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Research Paper

Investigation into drug release from colon-specific azoreductase-activated steroid prodrugs using in-vitro models

Juan F. Marquez Ruiz[a], Kinga Kedziora[a], Henry Windle[a], Dermot P. Kelleher[b] and John F. Gilmer[a]

[a]School of Pharmacy and Pharmaceutical Sciences and [b]Department of Clinical Medicine, Trinity College, Dublin, Ireland

Abstract

Objectives The aim of this study was to investigate drug release from a double steroid prodrug, OPN501, which incorporates a phenylpropionate linker, and its phenylacetate analogue. The prodrugs, which were designed to deliver prednisolone to the colon for the treatment of inflammatory bowel disease, are based on a novel design that requires sequential azoreductase activity and cyclization of an amino ester to trigger drug release. We sought to explain the divergent effects of the two compounds in anti-inflammatory models and to justify the selection of OPN-501 for clinical development.

Methods The compounds were incubated in mouse colonic contents (10%) fermented in brain heart infusion under anaerobic conditions. The disappearance of the prodrugs and release of prednisolone was monitored by HPLC. We then developed a method for assessment of prodrug activation using suspensions of *Clostridium perfringens*, an anaerobe from the human colon. The cyclization of the compounds was studied in various media, assessing the influence of pH and bulk solvent polarity on cyclization rate using HPLC and NMR.

Key findings The prodrugs were activated via multiple pathways releasing prednisolone in mouse colonic ferment. The compounds released prednisolone by reduction–cyclization in *C perfringens* suspension. The active OPN-501 generated a stoichiometric amount of prednisolone following azoreductase activation, whereas its analogue did not. The pH rate profile for the cyclization of the amino intermediates of the two compounds revealed significant differences in rate at pH values relevant to the inflamed colon, which explain in part the different amounts of drug produced.

Conclusions The steroid prodrug OPN-501 has optimal drug release characteristics for colon targeting because of a kinetic advantage of a six-membered ring formation in the aminolysis reactions of anilides. The results are relevant to the development of OPN-501 but also to cyclization strategies in prodrug design especially for colon targeting.

Keywords azoreductase; colon; cyclization; prodrug; steroid

Introduction

Glucocorticoids are the most important therapies for controlling acute ulcerative colitis and Crohn's disease.[1] Their long-term use is unfortunately limited by their tendency to cause unpleasant and serious side effects, which are well known.[2] Site-specific delivery of these agents to the lower gut has the potential to reduce systemic exposure and side effects, decrease dose and improve efficacy. There has accordingly been a lot of work investigating formulation and prodrug approaches to achieving what might be considered a topical effect from the oral route.[3–5] Despite some interesting developments there remains a strong clinical appetite for a steroid for inflammatory bowel disease (IBD) treatment with an improved safety profile.

The prodrug approach to colon targeting is plausible because of the success of 5-aminosalicylic acid prodrugs such as olsalazine, ipsalazide and basalazide.[6–10] These transit to the colon and are activated by azoreductase activity associated with the luxuriant colonic microflora. The clinical use of these agents validates azoreductase as a practical vector in IBD patients and it underlines its ideal distribution in the human gastrointestinal

Correspondence: John F. Gilmer, School of Pharmacy and Pharmaceutical Sciences, Trinity College, Dublin 2, Ireland.
E-mail: gilmerjf@tcd.ie

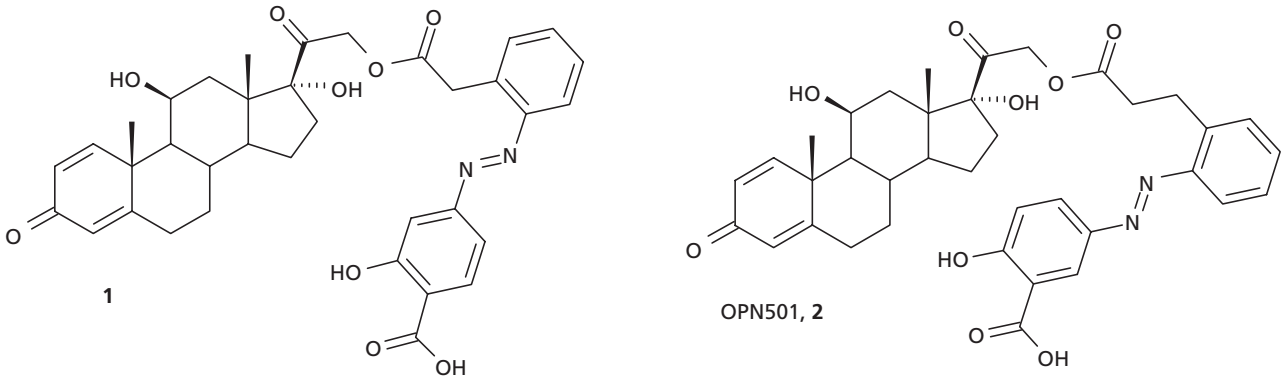

Figure 1 Structural formulae for phenylacetate **1** and phenylpropionate **2** (OPN501).

tract for colon targeting. Colonic azoreductase activity has therefore been investigated to target non-salicylate anilides to the colon.[11] We recently reported a novel cyclization-dependent prodrug system (Figure 1) that extends the possibilities for azoreductase-mediated drug release to alcohol-bearing drugs, including steroids.[12] The mechanism of drug release is illustrated in Figure 2. Reduction of the azo carrier by enzymes associated with colonic bacteria produces an amino ester (e.g. **6**), which is poised to undergo intramolecular aminolysis causing drug release. Cyclization strategies in prodrug design are popular because of the robust chemical predictability of the event.[13] In the prodrug design the intramolecular arrangement promotes anilide aminolysis, which is a very slow bimolecular reaction. There are in addition a number of competing possibilities for ester hydrolysis on the intact prodrug or its amino ester reduction product, and these are also shown. Overall, the design exploits the facile cleavage of azo bonds in the colon and the predictable aminolysis of the amino ester to achieve site-specific drug release. Compound **2** is in nonclinical development. It is not absorbed from the gastrointestinal tract in animal models, consistent with its transport characteristics in epithelial models.[12] It exhibits similar anti-inflammatory efficacy to prednisolone but causes significantly lower suppression of the hypothalamic–pituitary–adrenal (HPA) axis.[12] Compound **1** is an analogue of **2** that incorporates a shorter phenylpropionate linker. It was inactive in an in-vivo anti-inflammatory model indicating that it did not release prednisolone.[12]

The objective of this study was to find out why **2** is pharmacologically active but **1** is not. Therefore we studied the rates of drug release and disappearance kinetics of **1** and **2** under conditions designed to simulate colonic conditions. We developed and validated a generally useful assay that employs a suspension of *Clostridium perfringens* from the human colon in order to study the activation and drug release from **1** and **2**. We had to do this because there were no methods available that would have allowed us to measure the intact drugs and their metabolites. The relative rates of aminolysis of the intermediates **3** and **6**, the anilide products of azoreductase activity, were then studied. We propose that the relative effects of **1** and **2** arise because of differences in rates of cyclization under conditions that justify the selection of **2** for clinical development.

