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Chapter 3

Precision cut intestinal slices are an appropriate ex vivo model to study NSAID-induced intestinal toxicity in rats

Xiaoyu Niu, Inge A.M. de Graaf, Hendrik A. van der Bij, Geny M.M. Groothuis

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used therapeutic agents, however, they are associated with a high prevalence of intestinal side effects. In this investigation, rat precision cut intestinal slices (PCIS) were evaluated as an ex vivo model to study NSAID-induced intestinal toxicity.

Firstly, PCIS were incubated with 0-200µM diclofenac (DCF), one of the most intensively studied NSAIDs, to investigate whether they could correctly reflect the toxic mechanisms. DCF induced intestinal toxicity in PCIS was shown by morphological damage and ATP depletion. DCF induced endoplasmic-reticulum (ER) stress, mitochondrial injury and oxidative stress were reflected by up-regulated HSP-70 (heat shock protein 70) and BiP (binding immunoglobulin protein) gene expression, caspase 9 activation, GSH (glutathione) depletion and HO-1 (heme oxygenase 1) gene up-regulation respectively. Furthermore, DCF intestinal metabolites, which gave rise to protein adduct but not toxicity, were detected in PCIS.

Secondly, PCIS were incubated with various concentrations of five NSAIDs. Typical NSAID-induced morphological changes were observed in PCIS. The ex vivo toxicity ranking (diflunisal>diclofenac=indomethacin>naproxen>>aspirin) showed good correlation with published in vitro and in vivo data, with diflunisal being the only exception.

In conclusion, PCIS correctly reflects the various mechanisms of DCF-induced intestinal toxicity, and can serve as an ex vivo model for the prediction of NSAID-induced intestinal toxicity.

Key words: NSAID; precision cut intestinal slices; toxicity; metabolism; toxicity ranking; diclofenac
1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs), commonly administered for rheumatic and arthritic diseases (Aithal and Day, 2007), are notorious for their high prevalence of side effects in the small intestine including bleeding, ulceration, inflammation or perforation. NSAID-associated enteropathy is a significant clinical issue due to its high morbidity and mortality rates (Davies et al., 2000, Scarpignato and Hunt, 2010). In the present study, rat precision cut intestinal slices (PCIS) were evaluated for their potential to reflect various reported mechanisms of diclofenac-induced intestinal toxicity, and as an *ex vivo* model to predict NSAID-induced toxicity in rat intestine.

Multiple toxic mechanisms have been reported contributing to the NSAID enteropathy, and these mechanisms are shared by most of the NSAIDs. In the present study, one of the most extensively studied NSAID, diclofenac (DCF), was used as a model compound to test the PCIS system. It has been suggested that the topical effects of DCF in the intestine are the result of a multiple hits pathogenesis involving several factors. When the luminal enterocytes are exposed to DCF, electrophile stress (induced by biliary or intestinal metabolites), endoplasmic reticulum (ER) stress, mitochondrial injury, and oxidative stress are induced, which finally lead to cell death (Mahmud et al., 1996, Boelsterli et al., 2013).

A number of experimental approaches both *in vivo* and *in vitro* have provided evidences for the involvement of multiple mechanisms. *In vivo* studies in rodents indicated that electrophile stress by DCF metabolites formed in the liver and excreted in the bile (Seitz and Boelsterli, 1998, LoGuidice et al., 2012), or intestinal CYP-mediated oxidative metabolism (Zhu and Zhang, 2012), could result in enterocyte protein adduct formation, which were responsible for intestinal ulceration. Other reports suggested that ER stress was involved in NSAID intestinal toxicity, as reduced NSAID enteropathy was found in Chop (CCAAT-enhancer-binding protein homologous protein)-knockout and HSF1 (heat shock transcription factor 1)-null mice (LoGuidice et al., 2010, Asano et al., 2009). NSAID induced mitochondrial changes were observed in the rat intestine by electron microscopy (Somasundaram et al., 1997), and DCF-induced enterocyte demise via the mitochondrial permeability transition pore (mPT) opening was found in mice (Ramirez-Alcantara et al., 2009, LoGuidice et al., 2010). Genetic deletion of Bach1 (BTB and CNC homologue 1), a transcriptional repressor of HO-1 (heme oxygenase 1), fully protected mice from an ulcerogenic dose of indomethacin, indicating the involvement of oxidative stress (Harusato et al., 2011).

More insight of the mechanism was gained by *in vitro* approaches using cell lines or isolated mitochondria from several tissues including rat intestinal epithelium, showing NSAID-induced uncoupling of mitochondrial oxidative phosphorylation, the formation of reactive oxygen species, and mPT pore opening-mediated cell death (Lichtenberger et al.,...
In addition, increased cellular Ca\textsuperscript{2+} concentration, induction of oxidative stress and apoptosis were observed in primary gastric mucosal cells when cultured with NSAIDs (Tanaka et al., 2005, Tsutsumi et al., 2004). Involvement of reactive oxygen species in indomethacin-induced apoptosis was shown in the colon cancer cell line Caco-2 (Omatsu et al., 2009) and in a rat intestinal epithelial cell line (RIE-1) (Omatsu et al., 2010).

