Bioorganic & Medicinal Chemistry Letters 23 (2013) 1693–1698

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

A nitrophenyl-based prodrug type for colorectal targeting of prednisolone, budesonide and celecoxib

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ARTICLE INFO

Article history:
Received 5 December 2012
Revised 11 January 2013
Accepted 16 January 2013
Available online 8 February 2013

Keywords:
Prodrug
Colon
Targeting
Nitroreductase
Cancer
Celecoxib
Budesonide
Prednisolone

ABSTRACT

Celecoxib is a COX-2 inhibitor drug that can be used to reduce the risk of colorectal adenocarcinoma. Glucocorticoids are used in the treatment of inflammatory bowel disease. A limitation to the use of both drug types is that they undergo absorption from the intestinal tract with serious side effects. The prodrug systems introduced here involve forming a nitro-substituted acylsulfonamide group in the case of celecoxib and a nitro-substituted 21-ester for the glucocorticoids. Drug release is triggered by the nitro reductase action of the colonic microflora, liberating a cyclization competent species. The release of the active parent drugs was evaluated in vitro using *Clostridium perfringens* and epithelial transport through Caco-2 monolayer evaluation was carried out to estimate the absorption properties of the prodrugs compared to the parental drugs.

Oral drug delivery to the colon is pursued in order to improve therapy for colorectal diseases or as a means to increase bioavailability of drugs that would be degraded in the small intestine.[1,2] The conventional pharmaceutical strategy for achieving colon targeting involves the use of slowly biodegradable polymers that delay drug release until the oral caecal transit time. The variability in this parameter has led to the development of more sophisticated formulation approaches in which the matrix exhibits more or less specific sensitivity to a chemical or biological property of the colon, for example a property associated with its dense microflora.[3] The candidate drug may also be linked covalently to the biodegradable polymer through a biodegradable bond.[4]

The more explicit prodrug approach to colorectal delivery involves attaching a group that suppresses intestinal absorption using a form of covalent linkage that is susceptible to cleavage in the colon.[5] The most well known examples of this are the azo-linked derivatives of 5-amino salicylic acid such as olsalazine and balsalazide, which were inspired by the older sulfasalazine. The latter remains an important compound not just for ulcerative colitis but also for arthritis. There have been several investigations into prodrugs that target sugar metabolism of bacteria, for example steroidal glucuronides and glycosides, cyclodextrin and even dextran conjugates.[6,7] Amino acid carriers have been investigated more recently for colorectal targeting of ursodeoxycholic acid (UDCA), celecoxib and naproxen.[8,9] Sulfate conjugates of UDCA have been explored for similar reasons.[10] As of yet none of these prodrug approaches can rival the clinical success of the azo prodrugs of 5-ASA. It may be that the reductive capacity of the colon more clearly distinguishes it from the small intestine than other potential vectors. The azoprodrug approach is directly applicable only to primary aromatic amino drugs such as 5-ASA. However, we showed, that by incorporating an azoreductase sensitive linker, it is possible to adapt the approach to drugs bearing a primary alcohol group as shown in Figure 1. These conjugates are less prone to intestinal absorption than the parent compounds but they can be specifically activated by murine colonic microflora and by *Clostridium perfringens*, an obligate anaerobe found widely in the human colon. A prednisolone analogue was as efficacious as its parent in the murine dextran sodium sulfate model of ulcerative colitis but caused less thymic atrophy, indicating reduced systemic steroid exposure.[11] We subsequently showed that the approach could be applied to the more sterically hindered steroid budesonide and to celecoxib, a sulfonamide COX-2 inhibitor, through the use of an acylsulfonamide link.[12]

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http://dx.doi.org/10.1016/j.bmcl.2013.01.060