Materials and Methods

All chemicals were obtained from the Sigma-Aldrich Chemical Company (Dublin, Ireland). Brain heart infusion (BHI) and BHI-agar was obtained from Sigma Aldrich (Dublin, Ireland). *C. perfringens* and *Bacteroides fragilis* were obtained from the Health Protection Agency Culture Collections (Centre for Emergency Preparedness and Response, Porton Down, Salisbury, UK) and *Escherichia coli* was from New England Biolabs (Ipswich, USA). Samples of fresh mouse colonic contents were obtained from OPSONA, Therapeutics Ltd (St James's Hospital, Dublin, Ireland).

General methods

Uncorrected melting points were obtained using a Stuart melting point SMP11 melting point apparatus. Spectra were obtained using a Perkin Elmer 205 FT Infrared Paragon 1000 spectrometer. Band positions are given in cm^{-1}. Solid samples were obtained by KBr disk; oils were analysed as neat films on NaCl plates. ^{1}H and ^{13}C spectra were recorded at 27°C on a Bruker Advance II 600 MHz spectrometer (600.13 MHz ^{1}H, 150.91 MHz ^{13}C) and Bruker DPX 400 MHz FT NMR spectrometer (400.13 MHz ^{1}H, 100.16 MHz ^{13}C), in either $CDCl_3$ or CD_3OD (tetramethylsilane as internal standard). For $CDCl_3$, ^{1}H NMR spectra were assigned relative to the tetramethylsilane (TMS) peak at 0.00 δ and ^{13}C NMR spectra were assigned relative to the middle $CDCl_3$ triplet at 77.00 ppm. For CD_3OD, ^{1}H and ^{13}C NMR spectra were assigned relative to the centre peaks of the CD_3OD multiplets at 3.30 δ and 49.00 ppm, respectively. Coupling constants were reported in hertz (Hz). For ^{1}H NMR assignments, chemical shifts are reported: shift values (number of protons, description of absorption (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant(s) where applicable, proton assignment). High-resolution mass spectrometry (HRMS) was performed on a Micromass mass spectrophotometer (EI mode) at the Department of Chemistry, Trinity College. Thin-layer chromatography (TLC), was performed on silica gel Merck F-254 plates. Compounds were visually detected by absorbance at 254 nm and/or vanillin staining. The HPLC system used in this study consisted of a 600 controller pump (Waters), 2996 photodiode array detector (Waters), 717 plus autosampler (Waters) and an on-line degasser. The Empower

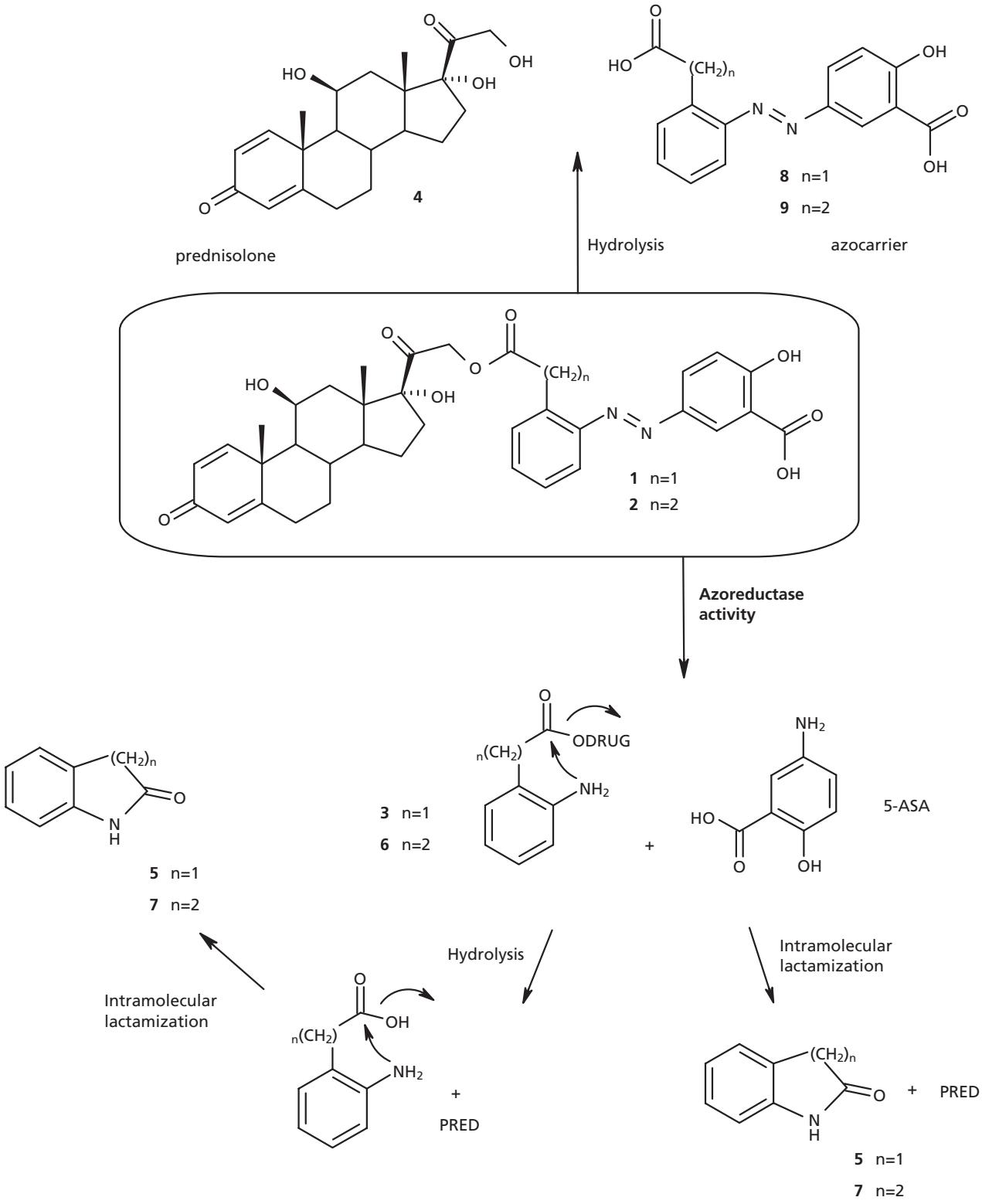

Figure 2 Competition between intramolecular lactamization and hydrolysis in **3** and **6**.

software was used for system control, data acquisition and processing. The HPLC separation was achieved using X Bridge C18 column (6 × 250 mm, 5 μm particle size). Mobile phase with gradient elution 10–90% acetonitrile in ammonia formate buffer (0.5% NH_4OH, pH 10) over 20 min was used. The assay was performed at room temperature and flow rate of 1.5 ml/min. Detector $\lambda = 245$ nm. The method was validated according to the principles of ICHQ2R.