Since animal experiments are costly and not in compliance with the 3R (reduction, replacement, refinement) concept, \textit{in vitro} models are needed to study drug-induced enteropathy. The existing \textit{in vitro} cell models are not representative for the intact intestine, each of them only reflects certain isolated aspects of the mechanisms. Therefore, it would be desirable to have a model which can integrate the various aspects of NSAIDs enteropathy (electrophile stress, ER stress, oxidative stress, mitochondrial injury), more closely emulates the intestine, and resembles the \textit{in vivo} situation. Ideally such a model would reflect the multiple toxic mechanisms and be predictive for the potential toxicity of the tested compounds.

PCIS are a multicellular three-dimensional \textit{ex vivo} model. It has been used intensively to study drug metabolism, as well as metabolism inhibition and induction in animals and humans (Martignoni et al., 2006a, Martignoni et al., 2006b, van de Kerkhof et al., 2005, van de Kerkhof et al., 2007, van de Kerkhof et al., 2008, de Kanter et al., 2002, de Kanter et al., 2004, Khan et al., 2009, Chow et al., 2010). Due to the presence of various cell types in an architectural organization, and the preserved function of metabolic enzymes, transporters, and cofactors, this model closely represents the functional intestine. The PCIS model has therefore the potential to study mechanisms of drug-induced intestinal injury. In addition, by the approach to use both human and animal tissue, it helps gaining a better understanding of species-specific toxicity (de Kanter et al., 2004, Niu et al., 2013). Furthermore, slices can be obtained from duodenum, jejunum, and ileum separately, which make it possible to determine the different sensitivities of these different parts of the intestine.

In the present study, the rat PCIS are evaluated as a model for NSAID-induced intestinal toxicity for the first time. First of all, it was examined whether PCIS could reflect the above-discussed mechanisms underlying NSAIDs enteropathy, using DCF as a model compound. Subsequently, the study was extended to four other NSAIDs (diflunisal, indomethacin, naproxen and aspirin). The toxicity was assessed by determining ATP level and cell death as observed microscopically. \textit{Ex vivo} toxicity ranking of these four compounds and diclofenac was compared with published \textit{in vivo} and \textit{in vitro} data.
2. Materials and Methods

2.1 Preparation of rat PCIS

Male Wistar rats, obtained from Charles River (Sulzfeld, Germany), with body weight between 316 g and 422 g, were used in this study. The rats were kept in a temperature and humidity-controlled room with a 12 hours light/dark cycle with food and tap water ad libitum (Harlan Laboratories B.V., Horst, the Netherlands). The Animal Ethics Committee of the University of Groningen approved the experiments.

PCIS were prepared as described in detail by de Graaf (de Graaf et al., 2010). In brief, rats were sacrificed under anesthesia with isoﬂurane/O$_2$. The intestine was preserved in ice-cold oxygenated Krebs-Henseleit buffer (pH 7.4). Jejunum tissue segments were embedded in 3% agarose (Sigma-Aldrich, Steinheim, Germany) in 0.9% NaCl, using a tissue embedding unit. Subsequently, the intestinal segment was sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold Krebs-Henseleit buffer saturated with carbogen (95% O$_2$ and 5% CO$_2$). PCIS (350-450 µm thick and 3-4 mg wet weight) were stored in ice-cold Krebs-Henseleit buffer until incubation.

2.2 Incubation of the PCIS with NSAIDs and metabolism inhibitors

The PCIS were incubated individually in 12 wells plates (Greiner Bio-one GmbH, Frickenhausen, Austria) as described by de Graaf (de Graaf et al., 2010). Each well contained 1.3 ml Williams Medium E (WmE) with Glutamax-I (Gibco, Paisley, UK) supplemented with 25mM D-glucose (Merck, Darmstadt, Germany) and 50 µg/ml gentamicin (Gibco, Paisley, UK) and 2.5 µg/ml fungizone (amphotericin B) (Gibco, Paisley, UK). The plates were placed in plastic boxes and shaken (90 times per minute) in an incubation cabinet at 37°C in an atmosphere of 95% O$_2$ and 5% CO$_2$.

The stock solutions of diclofenac sodium salt, diflunisal, indomethacin, naproxen and aspirin (Sigma-Aldrich, St. Louis, MO, USA) were prepared in dimethyl sulfoxide (DMSO, VWR, Fontenay-sous-Bois, France) and stored at 4°C. The DMSO concentration was the same in the treated groups and their corresponding vehicle controls and kept below 0.5%. The applied concentrations of DMSO in this paper did not affect the viability of the PCIS (results not shown). The slices were incubated with diclofenac (50, 100, 200, 500, 1000µM), diflunisal (50, 100, 500, 1000µM), indomethacin (50, 100, 500, 1000µM), naproxen (0.1, 0.5, 2.5, 5mM) and aspirin (0.1, 0.5, 1, 2, 5, 10mM).

CYP inhibitors ketoconazole and cimetidine or the UGT inhibitor borneol (Sigma-Aldrich, St. Louis, MO, USA) were used in this study to inhibit the DCF metabolism. PCIS were incubated for 5 hours with 200µM DCF in the presence or absence of ketoconazole.
(10µM), cimetidine (5mM) or borneol (0.5mM) respectively. The ATP content in the PCIS was measured after the incubation.

2.3 ATP and protein content of slices

The viability of slices was determined by measuring the ATP content after 5 hours incubation using the ATP Bioluminescence Assay kit CLS II (Roche, Mannheim, Germany) as described previously (de Graaf et al., 2007). The ATP content was corrected by the amount of protein of each slice. The protein content of the PCIS was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Munich, Germany) with bovine serum albumin (BSA, Sigma-Aldrich, Steinheim, Germany) for the calibration curve (de Graaf et al., 2007).