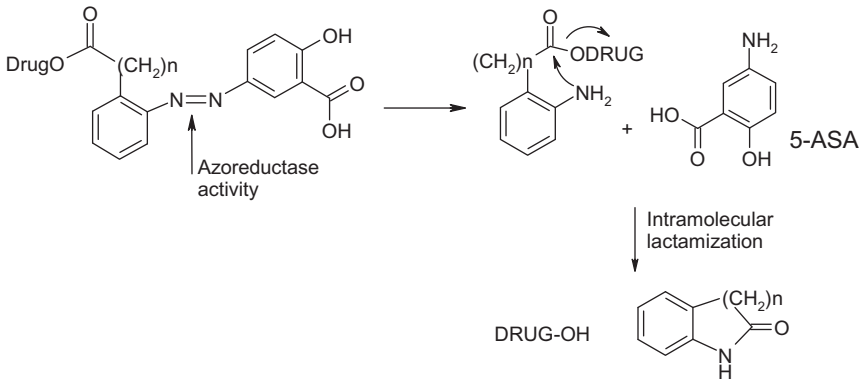
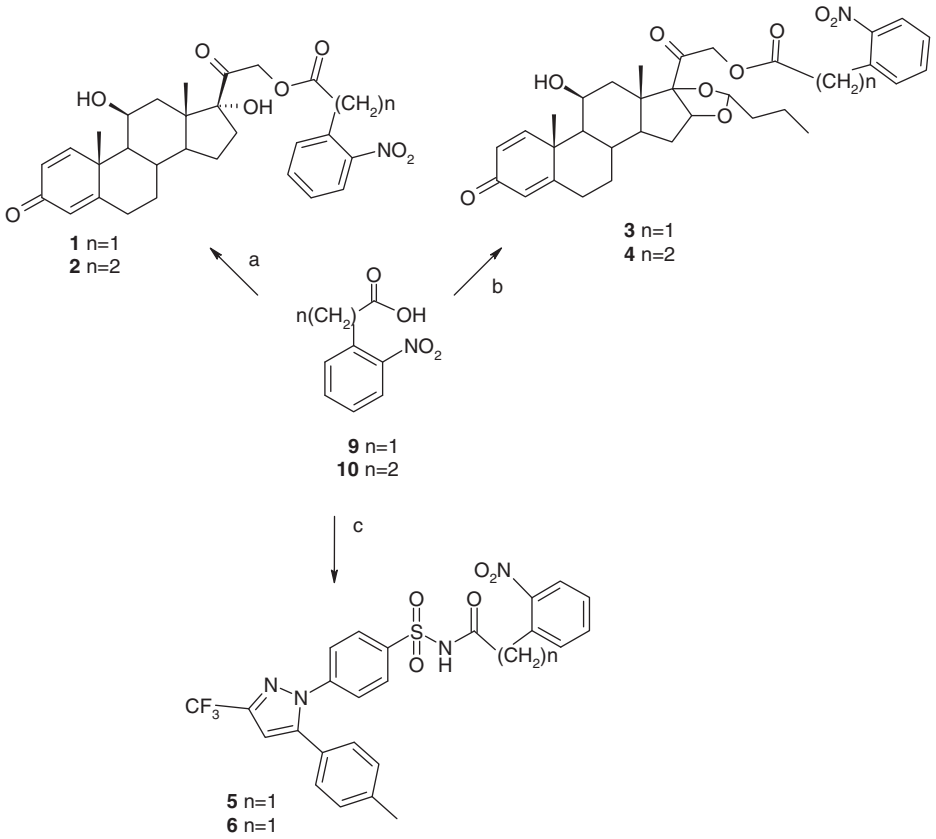