(2-*tert*-Butoxycarbonylamino-phenyl)-acetic acid 2-(11,17-dihydroxy-10,13-dimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3*H*-cyclopenta[α]phenanthren-17-yl)-2-oxo-ethyl ester (BOC-3)

To a solution of 3-(2-*t*-butoxycarbonylmethyl-phenyl)-acetic acid (**13**) (0.02 g, 0.79 mmol) in DCM (5 ml), DMAP (1 equivalent, 0.009 g, 0.79 mmol) and DCC (1 equivalent, 0.016 g, 0.79 mmol) were added followed by prednisolone (1.1 equivalent, 0.03 g, 0.86 mmol). After 3 h the reaction was complete. DCU was removed by filtration. The solvent was removed under reduced pressure to afford a yellowish oil. This was flash columned using hexane : ethyl acetate (2 : 1) to yield the product as off-white crystals (0.03 g, 64%), m.p. 142–144°C. IR_{vmax}(KBr): 3327.27, 1722.56, 1626.17 and 1573.84 cm^{-1}. ^1H NMR δ ($CDCl_3$): 8.56 (1H, s, H'6), 7.32 (2H, m, H'4+H2), 7.23 (1H, m, H5'), 7.11 (1H, m, H3'), 6.17 (1H, d, *J* 10.04 Hz, H1), 5.91 (1H, s, H4), 5.60 (2H, d, *J* 7.56 Hz, C21CH_2), 4.26 (1H, s), 3.80 (2H, s), 1.45 (9H, s, BOC), 2.30–0.78 (prednisolone envelope, 19H). ^{13}C NMR δ ($CDCl_3$): 205.25, 185.16, 170.54, 170.40, 156.72 156.60, 153.51, 136.84, 130.78, 128.64, 127.34, 127.02, 124.71, 121.55, 88.63, 78.83, 68.26, 67.91, 55.34, 51.03, 47.49, 47.09, 43.75, 33.35, 32.77, 32.06, 31.42, 28.10, 25.53, 24.46, 23.57, 16.49. HRMS: Found: $(M\text{-}Na)^+$ = 616.2894. Required: $(M\text{-}Na)^+$ = 616.2886.

3-(2-*tert*-Butoxycarbonylamino-phenyl)-propionic acid 2-(11,17-dihydroxy-10,13-dimethyl-3oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3*H*-cyclopenta[α]phenanthren-17-yl)-2-oxo-ethyl ester (BOC-6)

To a solution of 3-(2-*t*-butoxycarbonylmethyl-phenyl)-propionic acid (**14**) (0.15 g, 0.55 mmol) in DCM (6 ml), DMAP (1 equivalent, 0.067 g, 0.55 mmol) and DCC (1 equivalent, 0.113 g, 0.55 mmol) were added followed by prednisolone (1 equivalent, 0.2 g, 0.55 mmol). The reaction was left stirring overnight and TLC (EtOAc) showed completion. DCU was removed by filtration and solvent was evaporated under reduced pressure to afford an oil. The crude was flash columned using DCM : ethyl acetate (7 : 3) to yield the product as white crystals (0.31 g, 90%), m.p. 152–154°C. IR_{vmax} (KBr): 3443.05, 1722.69, 1657.72 cm^{-1}. ^1H NMR δ ($CDCl_3$): 7.6 (1H, m, H-6'), 7.33 (1H, d, *J* 10.04 Hz, H2), 7.22 (1H, t, *J* 7.27 Hz, H-4), 7.16 (1H, dd, *J* 9.04 & 1.51 Hz, H'5), 7.09 (1H, t, *J* 7.53 & 1.26 Hz, H'3), 6.3 (1H, dd, *J* 10.29 & 1.8 Hz, H4), 6.02 (1H,s), 4.93 (2H, q, *J* 17.13 Hz, 21-CH_2), 4.49 (1H, s), 4.15 (1H, q, *J* 7.27 Hz), 1.55 (9H, s, BOC), 1.30 (1H, t, *J* 7.28), 1.0 (3H, s), 2.93–0.98 (prednisolone envelope 21H). ^{13}C NMR δ ($CDCl_3$): 204.85, 186.78, 171.26, 170.54, 156.74, 153.92, 135.92, 131.46, 129.59, 127.73, 127.16, 124.57, 123.60, 122.31, 89.89, 80.68, 69.99, 68.67, 55.37, 51.39, 48.05, 39.41, 34.67, 34.54, 34.12, 32.06, 31.33, 28.37, 25.33, 23.86, 21.09, 16.72. HRMS: Found: $(M\text{-}Na)^+$ = 630.3062. Required: $(M\text{-}Na)^+$ = 630.3043.

Lactamization studies

Stock solutions of BOC-3 and BOC-6 were prepared at 10 mM in MeOH. The lactamization samples were prepared by adding 80 µl of trifluoroacetic acid (TFA) into 30 µl of the stock solution; the reaction was left for 6 min before evaporation of the solvents with nitrogen. After that MeOH (50 µl) was added and 950 µl of the buffer solution at different pH values at 37°C and monitored by HPLC. The buffers were prepared using a universal borate/citrate/phosphate buffer system.

To study the effect of the ionic strength on the lactamization process of **3** and **6**, solutions were prepared with ionic strength values in the range 0.06–0.6, at constant pH (7.4).

Borate buffer pH 7.4 was prepared by mixing 2.5 ml solution A (containing boric acid, citric acid, sodium chloride, I = 0.06 M) and 4.5 ml solution B (containing potassium phosphate I = 0.06 M) and ionic strength of 0.06 M. Borate buffers at ionic strength 0.12, 0.24 and 0.6 M were prepared by addition of NaCl.

Solution A: 0.005 M of citric acid monohydrate, 0.02 M of boric acid and 0.059 M of NaCl in distilled water.
Solution B: 0.01 M of tri-potassium *ortho*-phosphate in distilled water. The solutions were mixed in the necessary proportion to achieve the required pH.

It is possible to make a lineal correlation to make the different solutions at different pH values using the formula below, substitution of the pH value in the equation gave the volume of the solution required to obtain the desired pH value.

$$\text{Volume of solution A (ml)} = 6.68 - 0.56 \times \text{pH}$$

$$\text{Volume of solution B (ml)} = -2.00 + 0.8670 \times \text{pH}$$

To find the buffer salt independent relationship between pH and cyclization rate we needed to generate a range of buffers at constant ionic strength.

In this case the buffer solutions were prepared as indicated below:

Solution 1: 0.05 M citric acid monohydrate, 0.2 M boric acid and 0.59 M NaCl in distilled water.
Solution 2: 0.1 M tri-potassium *ortho*-phosphate in distilled water.
Solution 1a: 0.025 M citric acid monohydrate, 0.1 M boric acid and 0.59 M NaCl in distilled water.
Solution 2a: 0.05 M tri-potassium *ortho*-phosphate and 0.3 M NaCl in distilled water.
Solution 1b: 0.02 M citric acid monohydrate, 0.04 M boric acid and 0.59 M NaCl in distilled water.
Solution 2b: 0.02 M tri-potassium *ortho*-phosphate and 0.48 M NaCl in distilled water.
Solution 1c: 0.005 M citric acid monohydrate, 0.02 M boric acid and 0.59 M NaCl in distilled water.
Solution 2c: 0.01 M tri-potassium *ortho*-phosphate and 0.54 M NaCl in distilled water.