2.4 Caspase 9 assay

The protocol was adapted from the Caspase-Glo 9 assay (Promega. Madison, WI, USA). Three intestinal slices were collected in a safe-lock vial containing 600 µl WmE. The samples were homogenized immediately after sampling by mini-beads-beating for 45 seconds and centrifuged for 2 minutes (4 °C, 16100 rcf). 5 µl sample supernatant was pipetted in one well of a 96 wells plate (Costar, Corning, NY, USA) together with 40 µl Caspase-Glo 9 Reagent (Promega. Madison, WI, USA) and 55 µl WmE medium. Subsequently, the plates were shaken for 2 minutes, incubated for 30 min at room temperature in dark and the luminescence was measured using a luminescence plate reader (LumicountTM, Packard Instrument Company Inc., Downers Grove, IL, USA).

2.5 Glutathione assay

To assess the redox status in the slices, both reduced and total glutathione were measured, as described in detail by Hadi (Hadi et al., 2012). In brief, four slices (from one treatment) were collected into one cup, washed by cold 0.9% NaCl, snap frozen in liquid nitrogen, stored in the -80°C freezer until further use. Each sample was homogenized by mini-beads-beating in 400 µl 50mM Tris-HCl/1mM EDTA buffer, subsequently centrifuged for 2 minutes at 16100 rcf. The pellet was stored for protein measurement. The supernatant was used for the measurement of total glutathione (GSSG+GSH) and reduced glutathione (GSH). For the determination of the total glutathione content, 7.5 µl 20 U/ml glutathione reductase (Sigma-Aldrich, St. Louis, MO, USA) and 15 µl 1mM NADPH (Roche Diagnostics GmbH, Mannheim, Germany) were added to 150 µl supernatant. The mixture was incubated in a water bath (GFL, Burgwedel, Germany) at 37°C for 15 minutes to reduce all the oxidized glutathione (GSSG) into the reduced form. Subsequently, the protein was precipitated by adding 50% w/v trichloroacetic acid (Merck, Darmstadt, Germany) and centrifuged for 5 min at 2000 rcf. Reduced glutathione was determined by adding 200 µl Ellman’s reagent (5,5'-
PCIS are an appropriate model to study NSAID intestinal toxicity
dithiobis-(2-nitrobenzoic acid)) (Sigma-Aldrich, St. Louis, MO, USA) to 50 µl sample supernatant, and the absorbance was measured at 405 nm. Appropriate GSH and GSSG (Sigma-Aldrich, St. Louis, MO, USA) calibration curves were used to calculate the GSH and GSSG concentration. The glutathione levels were corrected by the measured protein amount using Bio-Rad DC Protein Assay (Bio-Rad, Munich, Germany).

2.6 Gene expression

Three slices (from one treatment group) were collected into one cup, snap frozen in liquid nitrogen, and stored at -80°C until further use. RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to manufacturer’s instructions. RNA concentration was measured with a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilminton, DE, USA) at 230 nm. All RNA samples were diluted to a concentration of 2 ng/µl. From 0.5 µg RNA, cDNA was synthesized using the Promega Reverse Transcription System (Promega, Madison, WI, USA). The following solutions were added to 8.16 µl 2 ng/µl RNA: 10 µl MgCl₂ (25mM), 2.5 µl 10 x Reverse Transcription buffer, 2.5 µl dNTP’s (10mM), 0.8 µl random primers (0.5 µg/µl), 0.5 µl RNasin (40 U/µl) and 0.5 µl AMV-Reverse Transcriptase (25 U/µl). The samples were placed in a Mastercycle Gradient (Eppendorf AG, Hamburg, Germany) to start the cDNA synthesis with cycles at 25°C/10 min; 45°C/60 min and 95°C/5 min. Afterwards, the cDNA samples were stored at -20°C. For RT-PCR, 1.25 µl cDNA was pipetted on a 384 wells real-time PCR plate (Applied Biosystems, Carlsbad, CA, USA). Subsequently, 18.8 µl of a mixture containing 0.4 µl forward primer (50µM), 0.4 µl reverse primer (50µM), 8 µl RNase free water and 10 µl SYBR Green mix (Abgene, Epsom, UK), was added to each sample. The plate was covered by an ADI PRISM Optical Adhesive Cover and centrifuged for 5 minutes at 900 rcf. Samples were analyzed with a real-time PCR ABI PRISM® 7900HT Sequence Detection System according to the protocol for real-time PCR with SYBR Green using the SDS 2.4 software (Applied Biosystems, Carlsbad, CA, USA). The following primers were used:

GAPDH: 5'-CGCTGGTGAGTATGTCG -3'; 3'-CTGTGGTCATGAGCCCTTCC -5'.
Hsp-70: 5'- GGTGGCAGTGGCTAGGGTGTT -3'; 3'-GGTGCAAGGCGGTCAGGTT -5'.
HO-1: 5'-CTCGCATGAACACTCTGGAGAT -3'; 3'-GCAGGAAGGCCTGTTAGC -5'.
BiP: 5'-CTGGGTACATTTGATCTGACTGG -3'; 3'-GCATCCCTGGGCTTCCAGCCATTC -5'.