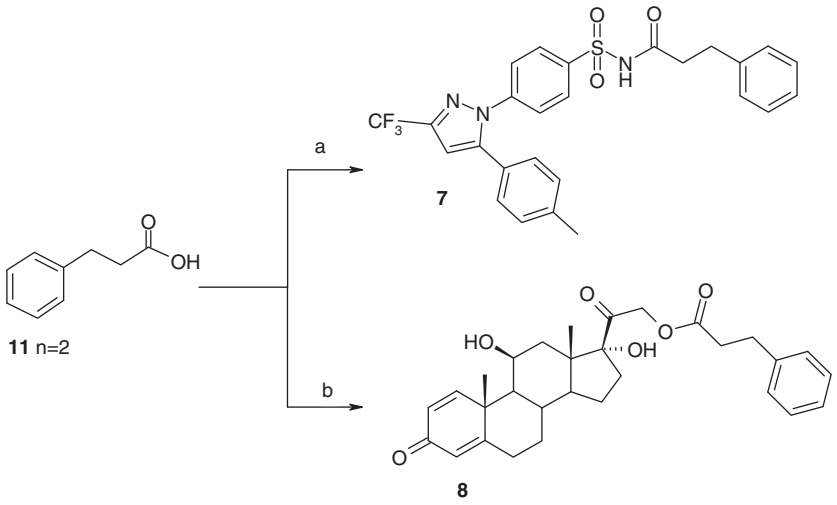
Figure 1. Azoreductase-cyclization activation mechanism.



Scheme 1. Reagents: (a) Prednisolone, DMAP, DCC in ACN; (b) budesonide, DMAP, DCC in ACN; (c) celecoxib, EDC, DMAP in ^{t}BuOH/ClCH$_2$CH$_2$Cl.

In this study we have further extended the cyclization approach using a nitroreductase activation design. We have focused on nitro substituted phenyl acetic and propionic acid esters of three drugs that would benefit from colorectal targeting-budesonide, prednisolone and celecoxib (Scheme 1). The passive permeability of the compounds was assessed using the predictive Caco-2 monolayer model while their capacity to trigger drug release was investigated using *C. perfringens* as a model intestinal anaerobe. The stability of the prodrugs towards hydrolysis under simulated gastric and duodenal conditions was tested in order to predict off-target drug release.

The pro-moieties/carriers selected for attachment to the steroids prednisolone, budesonide and celecoxib were 2-nitrophenyl acetic acid **9**, which was commercially available, and 2-nitrophenyl propionic acid (**10**), which was prepared as described.[13] The starting material for this was 2-nitrobenzoyl bromide which was treated with dimethylmalonate under basic conditions to obtain dimethyl-2-(2-nitrobenzyl)-malonate, which was heated under acidic conditions to yield **10** (Scheme 1). The esterification reactions between the carboxylic acid group of **9** and **10** and the 21-OH groups of the prednisolone and budesonide to obtain the prodrugs **1**, **2**, **3** and **4** was carried out using DCC and DMAP in ACN.[14] The synthesis of the celecoxib prodrugs **5**[15] and **6** was achieved using as coupling agent (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC). In order to evaluate the intrinsic stability of the ester/acylsulfonamide groups towards the *C. perfringens* and the role of the nitro group in ester scission and prodrug activation we prepared two analogs without a nitro group **7** and **8** (Scheme 2).



Scheme 2. Synthesis of **7** and **8**; Reagents: (a) celecoxib, EDC, DMAP in ^{t}BuOH/$ClCH_2CH_2Cl$; (b) prednisolone, DMAP, DCC in ACN.

The candidate compounds were tested for prodrug activation using a suspension of *C. perfringens*.[16] The day before the experiment *C. perfringens* were inoculated on agar plates containing brain heart infusion medium (BHI). On the day of experiments *C. perfringens* colonies were scratched from the agar plates and inoculated in BHI media to obtain cell density between 0.9 and 1.1 at 600 nm.

Test compound was introduced into suspension at 50 μM in 1% DMSO. The negative control consisted of BHI media with **3–6** at the same concentration.

Immediately after the compounds were added to the *C. perfringens*, an aliquot was taken (200 μl) and added to ACN and samples were centrifuged at 10,000 rpm for 10 min. Aliquots were taken at time points 0, 2, 4, 6 and 24 h while maintaining anaerobic conditions. Supernatant was transferred to a tube and stored at −20 °C until analysis by a HPLC method that could separate BHI components from the prodrug and expected byproducts of activation.