The solutions were mixed in the necessary proportion to achieve the required pH using the above formula. In this way we prepared four solutions at constant ionic strength at each pH. The disappearance kinetics were monitored at each dilution, the rate estimated from the exponential decay and then the resulting k_{obs} values plotted against buffer concentration. Plots of k_{obs} versus buffer concentration produced straight lines which were used to estimate k_{obs} at zero buffer.

Culture of mouse colon contents

The azoreductase activity of mouse colon contents towards **1** and **2** was tested under anaerobic conditions at 37°C. Bacteria and faeces were scraped from the regions of the colon of BALB/C mice until 1.5 cm from the anus, weighed and inoculated in BHI media to obtain a suspension 10% in weight of colon contents. Compounds **1** and **2** stock solutions were added (20 µl) into fermented suspension (1 980 µl) in a sterile 20 ml universal tube to reach 50 µM concentration and 1% dimethyl sulfoxide (DMSO). The negative control consisted of BHI media with **1** and **2** at the same concentration, also introduced in DMSO. Immediately after the prodrugs were added to the colon contents a 200 µl sample was taken and added to 400 µl of acetonitrile in a 1.5 ml eppendorf; samples were then centrifuged at 10 000 rpm for 10 min and supernatant transferred to a tube and stored at −20°C until analysis. Samples were taken at time points: 0, 1.5, 3, 5 and 22 h, while carefully maintaining the ferment under anaerobic conditions. Samples were analysed by HPLC for remaining prodrug and products. The cyclization reaction was also performed at 4°C to validate the extraction and sample storage conditions. The $t^1/_2$ for prodrug disappearance under these conditions was >1000 min in both cases.

Clostridium perfringens screening assay

A 20-µl volume of test substance (2 mM in DMSO) was added to BHI broth (1 980 µl) inoculated with *C. perfringens* in a sterile 20 ml universal container resulting in substrate concentrations of 15–50 µM (1% DMSO). Immediately after addition of the test compounds, a sample was taken (200 µl) and added to 400 µl of acetonitrile in a 1.5 ml minifuge tube. Samples were then centrifuged at 10 000 rpm for 10 min. The resulting supernatants were recovered and stored at −20°C until required for analysis. Samples were taken every 2 h up to 8 h and a final sample taken at 24 h. At all times the bacterial suspensions were maintained under strictly anaerobic conditions using a Mart Anoxomat system (Mart Microbiology B.V., Drachten, Netherlands) (gas mix: 10% CO_2, 10% H, 80% N) and the cells were kept in suspension by incubating the gas jars in a shaking incubator (120 rpm, at 37°C). The negative controls consisted of BHI broth containing the appropriate test compounds at the same concentration but without the addition of *C. perfringens*. The assay was also performed in the presence of the azoreductase inhibitors menadione (100 mM–1 mM) and iodosobenzoic acid (1 mM).

Data handling and statistical analyses

All data manipulations were performed using GraphPad Prism. The experiments using mouse colon contents were performed three times. The data points represent the mean concentration of each species and error bars the standard deviation of these determinations. The rate constants for cyclization at zero buffer were estimated as described; the error associated with these values corresponds to the SD of the y-intercept.

Results and Discussion

Activation and hydrolysis in mouse colon contents

To mimic colonic conditions in the murine inflammation model, which had been previously used to assess the anti-inflammatory activity of the two prodrugs, mouse colon contents were allowed to incubate *in vitro* for 24 h in BHI (37°C) under anaerobic conditions before the introduction of **1** or **2**. Samples were taken periodically, quenched and analysed by a validated HPLC method, which permitted the separation and quantitation of components identified in Figure 2. Compounds **1** and **2** underwent processing in the colonic suspension with the appearance of prednisolone (**4**) (progress curves appear in Figure 3). The prodrug disappearance (and prednisolone evolution) could be attributed to a mixture of azo-reductase activity leading to the formation of the cyclization products (**5** and **7**) and ester hydrolysis producing azo acids **8** and **9** (Figure 2), which were themselves consumed through azo reduction. The amino products of azoreductase

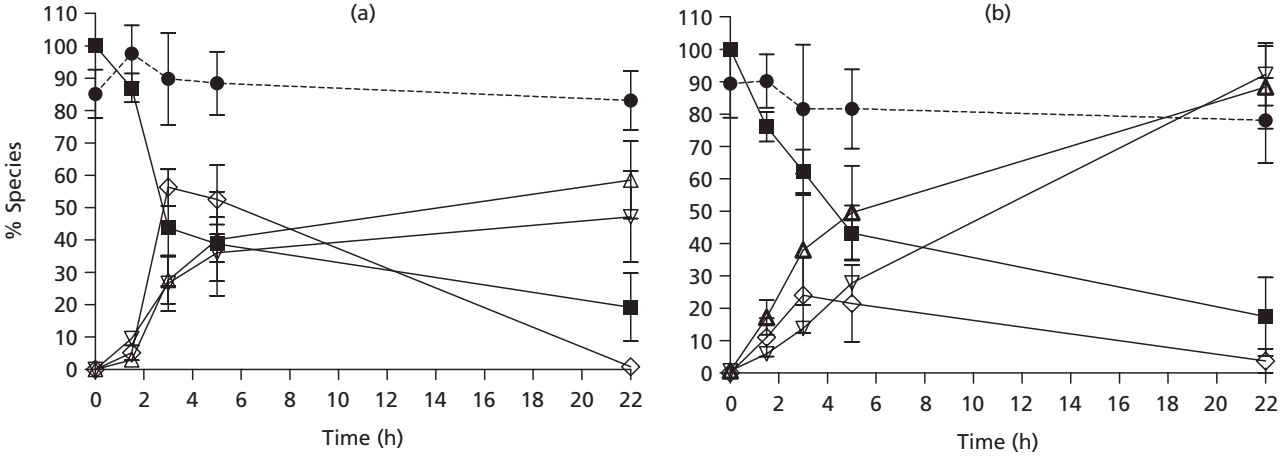

Figure 3 Progress curves for the disappearance of **1** and **2** following incubation in cultured mouse colon contents (MCC) (10%) in pH 7.4 buffer under anaerobic conditions at 37°C: A. **1** incubated in MCC (■), **1** in BHI (●), prednisolone (△), **5** (▽), **8** (◇); B. **2** (■), **2** in BHI (●), prednisolone (△) B. **2** incubated in MCC (■), **2** in BHI (●), prednisolone (4) (△), **7** (▽), **9** (◇).

activity on **1** and **2** (**3** and **6**) were not detected. Ester hydrolysis was apparently more prominent in the case of **1** (60% at max in molar terms) than **2** (~20% at max). This may reflect greater susceptibility towards hydrolysis in the case of **1** or slower competing processes such as azoreduction. Ester hydrolysis appeared to be enzymatically mediated because **1** and **2** were stable under similar conditions of pH and temperature in BHI. The esterase activity may have been associated with the bacterial component of the preparation or with luminal or tissue-derived esterases from the intestinal wall artifactually introduced during sampling. The intestine of rodents has significantly higher esterase activity than the human intestine.[14,15] Notably, the disappearance of **2** was associated with significantly greater prednisolone evolution and the expected cyclization product **7** than from **1** and its cyclization product **5**. The latter observation suggested that differences in prednisolone release were caused by differences in rates of reduction or lactamization, although in the case of **1** there was presumably less intact prodrug available to undergo reduction/cyclization because of its greater susceptibility to hydrolysis. It was decided to make a closer examination of the relative rates of cyclization of the intermediates **3** and **6** arising from azo reduction in each case.