To quantify the expression of the genes the comparative threshold method (Ct) was used. The obtained Ct values of each sample were related to the Ct value of GAPDH (ΔCt), and the Ct value of housekeeping gene GAPDH in the vehicle control group (19.8±0.9) was not changed due to the treatment of DCF 200µM (19.6±0.6). The ΔCt of the treated slices was then compared to the ΔCt of the controls (ΔΔCt). The fold induction was calculated by the formula: $2^{\Delta\Delta Ct}$. 
2.7 Metabolism of DCF

After the incubation, 500 µl medium from each well was collected and acidified by adding 10 µl 2M HCl (Merck, Darmstadt, Germany) for HPLC analysis, and 100 µl was injected into a HPLC. Samples were separated by a 5 µm Hypersil Gold column (150 x 4.6 mm) (Thermo Technologies, Bellefonte, PA, USA) with 25% acetonitrile/0.05 M ammonium formate (pH 6.2) as eluent, 1.0 ml/min. The retention time was 30 minutes. Peaks were identified by UV detection at 282 nm. DCF and its metabolites were identified by adding standard DCF, 4’-hydroxyl diclofenac (4’-OH DCF), 5-hydroxyl diclofenac (5-OH DCF) and acyl glucuronide diclofenac (DAG) as the references and quantified using appropriate standard curves.

2.8 Assessment of drug-protein adducts

Immunohistochemical staining for DCF-related protein adducts was performed by using a rabbit polyclonal antibody (a gift from Prof. N.P.E Vermeulen, University of Amsterdam) raised against a DCF keyhole limpet hemocyanin conjugate as the primary antibody (Pumford et al., 1993). After incubation, the PCIS were collected, washed with WmE and fixed in 4% (w/v) formaldehyde solution for 24 hour at 4°C. Thereafter, PCIS were dehydrated, embedded in paraffin and sectioned (4 µm). After deparaffinization and rehydration, sections were first incubated for 20 min at 95 ºC in citrate buffer (10mM, pH=6) for antigen retrieval. To block nonspecific Fc-receptor binding, sections were incubated with normal goat serum (1:20 dilution in 2% BSA/PBS) for 30 min. Thereafter, the sections were exposed for 60 min to the first antibody (1:8000 dilution). Endogenous peroxidase activity was inhibited by incubation of the sections with 0.3% H2O2 (VWR, Fontenay-sous-Bois, France) in methanol for 30 min. After 20 min of endogenous biotin blocking (Dako, Carpinteria, CA, USA), sections were incubated for 30 min with biotinylated goat anti rabbit IgG (1/200, Dako, Denmark). Second antibody labeled proteins were visualized with a vector stain immunoperoxidase ABComplex kit (Vector Laboratories Inc., USA), following the manufacturer’s instructions. The tissue was then stained with AEC (containing 0.03% H2O2, Sigma-Aldrich, Steinheim, Germany) for 20 min and counterstained in hematoxylin for 1 min. Slides were covered with a cover glass using glycerin/gelatin (Merck, Darmstadt, Germany).

2.9 Morphology and scoring

Three PCIS were collected into one well and fixed in 4% (w/v) formaldehyde solution (Klinipath, Duiven, NL) for 24 hours at 4°C. Thereafter, PCIS were embedded in paraffin and sectioned (4 µm). Hematoxylin (Sigma-Aldrich, Steinheim, Germany) and eosin (Merck, Darmstadt, Germany) staining was performed as described previously (de Graaf et al., 2010). The integrity of the slices was quantified by a scoring method as published by Roskott et al. (Roskott et al 2010). Six aspects including viability and shape (columnar or flat) of the
epithelial cells, intactness of the stroma and crypts, flattening of the villi and damage of the muscle were scored from 0 to 3 (compared to the intestine in vivo). A total score (0-18) was obtained by summarizing these six parameters. An increased score represents decreased integrity of the slice. All samples were evaluated blinded.

2.10 Statistics

Each experiment was performed with a minimum of three rats, using three PCIS for each experimental condition from each intestine. The number of rats used for each experiment is indicated in the figure legend. Multiple comparisons between control group and the treated groups were performed by One-way ANOVA with Dunnett post-hoc correction, in GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, CA, USA). For the normalized gene expression data, one sample t-test was used. A level of confidence of 95% was chosen to denote a significant difference between means.

3 Results

3.1 DCF-induced toxicity in PCIS

PCIS were incubated with 0-200µM DCF for 5 hours. The toxicity of DCF was indicated by morphological changes and ATP depletion. After 5 hours incubation, the intestinal epithelial layer remained intact in vehicle control slices in the absence of DCF. Slices treated with 50µM DCF showed similar morphology as controls. Damaged epithelial lining and aberrant crypts were induced by 200µM DCF (Fig. 1a). A concentration-dependent toxicity of DCF to PCIS was shown by a decrease in ATP content as well. At 200µM concentration, DCF significantly reduced the ATP content compared with the non-DCF-treated vehicle controls (Fig. 1b). Morphological integrity of 200µM DCF treated slices was significantly decreased compared with vehicle treated controls, as indicated by the higher morphology score (Fig. 1c).

3.2 The mechanisms underlying DCF-induced intestinal toxicity reflected in PCIS

PCIS were incubated with DCF at a non-toxic concentration of 50µM or at a toxic concentration of 200µM for 5 hours. Ex vivo biomarkers were used to indicate the induced electrophile stress (reactive metabolites and their protein adducts), mitochondrial stress, ER stress and oxidative stress.