The nitro prodrugs were activated in the bacterial suspensions (Fig. 2A–F) with substrate consumption observed within 6 h in the prednisolone cases (**1**,**2**) and within 4 h in the celecoxib cases ($t_{1/2}$ ~2 h). Processing was slower in the case of the budesonide prodrug **3** and incomplete in the case of the propionate **4**. Budesonide is probably more susceptible to steric hindrance of reduction and/or cyclization than prednisolone. All of the compounds were stable when incubated at the same temperature in BHI (i.e., in the absence of bacteria). Furthermore, **7** and **8**, which lack the nitro group, were not processed to a significant extent in the bacterial model proving that nitro group metabolism triggered drug release in the other cases (Fig. 3).

Although the nitroester compounds were presumably being reduced we were unable to detect amino intermediates in this process or the corresponding lactamization byproducts when the HPLC effluent was monitored by UV or MS. Samples of oxindole and dihydroquinolone (DHQ) were available from our previous work which allowed us to confirm their absence. Under reverse phase HPLC gradient conditions, the suspensions of *C. perfringens* and **1** and **2** contained unidentified components at shorter retention than oxindole (in the case of **3**) and DHQ (in the case of **4**).

Aromatic nitro compounds may be reduced to the corresponding amines by aerobic and anaerobic bacteria. Under anaerobic conditions the complete reduction to aromatic amine involves a six electron transfer via nitroso (2-electron) and hydroxyl amino (4-electron) intermediates. The latter is cyclization competent. In the incipient five-membered ring case, the expected product is the *N*-acyl compound (cyclic hydroxamate), whereas at smaller chain length the OH group of the hydroxylamine is expected to cyclise.[17] In a potential ADEPT application, Liu and Hu showed that certain nitro ester analogs of 2′-deoxy-5-fluorouridine could be reduced by isolated *Escherichia coli* nitroreductase causing spontaneous cyclization and DFU release.[18] However, whole cell reduction by anaerobes may be more likely to produce the amino (6 electron) products.

In order to identify the byproduct of prodrug activation by *C. perfringens* we sought to establish in vitro synthetic conditions that could replicate it. Since 4-electron reduction seemed a likely possibility and since dithionite is reported to produce hydroxylamines from nitroimidazoles, we tried reductions several times with **1** and **2** in the presence of sodium dithionite under various states of oxygen availability and in various solvents (ACN, IPA, H_2O).[19] Unfortunately, these conditions produced as byproducts, prednisolone and oxindole or DHQ, the expected cyclization product from complete reduction of the nitrogroup to amine, indicating that in the presence of sodium dithionite, the last step in the reduction process occurred more rapidly than the cyclization of the hydroxylamine intermediates.

We focussed subsequent efforts to produce a hydroxylamine or its cyclic hydroxamate product on the methyl ester of nitrophenylacetic acid as this was more synthetically accessible than the prodrugs. The methyl ester was treated with $NaBH_4$ in aqueous dioxane in the presence of palladized charcoal, conditions reported to favour the production of hydroxylamines from aromatic nitro compounds, rather than the aniline products. Under these conditions nitrophenyl acetic acid methyl ester produced a major product with identical relative retention time (prednisolone) to the unknown byproduct of activation of **1**. This was identified by NMR and MS as 1,3-dihydro-1-hydroxy-2*H*-indole-2-one, the cyclic hydroxamate resulting from cyclization of the hydroxylamine product of incomplete reduction of the nitro ester.

