21.0 for Windows®. The data was analyzed using parametric tests. C_{max}, t_{max} and $AUC_{0–24\ h}$ of diclofenac and sulfapyridine for the test and control groups were compared using Student *t*-tests.

3. Results

The mean plasma concentration–time profiles of diclofenac and sulfapyridine for the rats dosed orally with the prodrugs or with the free drugs are shown in Figures 2 and 3 respectively. The calculated C_{max}, t_{max} and $AUC_{0–24\ h}$, are shown in Table 1. From Figures 2-3 and Table 1, it can be seen that:

i. The drug concentration-time profiles for the prodrugs are significantly different to the profiles of the corresponding 'free' compounds diclofenac and sulfapyridine

ii. Most obviously, C_{max} occurs at a much later time point for the prodrugs. Thus t_{max} is significantly later for the prodrugs ($p<0.05$)

iii. The C_{max} of both prodrugs were lower than those of the corresponding free compounds ($p<0.05$), the difference being more marked for diclofenac.

iv. The AUCs of the two drugs were affected to different extents following prodrug administration. While the AUC of the diclofenac prodrug was about half that of free diclofenac ($p<0.05$), the AUC of sulfapyridine was almost the same for the prodrug and the free compound ($p>0.05$).

4. Discussion

The free active drug diclofenac was rapidly absorbed, its t_{max} being similar to previously reported values (Zhang et al., 2011).The free sulfapyridine also started being absorbed soon after its administration, although its absorption occurred at a slower rate compared to diclofenac. This is probably related to its poor solubility in water, which is less than 0.1g/100mL.

In contrast to the free diclofenac and sulfapyridine, the prodrugs have to be metabolized in the gastro-intestinal tract to release the drugs, which would then be absorbed into the systemic circulation. Thus, as expected, the prodrugs' t_{max} occurred much later, at around a mean of 10h post drug administration. Both diclofenac-β-cyclodextrin and sulfasalazine are expected to remain intact in the rat upper gastro-intestinal tract, and only be metabolized once they reach the caecum and/or the colon, where release and absorption of diclofenac and sulfapyridine would take place. As mentioned earlier, sulfasalazine is known to be metabolized in the colon and the appearance of its metabolite, sulfapyridine, in the systemic circulation can provide an indication of gastro-intestinal transit time. The fact that the t_{max} of both prodrugs is statistically the same (about 10 hours, $p>0.05$) shows that maximal diclofenac release and absorption occurred at around the same time post-dose, and therefore likely to be at the same site in the gastro-intestinal tract as the sulfasalazine. This confirms that diclofenac-β-cyclodextrin is metabolized and the active drug diclofenac is released in the colon following oral administration in rats.

The lower C_{max} of both prodrugs and a more extended absorption profile compared to the free drugs is also explained by the fact that the prodrugs have to be metabolized by the gut microbiota prior to absorption. This metabolic step may be rate-limiting drug absorption, and may have advantages in the avoidance of peaks and troughs in plasma drug levels.

The lower AUC (by 2 times, $p<0.05$) of diclofenac from the prodrug compared to the free drug indicates a lower bioavailability of diclofenac from the prodrug. This could be due to incomplete absorption of the released diclofenac or incomplete metabolism of diclofenac-β-CD in the rat colon. Analysis of the rat faeces for prodrug and metabolite levels could have provided an indication of whether the reason was incomplete absorption of diclofenac or incomplete metabolism of the prodrug. It is possible that insufficient enzymes are present in the rat colonic milieu for the amount of diclofenac-β-CD that was orally administered. Experiments where lower doses of diclofenac-β-CD are orally administered to rats would show whether this hypothesis is true. It is also possible that the duration of residence of the

prodrug in the colon was insufficient for either complete enzymatic degradation of the prodrug or for complete absorption of the released active drug, due to colonic emptying. In contrast, to the lower diclofenac bioavailability from the CD prodrug, sulfapyridine was completely absorbed from its prodrug as shown by the similar AUCs (59.4 µg.h/mL of the prodrug vs 61.8 µg.h/mL of the free compound). This reflects previous reports of high absorption of sulfapyridine following administration of sulfasalazine (Azad Khan and Truelove, 1980; Buggé et al., 1990). The difference in *in vivo* bioavailabilities of diclofenac and sulfapyridine from their prodrugs correlates with *ex vivo* studies which showed a much slower rate of release of diclofenac from diclofenac-β-cyclodextrin compared to the very fast release of sulfapyridine from sulfasalazine (Vieira et al., 2014).

5. Conclusion

In this *in vivo* study, we show that oral administration of diclofenac-β-cyclodextrin prodrug conjugate results in the absorption of the active drug diclofenac in the colon. The site of prodrug metabolism was confirmed using the marker sulfasalazine. This study demonstrates the feasibility of this new cyclodextrin prodrug to target and release diclofenac specifically in the colon following oral administration.

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