PCIS incubated with 50µM DCF produced a similar amount of metabolites as slices incubated with 200µM DCF (Fig. 2a). Different concentrations of the inhibitors ketoconazole, cimetidine and borneol were incubated with the PCIS, and non-toxic concentrations, which did not have any effect on the ATP, were selected (data not shown). The addition of these
non-toxic concentrations of the metabolism inhibitors ketoconazole (10µM), cimetidine (5mM) or borneol (0.5mM) resulted in a further ATP decrease compared to DCF (200µM) alone (Fig. 2b). The addition of borneol significantly reduced the DAG formation from 0.190±0.004 nmol to 0.035±0.020 nmol in the medium. However, the amount of phase 1 metabolites (4'-OH DCF and 5-OH DCF) was close to the limit of detection, so any further decrease of the formation could not be accurately measured with the HPLC system used. Protein adduct formation was observed in PCIS after immunochemical detection using an anti-diclofenac antibody after exposure to both 50µM and 200µM DCF (Fig. 2c).

Mitochondrial damage was indicated by caspase 9 activation. DCF at a concentration of 200µM significantly induced caspase 9 activity, while the tested non-toxic concentration (50µM) did not influence this biomarker (Fig. 3). ER stress was indicated by significantly up-regulated mRNA expression of HSP-70 (heat shock protein 70) and BiP (Binding immunoglobulin protein) after incubation with 200µM DCF (Fig. 4). Oxidative stress was indicated by GSH depletion, 200µM DCF significantly decreased the reduced glutathione level (Fig. 5a), but did not change the total glutathione level, as oxidized glutathione increased concomitantly (Fig. 5b). In addition the HO-1 gene expression was found to be higher in the DCF 200µM treated group, however, due to the relatively high variation this increase was not significant at the 5% level (the p value is 0.1) (Fig. 5c). A low concentration of 50µM DCF did not influence these parameters except HSP-70 gene expression.

**Fig. 1.** DCF induced toxicity in PCIS. (a) Hematoxylin-eosin (HE) staining showing DCF induced morphological changes in PCIS. N=3 (Three independent rat experiments). The results of a representative experiment are shown. (b) DCF induced concentration-dependent ATP decrease in PCIS. The ATP content of the slices is corrected by the protein content. Data represent the average ± SEM (n≥4). *p < 0.05 in comparison with the control group (incubation 5 hours without DCF). (c) Scoring of DCF-induced morphological changes. Data represent the average ± SEM (n=3). *p < 0.05 in comparison with the control group (incubation 5 hours without DCF).
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Fig. 2. DCF intestinal metabolism and reactive metabolites mediated adduct formation.

(a) Main metabolites formation by PCIS after 5 hours incubation with 50µM or 200µM DCF. (b) The effects of the metabolism inhibitors ketoconazole, cimetidine and borneol on the ATP level in PCIS exposed to 200µM DCF. *p < 0.05 in comparison with the DCF alone group (incubation 5 hours with 200µM DCF). Data represent the average ± SEM (n≥3).

(c) Immunohistochemical (IHC) staining using anti-DCF antibody showing specific drug-protein covalent binding in rat PCIS exposed to 50µM or 200µM DCF (arrow). Three experiments (three rats) were performed, the results of a representative experiment are shown.

Fig. 3. DCF induced mitochondrial stress.
The effect of DCF on the caspase 9 activity in PCIS. Data represent the average ± SEM (n=4). *p < 0.05 in comparison with the control group.
Fig. 4. DCF induced ER stress.
The effects of DCF on HSP-70 (a) and BiP (b) gene expression in PCIS. The mRNA level is normalized by the housekeeping gene GAPDH, and expressed as the fold induction relative to the 5 hours vehicle treated controls. The Ct value in the vehicle control group of HSP-70 is 28.1±0.1, of Bip is 25.1±0.2. Data represent the average ± SEM (n=3). *p < 0.05 in comparison with the control group (incubation 5 hours without DCF).

Fig.5. DCF induced oxidative stress in PCIS.
(a) The effect of DCF on the intracellular glutathione (GSH) level in PCIS. (b) The effect of DCF on the total glutathione (GSX) level in PCIS. (c) The effect of DCF on the heme oxygenase-1 (HO-1) gene expression in PCIS. The mRNA level was normalized by the housekeeping gene GAPDH, and expressed as the fold induction relative to the 5 hours vehicle treated controls. The Ct value of HO-1 in control group is 24.6±0.5. Data represent the average ± SEM (n=3). *p < 0.05 in comparison with the control group.

3.3 *Ex vivo* prediction of NSAID-induced intestinal toxicity by PCIS

PCIS were incubated with five NSAIDs: diflunisal (0.05, 0.1, 0.5, 1mM), indomethacin (0.05, 0.1, 0.5, 1mM), diclofenac (0.05, 0.1, 0.5, 1mM), naproxen (0.1, 0.5, 2.5, 5mM) and aspirin (0.1, 0.5, 1, 2, 5, 10mM) for 5 hours. Intracellular ATP content was measured and expressed as the percentage of vehicle treated control slices. A concentration dependent decrease of ATP was seen in all five NSAIDs treated groups albeit to different levels. The lowest concentrations of four of the NSAIDs (except diflunisal) induced a small increase of ATP levels. The concentrations that induced a 50% reduction of ATP content compared to the
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Vehicle controls was calculated as TC50. Diflunisal decreased the ATP to 50% of the control at 0.18mM. Diclofenac and indomethacin had a similar TC50 of 0.29mM. The TC50 of naproxen was 2.96mM. Aspirin did not reduce the ATP by 50% even above 10mM (Fig. 6).