C. perfringens reduction or biodegradation of aromatic nitro compounds (e.g., trinitrotoluene) has been extensively studied.[20] The reaction may be mediated by ferrodoxin hydrogenase systems rather than by a specific nitroreductase enzyme. The fate of the resultant hydroxylamine would, under colonic conditions, depend on the relative susceptibility of the hydroxylamine to undergo cyclization and the reductive potential for sequential production of the corresponding amine. Clearly in the in vitro model conditions used, the cyclization reaction speed exceeded the rate of amine production. The processing of the compounds could be

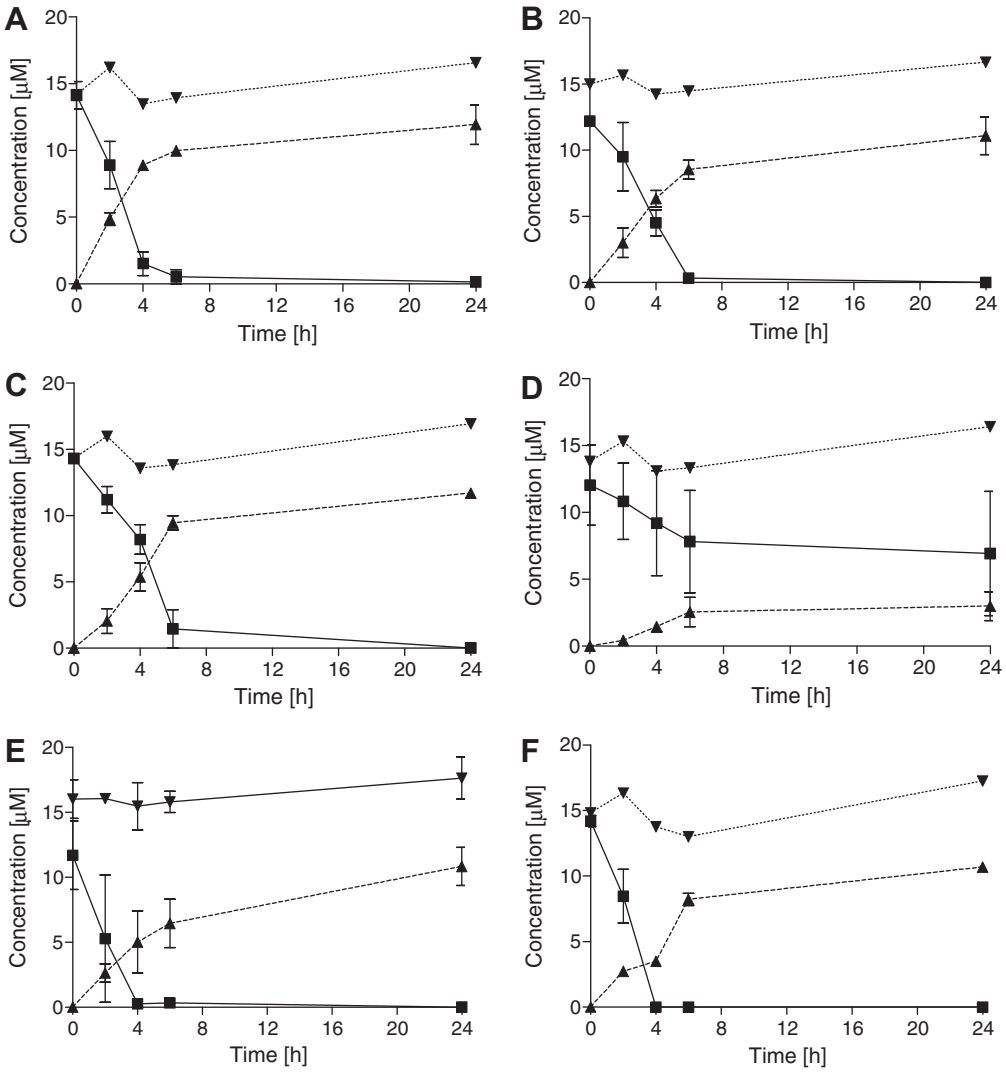


Figure 2. Progress curves following incubation of **1, 2, 3, 4, 5, 6** (■) in suspension of *C. perfringens* under anaerobic conditions at pH 7.4 and 37 °C. (A) **1** (■), prednisolone (▲), and **1** in BHI (▼); (B) **2** (■), prednisolone (▲), and **2** in BHI (▼); (C) **3** (■), budesonide (▲), and **3** in BHI (▼); (D) **4** (■), budesonide (▲), and **4** in BHI (▼); (E) **5** (■), celecoxib (▲), and **5** in BHI (▼); (F) **6** (■), celecoxib (▲), and **6** in BHI (▼) ($n = 3$).