Development of an azoreductase assay using bacterial suspension

To identify bacteria with appropriate levels of azoreductase activity, BHI-agar plates containing either the dye Direct Blue 15 (300 μM) or the prodrug olsalazine (250 μM) were inoculated with *E. coli* (K12), *C. perfringens* (NCTC 8237) or *B. fragilis* (NCTC 9343). These organisms are known to possess azoreductase activity.[16,17] The plates were incubated under strictly anaerobic conditions at 37°C, together with control plates without bacteria. After 24 h, the plates cultivated with *C. perfringens* were completely decolourized indicating substrate consumption. There was significant but incomplete substrate consumption in the plates inoculated with *B. fragilis*, while *E. coli* plates remained unchanged.

Since *C. perfringens* demonstrated greater azoreductase activity than *B. fragilis*, further assay development work was undertaken with *C. perfringens*. This organism is widely distributed in the human colon; it has high levels of an azoreductase that exhibits wide crossed immuno reactivity with other human flora azoreductase; it can process substrate without the need for additional co-factors; and its azoreductase is secreted, obviating the need for cell fractionation.[17–21] Colorimetirc methods have been reported for monitoring substrate consumption that use *C. perfringens* as an azoreductase source;[21] however, we required a selective assay that would permit us to monitor steroid and cyclization adduct production, in addition to substrate disappearance. Initial experiments with the agar plates inoculated with *C. perfringens* were unsuccessful because of problems with accuracy and precision of extraction for the multiple analytes. The next steps were therefore: (i) to develop bacterial suspensions in BHI that could permit good precision and recovery of test compound and products by HPLC; (ii) to validate an extraction procedure; (iii) to determine the optimum substrate concentration; and (iv) to determine the optimum cell count as reflected in OD for substrate turnover.

Validation of the extraction method

A series of experiments were performed using different concentrations of **1**, **2** in BHI to validate an organic solvent extraction procedure. BHI solutions (1% DMSO) were prepared at three concentration levels (3.3, 16.5 and 50 μM) for each of the study compounds: olsalazine, prednisolone, steroid prodrugs, cyclization products 2-oxindole (**5**) and DHQ (**7**). These solutions were each repeatedly extracted ($n = 3$) with MeCN and analysed by a previously reported high pH HPLC method, which had been fully validated for water/MeCN solutions according to the principles of ICHQ2R.[12] The recovery (accuracy) and repeatability (precision) values for the extractions from BHI at the 3 μM level were, respectively, 90–110% and <10% (RSD), which was acceptable for the purposes of this kind of study.

Azoreductase assay with *C. perfringens*

Routinely, 24-h-old BHI-agar grown cultures of *C. perfringens* were harvested using sterile plastic loops and the cells suspended in BHI-broth (1.8 ml) at defined densities as reflected in OD at 600 nm. The relationship between bacterial cell density and the rate of substrate consumption was measured over the OD_{600} range 0.1–1.1 and at substrate concentrations in the range 20–500 μM. These variables were optimized using steroid prodrugs **1**, **2** and Direct Blue 15 as standards. Optimal kinetics were observed at bacterial densities in the OD_{600} range 0.9–1.1 and at substrate concentrations of 20–50 μM (Figure 4). Following co-incubation of bacteria and substrates for different periods of time, samples were removed, subjected to centrifugation in a minifuge at room temperature to remove intact bacteria and the supernatants stored at −20°C until required for analysis.

Compounds **1**, **2** were consumed rapidly under these assay conditions, producing prednisolone (**4**) and the respective cyclization products **5** (2-oxindole) and **7** (DHQ) (Figure 5). Half-lives for prodrug consumption could be estimated assuming first-order kinetics as **1**, 1.6 h (k_{obs} 0.424 h^{-1}, $r^2 > 0.999$) and **2**, 1.2 h (k_{obs} 0.584 h^{-1}, $r^2 > 0.99$). Differences in concentration between the prednisolone and cyclization products (**5**, **7**) did not achieve significance in either case indicating that reduction/cyclization was the predominant mechanism of drug release. There was no evidence of hydrolysis of the ester bond between steroid and the respective linkers. Compound **1** produced significantly less prednisolone than **2**c, consistent with the observations in the mouse colon contents and with their relative pharmacological effects.[12] Olsalazine and balsalazide[9] were also reduced under these conditions albeit more slowly. The role of azoreductase activity in the disappearance of **2** was confirmed by co-incubating with the azoreductase inhibitor 2-iodosobenzoic acid (1 mM), which led to significant attenuation in rate (Figure 4). Co-incubation with menadione did not significantly depress reduction rate at <1 mM.

Synthesis of BOC-3, BOC-6

The intermediates BOC-3 and BOC-6 to study intramolecular lactamization kinetics were synthesized as shown in Figure 6.

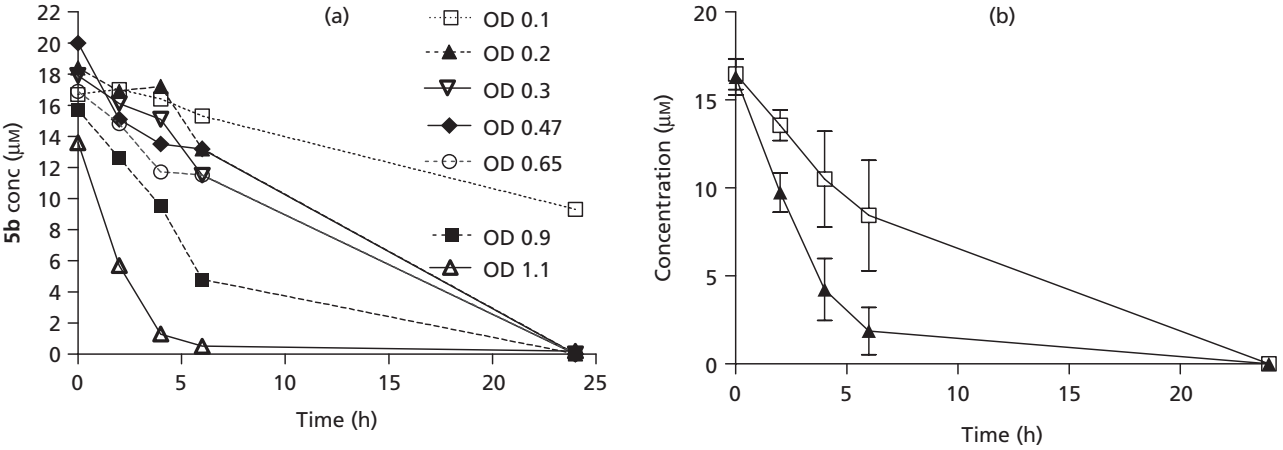

Figure 4 (a) Effect of *C. perfringens* cell count (OD) on rate of disappearance of **2** and (b) Disappearance of **2** (▲) in the presence of *C. perfringens* and in the presence of azoreductase inhibitor 2-iodosobenzoic acid (0.5 mM) (□).