**Fig. 6.** NSAIDs induced toxicity in PCIS. NSAIDs (diflunisal, indomethacin, diclofenac, naproxen, aspirin) induced concentration dependent decrease of ATP. Data are normalized to the vehicle controls (5 hours incubation without compounds). Data represent the average ± SEM (n≥5).

**Fig. 7.** HE staining showing morphological changes in PCIS after treatment with the various NSAIDs. (a-1) Control slices without incubation. (a-2) Slices incubated for 5 hours with only WmE. (b-1, c-1, d-1, e-1, f-1) Slices treated with non-toxic concentration of diflunisal, indomethacin, diclofenac, naproxen and aspirin. Toxic concentrations of 4 NSAIDs (b-2, c-2, d-2, e-2), except aspirin, induce severe loss of epithelial cells. Aspirin 10mM induces only mild toxicity (f-2). Three experiments (three rats) were performed, the results of a representative experiment are shown.
The toxicity of NSAIDs in PCIS was also shown morphologically by Hematoxylin-Eosin (HE) staining. Slices were incubated with a non-toxic and a toxic concentration of the five NSAIDs (based on the ATP data) (Fig. 7). Control slices without incubation present intact villi (Fig. 7a-1). In slices incubated for 5 hours with only WmE, the epithelial layer is intact, but the villi have flattened somewhat (Fig. 7a-2). Slices treated with a non-toxic concentration of diflunisal (50µM) (Fig. 7b-1), indomethacin (50µM) (Fig. 7c-1), diclofenac (50µM) (Fig. 7d-1), naproxen (0.1mM) (Fig. 7e-1), aspirin (500mM) (Fig. 7f-1) showed similar morphology as the control incubated for 5 hours in WmE. Toxic concentrations of four of the NSAIDs: diflunisal (1mM) (Fig. 7b-2), indomethacin (1mM) (Fig. 7c-2), diclofenac (0.5mM) (Fig. 7d-2), naproxen (5mM) (Fig. 7e-2), induced severe loss of epithelial cells. Aspirin induced only mild toxicity at the highest concentration of 10mM (Fig. 7f-2).

Toxicity rankings of the five NSAIDs were compared between the obtained ex vivo data (based on TC50) and published in vivo and in vitro data. In vitro data were obtained from published studies where NSAIDs were incubated with rat hepatocytes, yeast or rat liver mitochondria (Somasundaram et al., 1997, van Leeuwen et al., 2012, Jurima-Romet et al., 1994, Banos and Reyes, 1989). In vivo rankings were obtained from data on intestinal ulcer formation in rats (Wax et al., 1975, Brune et al., 1987). Ex vivo toxicity ranking was in line with the in vitro data. Good accordance was observed when PCIS ranking was compared with in vivo ranking, with diflunisal as an exception. Diflunisal is the most toxic NSAID ex vivo and in vitro, but one of the weaker toxicants in vivo (Table 1).

### Table 1 Comparison of ex vivo, in vivo and in vitro toxicity ranking of five NSAIDs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>TC50 (mM)</th>
<th>Ranking ex vivo</th>
<th>Ranking in vitro</th>
<th>Ranking in vivo</th>
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<td>1</td>
<td>4</td>
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<tr>
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<td>2</td>
<td>1</td>
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<tr>
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<td>3</td>
<td>2</td>
</tr>
<tr>
<td>naproxen</td>
<td>2.96</td>
<td>4</td>
<td>4</td>
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Ex vivo toxicity ranking in the present study is based on the TC50 values calculated from the ATP reduction curve. In vitro ranking was obtained from the observations in yeast, liver mitochondria and hepatocyte. In vivo ranking was obtained from the ulcer formation data in rats.
4 Discussion

Intestinal toxicity induced by NSAIDs is the result of various complex toxicological mechanisms involving many cellular targets (Boelsterli et al., 2013). Moreover, metabolism and transport strongly determine the cellular exposure to these agents and therefore are important determinants of NSAID toxicity. As a consequence, the evaluation of NSAID toxicity \textit{in vitro} requires a model that is competent in metabolism and transport.

PCIS contain all cell types that are present in the intestine, in their natural coherence with the extracellular matrix and neighboring cells (de Graaf et al., 2010). This model has been shown to maintain metabolic activity and exhibit stable transporter expression during culturing (Groothuis and de Graaf, 2013). For these reasons, it has the potential to serve as an adequate \textit{in vitro} model to evaluate NSAID toxicity. In this study we investigated whether PCIS could be a representative \textit{ex vivo} model to study NSAID-induced toxicity, reflecting the various toxicological mechanisms.

4.1 PCIS as an \textit{ex vivo} model for DCF-induced intestinal toxicity study

First of all, DCF induced a concentration dependent decrease of ATP in PCIS, a significantly reduced ATP content was shown when the concentrations reached 200µM, and massive cell death was observed in H&E stained sections. In rats, the ulcerogenic dose of DCF \textit{in vivo} was reported to be 1.5-50 mg/kg body weight (Seitz and Boelsterli, 1998). Assuming a luminal volume of 11 ml (Davies and Morris, 1993), the \textit{in vivo} luminal concentration can be estimated to be 0.1-3.6mM, which is in the range of the \textit{ex vivo} concentrations that were used in the present study. We chose 5 hours as the incubation time due to the following reasons: first of all, intestinal slices remain viable (ATP levels and morphology) and fully functional with respect to phase 1 and phase 2 metabolism at least during 5 hours incubation (van de Kerkhof et al., 2007). Secondly, our pilot experiments showed that DCF induced an ATP decrease after one hour incubation, while other parameters such as caspase activation and gene expression up-regulation were observed only after 2.5 hours and increased thereafter. Finally, the exposure time in the intestinal slices should be comparable with the \textit{in vivo} situation. Due to the enterohepatic circulation, the DCF intestinal exposure time \textit{in vivo} in rats after an oral dose is estimated to be longer than the transit time (about 100 min) (Quini et al., 2012). Therefore we concluded that 5 hours incubation is long enough to observe the DCF toxicity, and short enough to maintain the viability and functionality of the rat intestinal slices.