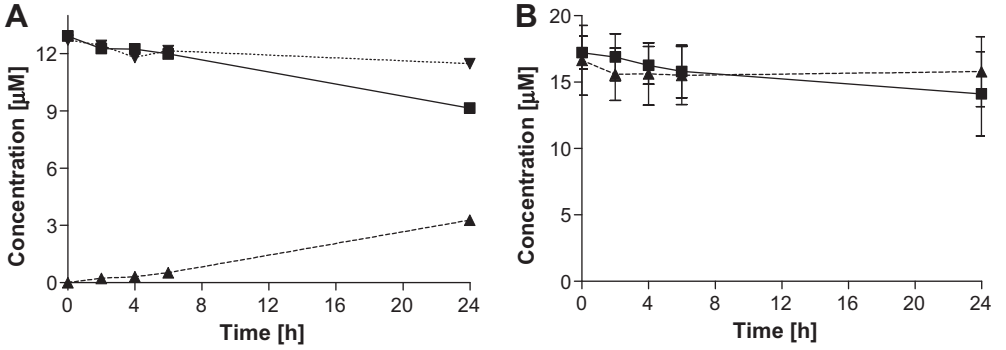


Figure 3. Progress curves following incubation of **7** and **8** (■) in suspension of *C. perfringens* under anaerobic conditions at pH 7.4 and 37 °C. (A) **7** (■), celecoxib (▲), and **7** in BHI (▼); (B) **8** (■) and **8** in BHI (▼)-no prednisolone was observed.

studied in conditions more closely related to the colon to predict byproduct identity in order to characterise potential off-target effects. However the model data taken together with the stability in simulated fluid suggest that the prodrugs would be selectively activated in the colon if able to resist absorption during transit. The potential intestinal permeability of **3**, **4**, **5** and **6** was then assessed using the Caco-2 assay (budesonide results were expected to be relevant to the properties of prednisolone). The transport of budesonide and celecoxib was tested for comparison. These clinically used drugs were at some point selected from amongst their

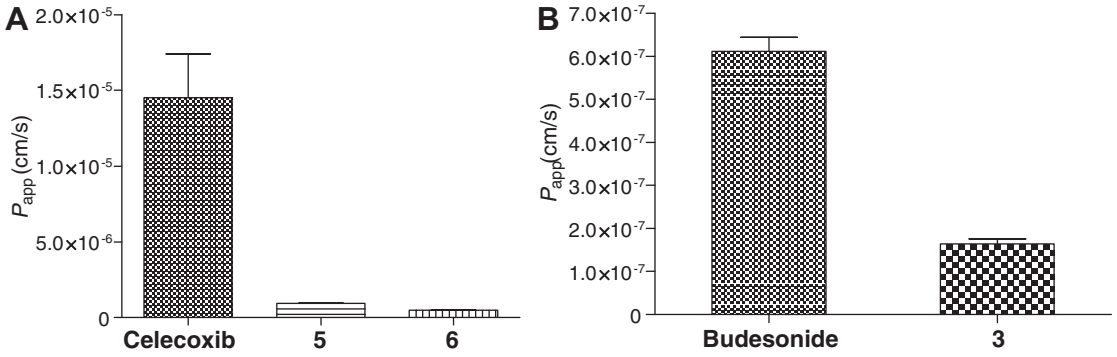


Figure 4. (A) Apparent permeability coefficient of **5**, **6** and celecoxib; (B) Apparent permeability coefficient transport of **3** and budesonide. There was no evidence of transport of **4** under similar conditions.