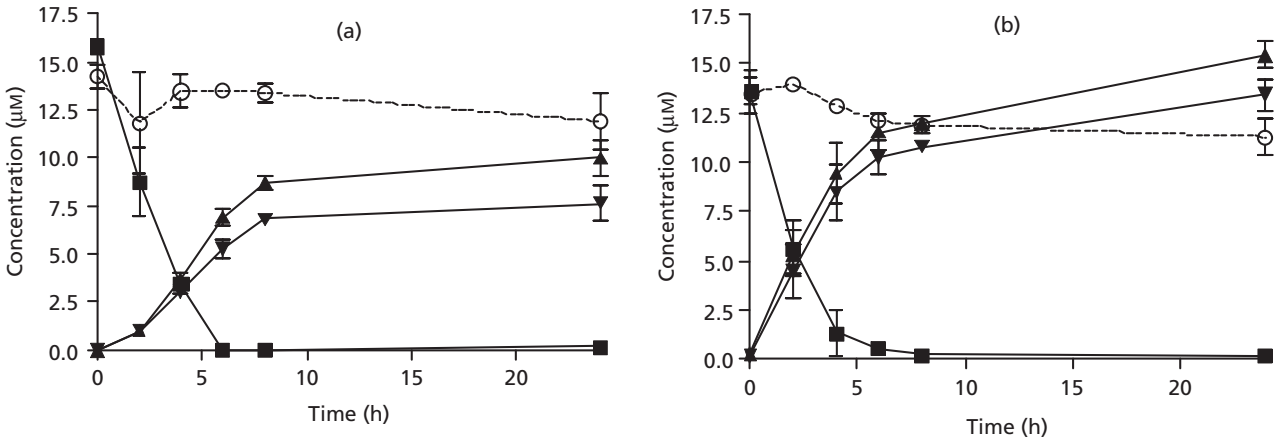

Figure 5 Progress curves for the disappearance of (a) **1** (■), (b) **2** and the evolution of prednisolone (▲), 2-oxindole (**5**) (a) and (b) DHQ (**7**) (▼), **1**,**2** in BHI (●) in the presence of *C. perfringens* (37°C, pH 7.4).

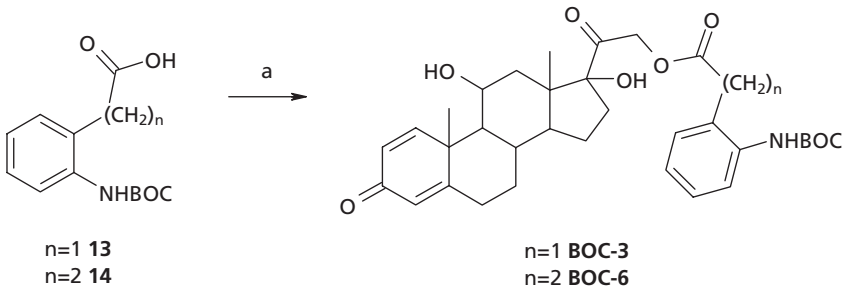

Figure 6 a: Prednisolone (**4**), DCC, DMAP in DCM.

BOC-protection of the amino group was necessary to prevent lactamization before or during the esterification with prednisolone. Compound **13** was produced by BOC protection of aminophenylacetic acid, which was in turn obtained by reduction (H_2/Pd/C) of commercially available nitrophenyl acetic acid. The synthesis of **14** was described previously.[12] The rate of cyclization of the 2-aminophenyl propionate ester of cortisone at several pH values was also reported in that study.

Nuclear magnetic resonance lactamization in $CDCl_3$

The lactamization of **3** and **6** was first monitored by NMR analysis in $CDCl_3$. The BOC intermediates were dissolved in deuterated chloroform and a drop of TFA added to trigger the lactamization. In the case of **6**, lactamization was complete by ^{1}H NMR after 128 min (stacked NMR spectra are shown in

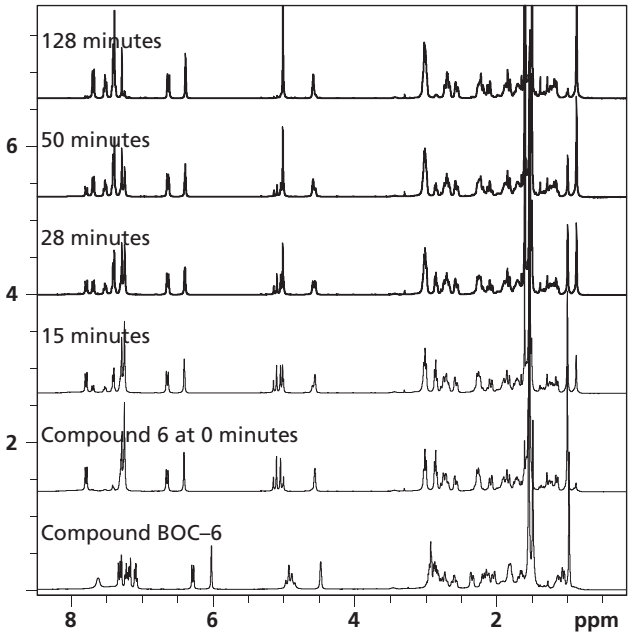

Figure 7 Lactamization of **6** monitored by NMR in $CDCl_3$.

Figure 7). Before deprotection, the CH_2 from C-21 of the prednisolone exhibits multiplicity forming a quadruplet at 4.93 ppm. Following removal of the BOC group the chemical shift of the CH_2 group at t_0 was 5.06 ppm and a quadruplet; this gradually transformed until it became a singlet at δ 5.01 ppm. Also there was confirmation of the lactamization when the CH_2 groups from C-23 and C-24, initially triplets at 3.02 and 2.98 ppm, respectively, changed to one peak at 3.00 ppm. When the experiment was repeated with **3** lactamization was not observed up to 1390 min. Under the experimental conditions, the compounds were in the fully protonated state (excess TFA) and in an anhydrous environment. The NMR experiment provided support for the identification of the lactamization products and it pointed to a significant difference in the behaviour of the two corresponding compounds that suggested an explanation for the failure of one to exhibit biological activity. The study was repeated more quantitatively under conditions that would more closely mimic those in the intestine.