Since energy depletion influences all cellular processes, ATP decrease often serves as an indicator for cell viability in \textit{in vitro} assays. When tested in hepatocytes, all the toxic NSAIDs depleted ATP, whereas the non-toxic ones did not (Masubuchi et al., 1998). Besides, diclofenac-induced depletion of ATP was the most sensitive indicator for cell death among
depletion of GSH, lipid peroxidation or [Ca^{2+}] changes in hepatocytes (Ponsoda et al., 1995). In vivo, DCF-induced ulcer formation in rat intestine was characterized by focal destruction and necrosis of villi, extending to the submucosa and muscularis mucosae (Satoh et al., 2014, Ramirez-Alcantara et al., 2009). Similar morphological changes were observed ex vivo. DCF induced necrosis of the epithelial cells at 200µM, while the villi and crypts were also partly destroyed.

From these data we conclude that PCIS as an ex vivo model reflect the observed toxicity of DCF in vivo.

### 4.2 PCIS reflect the mechanisms of DCF-induced intestinal toxicity

Electrophile stress, oxidative stress, ER stress and mitochondrial damage have been suggested to be the most important toxicodynamic determinants of the topical effects of DCF-induced intestinal toxicity in rats. In the present study, PCIS were incubated with a non-toxic concentration (50µM) and a toxic concentration of DCF (200µM), to investigate whether these mechanisms could be reflected in PCIS using ex vivo biomarkers.

**Electrophile stress**

DCF metabolism in rats in vivo occurs predominantly by glucuronidation (DAG formation) and oxidative biotransformation by Cytochrome P450 (hydroxyl DCF formation). Its electrophile stress is due to protein adduct formation and/or GSH conjugation of reactive metabolites. DAG has appeared to be responsible for the protein adduct formation in the liver (Kretz-Rommel and Boelsterli, 1993, Hargus et al., 1994, Wade et al., 1997). In addition, oxidative metabolism into 4’-OH DCF and 5-OH DCF results in the formation of the electrophilic intermediates diclofenac-2, 5-quinone imine and diclofenac-1′,4′-quinone imine (Tang et al., 1999, Tang, 2003), which can be conjugated with GSH and contribute to electrophile stress.

In the present study, we report for the first time that the three main metabolites 4’-OH DCF, 5-OH DCF and DAG, formerly shown to be produced by the liver, are also generated by the rat intestine itself. In addition, protein adduct formation was detected in the rat PCIS. However, the data indicate that these metabolites were not the inducer of the observed toxicity. First of all, when the slices were exposed to DCF in the presence of non-toxic concentrations of the metabolic enzyme inhibitors ketoconazole, cimetidine or borneol, enhanced toxicity was observed by further reduced ATP level. Reduced DAG formation was found in the presence of borneol, but reduction in phase I metabolites could not be detected due to limitation of the analytical method. Secondly, DCF 50µM produced similar amount of metabolites as the DCF 200µM. If the metabolites are the cause of the toxicity, we would
expect that more metabolites were formed at 200µM. Similar results were also found with human PCIS (Niu et al., 2014).

**Mitochondrial injury**

Mitochondrial injury is considered as another important component of the mechanisms underlying NSAID intestinal toxicity. DCF was reported to be an uncoupler of oxidative phosphorylation in the mitochondria, which can cause the dissipation of the mitochondrial inner transmembrane potential and trigger the opening of the mPT pore (Boelsterli et al., 2013). DCF induced caspase 9 activation was shown in hepatocytes (Gomez-Lechon et al., 2003) and melanoma cells (Albano et al., 2013). The functional studies on the intestinal mitochondrial injury were performed mostly in isolated liver mitochondria, due to the difficulty to get sufficient yield of mitochondria from the intestine. In the present study, we are able to assess mitochondrial damage in the intestine by measuring caspase 9 activity in PCIS. Significant caspase 9 activation induced by 200µM and not by 50µM DCF was shown in PCIS, which indicated DCF induced intestinal mitochondrial stress in the PCIS.

**ER Stress Response**

NSAID- induced ER stress was shown in mice, as BiP expression, a marker for ER stress (Lee, 2005), was highly increased after administration of DCF (100mg/kg, ip) (Ohyama et al., 2012). Also the induction of the expression of HSP-70 was shown as a response to ER stress (Gupta et al., 2010). In the present study, we show that 200µM DCF induced a significant up-regulation of BiP and HSP-70 gene expression, which revealed an ER stress response due to exposure to toxic concentrations of DCF.

**Oxidative stress**

HO-1 is a stress response protein and up-regulated HO-1 expression is used as an indicator for cellular oxidative stress (Takagi et al., 2008). It was reported that the NSAID indomethacin induced up-regulation of HO-1 mRNA and proteins in the small intestine in rodents in vivo. In the present study, HO-1 gene expression was up-regulated by exposure to 200µM DCF, indicating induced oxidative stress in PCIS.