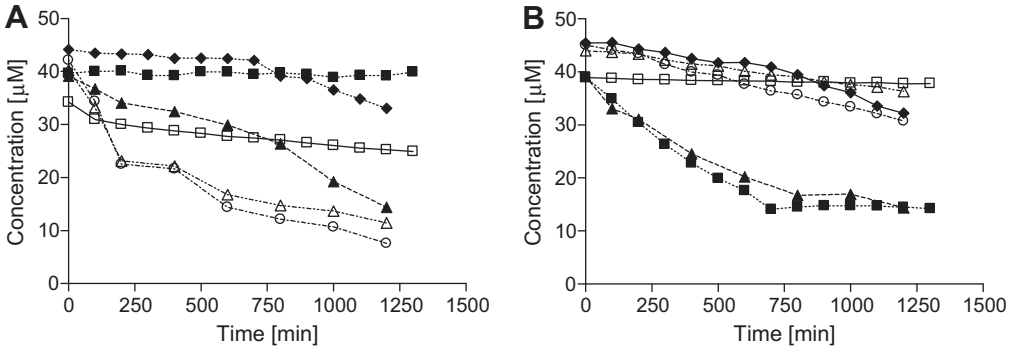


Figure 5. (A) Stability of **1** (■), **2** (Δ), **3** (♦), **4** (○), **5** (□) and **6** (▲) in simulated human gastric juices at 37 °C.[22] (B) Stability of compounds **1** (■), **2** (Δ), **3** (♦), **4** (○), **5** (□) and **6** (▲) in simulated human duodenal fluid at 37 °C.[22]

development rivals in part because of their permeability characteristics. Therefore we were confident that the relatively small structural changes involved in the prodrug design would be sufficient to significantly attenuate passive diffusion potential. Absorptive transport from apical to basolateral side (AP > BL) was evaluated for appearance of the test compound and in the case of the prodrugs, the potential metabolic byproducts. Aliquots were collected after 0, 30, 60, and 120 min from the acceptor reservoir and fresh buffer was added to maintain the initial volume. The donor solution was also collected after 120 min. Caffeine was tested in two wells in the AP > BL direction as a routine control. Trans-epithelial electric resistance (TEER) was over 350 Ω cm^2 in all wells used in the experiments prior to the addition of the test compounds.[21]

Compounds **3**, **5**, **6** possessed significantly lower permeability than the parents celecoxib and budesonide (Fig. 4). No transport of budesonide prodrug **4** could be detected. This data indicates that the compounds would be able to resist absorption and transit the intestinal tract if delivered orally. Nitroreductase-triggered cyclization and drug release may therefore be a generally effective approach to achieving colorectal drug targeting. The prodrugs were processed in the presence of *C. perfingens* present in the human colon, releasing celecoxib, prednisolone or budesonide. The stability of the candidate prodrugs in this study was tested using the USP gastric and intestinal fluid model, which mimics the enzymatic and chemical conditions in the stomach and small intestine.[22] Amounts of remaining compound was assessed using HPLC. The compounds were found to be stable over the expected orocaecal transit time (Fig. 5).[23] Finally, they are predicted by the standard Caco-2 permeability assay to be poorly absorbed relative to their parents suggesting that they might arrive as intact substrates for reductase activity associated with the microflora. Several aspects deserve further study: (i) the identity and biological profile of byproducts of the reduction/cyclization process under simulated colonic conditions; (ii) the potential for elaboration of the linker unit in order to influence (i) as well as pharmaceutical properties such as stability and permeability; the rates of reduction of the nitro group and its electronic and steric control.[24]

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$P_{app} = J/A_sC_0$

Where J is the rate of appearance of the compound on the acceptor compartment (μmol/s), C_0 is the initial concentration on the apical side (mM) as determined by the analysis of the donor solution, and A_s is the surface area of the monolayer (cm^2).
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