Lactamization studies in aqueous buffer

The rate of lactamization as a function of pH for compounds **3** and **6** was studied in a universal buffer system at pH range 2.5–8.5 ($\mu = 0.12$, 37°C). To commence lactamization, deprotection of **BOC-3** and **BOC-6** was carried out in DCM at 0°C with TFA for several minutes, the volatiles removed under a stream of nitrogen and the residue dissolved immediately in the appropriate buffer solution. HPLC was used to measure the disappearance of the parent and the appearance of prednisolone **4**, dihydroquinolone **7** or 2-oxindole **5**. Data for the disappearance of **3** and **6** could be analysed using first-order kinetics over the pH range and rates were determined by non-linear regression to an exponential decay. The lactamization rate was found to be highly buffer species dependent in some regions. In particular, the cyclization of **6** was highly sensitive to phosphate, which can act in a concerted manner as proton donor and acceptor. Therefore, the lactamization rate constants were determined at four different buffer salt concentrations at each pH value and the rate at zero buffer estimated by extrapolation. There was evidence of a hydroxide ion-catalysed hydrolysis of the ester group in both cases above pH 9; below this the disappearance of **3** and **6** could be exclusively attributed to cyclization. The two outstanding features of the pH rate profiles (Figure 8a, Table 1) are the markedly higher rate constants for the cyclization of **6** (the expected reduction product of **2**) compared with **3** and secondly the general independence of pH across the pH range for **3** and at <pH 7 in the case of **6**. The rate constants for the cyclization of **6** were 5–7 fold higher than for **3** at <pH **7** and only became similar at around pH 8 because of an apparent HO^--dependent fall in cyclization rate of **6**. The maximum rate of cyclization of **6** occurred in the pH range 3–4 followed by a slight inflection at pH 4.3 associated with the pKa of the anilide amino group in **6** (pKa 4.2). The cyclization of methyl esters of 3-(2-aminophenyl) propionic and acetic acid has been reported and there are similarities between the rate data for the cyclization of **3** and **6** and their methyl ester analogues.[22,23] The lactamization of the methyl esters is reported to be relatively independent of pH and faster for 3-(2-aminophenyl) propionate (leading to the 6-membered ring) than methyl (2-aminophenyl)acetate. A mechanism was proposed for intramolecular aminolysis of anilide esters by Fife and Duddy[22] based on the general scheme for ester aminolysis first proposed by Satterthwait and Jencks[24] and we assume this is relevant to the cyclization of steroid esters **3** and **6** (Figure 8b). The anilines are shown in equilibrium with their protonated forms. The weak nucleophilicity of the aromatic **6** means that there is a relatively small amount of the zwitterionic tetrahedral intermediates **10b** present. Direct breakdown of the tetrahedral zwitterion can occur with good leaving groups but not with the aliphatic alcohols such as prednisolone. In the case of the phenylpropionate **6**, formation of the neutral tetrahedral intermediate (**12b**) is likely to be rate determining (second-order reaction) and this may explain the observed decrease in rates at increasing pH. For the kinetically more favourable 5-membered ring closure, the concentration of zwitterion **10a** is expected to be higher and breakdown of the neutral tetrahedral intermediate is likely to be rate limiting. The slower breakdown of the intermediate in the general 5-membered case has been attributed to the inductive effect of the phenyl ring on the tetrahedral centre making C-O bond breaking more difficult than in the six-membered case. This difference in cyclization rate constants between **3** and **6** has a significant effect on half-lives that are relevant to release rates in the key pH region 4–6 (74 min vs 564 min at pH 4.1). In the case of ulcerative colitis, the pH values in stomach, proximal small intestine and ileum are similar to healthy subjects but the pH in the colon is lower in many individuals, especially when the disease is active.[25–27] Thus, pH values for the right colon/caecum from 2.3 to 3.4 have been recorded. Similar observations were made in Crohn's Disease patients and the pH values in the right and left colon had a mean value of 5.3 in both areas. Colonic pH values in IBD patients therefore fall in the region where the cyclization rate of the amino ester products of reduction of **1** and **2** are