In addition, 200µM DCF significantly reduced the GSH content of the PCIS by 50% compared to vehicle controls, indicating oxidative stress. The GSH is possibly scavenged by reactive oxygen species, since DCF was shown to inhibit mitochondrial complex I activity (Sandoval-Acuna et al., 2012), leading to increased superoxide production. In this case, GSH is oxidized into GSSG and the total glutathione remains constant. Another explanation for the GSH reduction could be the GSH conjugation with the electrophilic quinone imine.
intermediates after phase 1 metabolism of DCF. However, this seems less likely as it would have resulted in a reduction of total glutathione as well, which was not observed in the slices.

4.3 *Ex vivo* toxicity ranking

Since it was confirmed that the major toxicological pathways of DCF-induced injury were functional in PCIS, the study was extended with another four NSAIDs: diflunisal, indomethacin, naproxen and aspirin, which were all related to intestinal side effects (Stone et al., 1977, Tibble et al., 2000, van der Vijver et al., 2013, Watari et al., 2013, Kurokawa et al., 2013, Mizukami et al., 2011). Assuming NSAID-induced toxicity in the intestine is directly due to their local exposure, we hypothesized that PCIS could predict their toxicity *ex vivo*.

In the present study, the five NSAIDs induced a dose-dependent decrease of ATP content in PCIS. Interestingly, all these compounds, except diflunisal, showed an increase of the ATP content at low concentrations. A possible explanation for this hormetic response could be the anti-inflammatory effect of the NSAIDs, which may inhibit the inflammatory response that was induced during the slice preparation (unpublished data).

In our experiments, diflunisal appeared to be the most potent NSAID depleting the ATP content to 50% of the controls at 0.18mM. Indomethacin and diclofenac showed similar toxicity, with a TC50 value of 0.29mM. Naproxen induced mild toxicity to PCIS, and a lower TC50 of 2.96mM was found. Aspirin did not decrease ATP to 50% of controls even at the concentration of 10mM. Histomorphological examination of slices confirmed the ATP data.

To a large extent, the toxicity ranking found in PCIS was in agreement with the ulcer induction potency of these compounds *in vivo* (Table 1). The doses which induced intestinal ulcer in more than 50% of the tested rats were: aspirin (300 mg/kg) > diflunisal (200 mg/kg) > naproxen (80 mg/kg) > diclofenac (30 mg/kg) > indomethacin (10 mg/kg) (Wax et al., 1975, Brune et al., 1987). Indomethacin and diclofenac are the most potent ulcerogens *in vivo*, whereas they also appeared to be the most toxic NSAIDs in PCIS. Aspirin and naproxen have comparatively low ranking both in PCIS and *in vivo*. Diflunisal was an exception, low toxicity was observed *in vivo*, whereas it was the most toxic NSAID in PCIS.

*In vitro* toxicity rankings of these NSAIDs in yeast (van Leeuwen et al., 2012), isolated liver mitochondria (Somasundaram et al., 1997, Banos and Reyes, 1989) and rat hepatocytes (Jurima-Romet et al., 1994) were in line with what was found in PCIS. The most toxic NSAID was diflunisal in all the *in vitro* systems.

Factors involved in NSAIDs toxicity only *in vivo* but not *in vitro* may contribute to the discrepancy of the diflunisal toxicity found *in vitro* and *in vivo*. For example, enterohepatic recirculation plays an important role in the NSAID enteropathy *in vivo* by prolonging the
intestinal exposure. A correlation between biliary excretion of NSAIDs and ileal perforations in rats was reported (Brune et al., 1987). However, this does not explain the low diflunisal toxicity in vivo, as it undergoes higher enterohepatic circulation in rats than diclofenac or indomethacin, but provokes less toxicity (Beck et al., 1990). The capacity of impairing platelet aggregation, resulting in prolonged ulcer bleeding, was also suggested to attribute to NSAIDs enteropathy. Banos et al. showed dose-response alteration of platelet function by ten NSAIDs (Banos and Reyes, 1989). However, the potency of these NSAIDs to impair platelet aggregation cannot explain the discrepancy between our ex vivo ranking and the in vivo ranking either, as diflunisal showed less impairment of platelet aggregation than indomethacin and more than diclofenac in vivo. It should be noticed that potency for impairment of platelet aggregation also does not explain the in vivo toxicity ranking. Further studies are needed to reveal why diflunisal is much more potent in vitro than in vivo.

4.4 Conclusion

In conclusion, PCIS from rat intestine is a proper ex vivo model to study NSAID-induced intestinal toxicity. First of all, it reflects the different aspects of the multi-hits mechanism underlying DCF-induced intestinal toxicity. Moreover, the potency to produce both DCF phase I and phase II metabolites was shown for the first time in rat intestine, and DCF induced protein adduct formation was also found in PCIS. Furthermore, the toxicity ranking of five NSAIDs was in large agreement with previously published in vitro and in vivo ranking, with an exception of diflunisal. Diflunisal was considerably toxic in PCIS as in other in vitro systems but the least toxic one in vivo.

Since PCIS can be made from each region of the intestine, it offers the possibility to study drug-induced toxicity in different intestinal regions. Moreover it opens the possibility to study human specific toxicity using PCIS prepared from human intestinal tissue (de Graaf et al., 2010, Niu et al., 2014). Such an ex vivo model therefore may contribute to reduce animal experiments and may help to achieve a safer administration of new drugs to man.

References


PCIS are an appropriate model to study NSAID intestinal toxicity