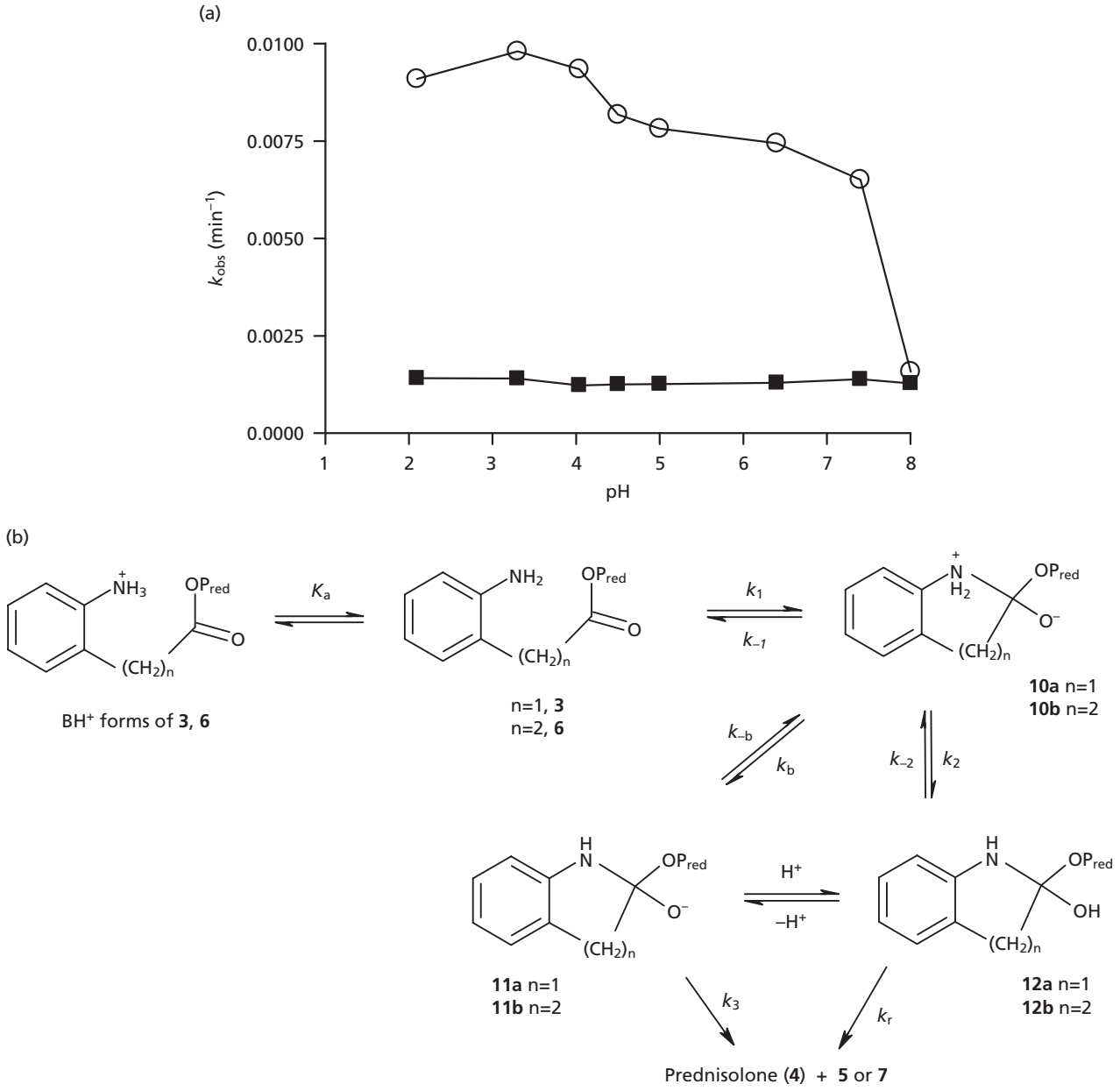

Figure 8 (a) Plot of k_{obs} vs pH for lactamization of **3** (■) and **6** (○) producing 2-oxindole (**5**) and dihydroquinolone (**7**), respectively, at 37°C and μ = 0.12 M adjusted with NaCl. Rate constants were obtained by extrapolation to zero buffer over $n = 4$ experiments at a range of buffer concentrations using a universal borate/citrate/phosphate system. (b) Mechanism for intramolecular aminolysis of **3** and **6** adapted from.[22]

most different indicating that in addition to its demonstrably better activity in animal models, **2** is a better candidate for human development based on its activation and drug release rate *in vitro*.

Lactamization studies: effect of the ionic strength and organic solvent

The relationship between ionic strength and rate of lactamization of **3** and **6** was studied in the range $\mu = 0.06$–0.6 while maintaining constant pH (7.4). The ionic strength throughout the human gastrointestinal tract is likely to vary significantly depending on diet and water intake. The USP simulated intestinal fluid ionic strength is 0.07.[28] As evident in Figure 9a the higher the ionic strength, the longer the time to complete the lactamization reaction although the effect was small (2-fold). We also evaluated the effect of increasing the proportion of methanol (5–90%) in PBS at pH 7.4 ($\mu = 0.12$ at 37°C) (Figure 9b, Table 2). We were interested in the relative behaviour of the compounds under conditions prevailing in the colon where free water content is low (13 ± 12, fed; 18 ± 26 fasted).[29,30] There was a marked 10–15-fold decrease in the rate of cyclization the cases of both **3** and **6** with increasing MeOH. This can attributed mechanistically to destabilization of the initial zwitterionic intermediate in the increasingly

Table 1 Kinetic data for the lactamization of compounds **3** and **6** at 37°*C* (μ = 0.15) in the pH range 2.4–8.5

pH	3			6		
	k_{obs} (min^{-1})	$t^1/_2$ (min)	sd*	k_{obs} (min^{-1})	$t^1/_2$ (min)	sd
2.4	0.0014	490.55	0.0001	0.0090	76.30	0.0005
3.4	0.0014	494.75	0.0001	0.0091	70.75	0.0040
4.10	0.0012	563.99	0.0001	0.0093	74.22	0.0017
4.50	0.0012	553.63	0.0001	0.0081	84.87	0.0070
4.95	0.0012	547.94	0.0001	0.0078	88.76	0.0080
6.49	0.0012	535.24	0.0003	0.0074	93.17	0.0006
7.40	0.0013	499.74	0.0004	0.0065	106.62	0.0015
8.08	0.0012	541.94	0.0002	0.0015	437.86	0.0018
8.5	0.0010	2243.19	0.0003	0.0012	573.32	0.0008

*sd is the standard deviation of the y intercept from plots of k_{obs} vs [buffer] estimated using GraphPad Prism5.

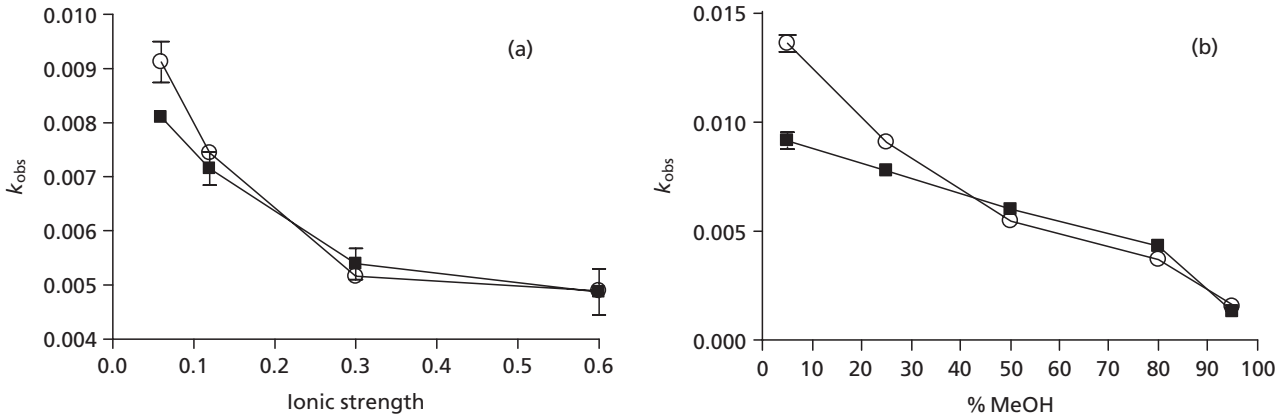

Figure 9 Lactamization rate (k_{obs}) vs (a) ionic strength (μ = 0.05–0.6) and, (b) % MeOH (5–95%) for **3** (■) and **6** (○) at 37°C at pH 7.4 (37°C).

Table 2 Kinetic data for the lactamization of **3** and **6** at pH 7.4 (37°*C*) at different proportions of MeOH

% MeOH	3			6		
	k_{obs}	$t^1/_2$ (min)	sd*	k_{obs}	$t^1/_2$ (min)	sd*
5	0.0091	75.97	0.0004	0.0135	51.00	0.0004
25	0.0077	89.41	0.0002	0.0090	76.36	0.0003
50	0.0059	116.0	0.0001	0.0054	127.1	0.00001
80	0.0043	159.6	0.0001	0.0036	187.9	0.00005
90	0.0013	513.8	0.0001	0.0016	432.6	0.00001

*sd is standard deviation of the k_{obs} values: $n = 3$.

apolar environment (Figure 8b). Interestingly the behaviour of the cyclization intermediates was most different at low organic (5%) and low ionic strength at which the pH rate profile was determined, suggesting that these are closest to colonic conditions considering the divergent pharmacological characteristics of **1** and **2**.

Conclusions

OPN-501 (**2**) produced more prednisolone than its analogue **1** in cultured mouse colon contents and in a newly developed assay employing *C. perfringens* suspension. To explain this, we studied the rates of cyclization of the amino intermediates (**3** and **6**) arising from the actions of anaerobic bacteria on **1** and **2**. There were significant differences in the rates of cyclization in the colonic pH range found in IBD patients and this may explain the relative yield of prednisolone from the two compounds in the culture media and their pharmacological activity.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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