Meta-analysis of the turnover of intestinal epithelia in pre-clinical animal species and human

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Abbreviations:

$A_{CYP3A}$: Relative activity of CYP3A4 enzyme

ACAT: Advanced compartmental absorption and transit

ADAM: Advanced dissolution absorption and metabolism

BrdUrd: Bromodeoxyuridine

CYP: Cytochrome P450

DDI: Drug-drug interaction

GI: Gastrointestinal

GM: Geometric mean

GSD: Geometric standard deviation

LI: labelling index

$k_{deg}$: Enzyme turnover rate

MBI: Mechanism-based inhibition

MIc: Cells in mitotis

PBPK: Physiologically-based pharmacokinetics

SD: Standard deviation

SFM: Segregated flow model

SS: Sum of squares

t: Time

$T_c$: Cell cycle time

$T_{\alpha}$: Turnover time

$T_S$: Duration of S-phase

WX: Weighted mean

X: Mean
Abstract

Due to the rapid turnover of the small intestinal epithelia, the rate at which enterocyte renewal occurs plays an important role in determining the level of drug metabolising enzymes in the gut-wall. Current physiologically-based pharmacokinetic (PBPK) models consider enzyme and enterocyte recovery as a lumped first-order rate. An assessment of enterocyte turnover would enable enzyme and enterocyte renewal to be modelled more mechanistically. A literature review together with statistical analysis was employed in order to establish enterocyte turnover in human and pre-clinical species. A total of 85 studies were identified reporting enterocyte turnover in 1,602 subjects in six species. In mice, the weighted combined geometric mean (WX) enterocyte turnover was 2.81±1.14 days (n=169). In rats, the weighted arithmetic mean enterocyte turnover was determined to be 2.37 days (n=501). Human exhibited a WX enterocyte turnover of 3.48±1.55 days for the gastrointestinal (GI) epithelia (n=265), displaying comparable turnover to that of Cytochrome P450 enzymes in vitro (0.96-4.33 days). Statistical analysis indicated human to display longer enterocyte turnover as compared to pre-clinical species. Extracted data was too sparse to support regional differences in small intestinal enterocyte turnover in man despite being indicated in mouse. The utilisation of enterocyte turnover data, together with in vitro enzyme turnover in PBPK modelling may improve the predictions of metabolic DDIs dependent on enzyme turnover (e.g. mechanism-based inhibition and enzyme induction) as well as absorption of nanoparticle delivery systems and intestinal metabolism in special populations exhibiting altered enterocyte turnover.
Introduction

Physiologically-based pharmacokinetic (PBPK) models of oral drug absorption and gut-wall metabolism may be implemented at varying degrees of complexity from a simple first-order rate of absorption into the enterocytes located in the gut-wall to more sophisticated segmented models of the intestinal tract, such as the advanced compartmental absorption and transit (ACAT) model and the advanced dissolution absorption and metabolism (ADAM); allowing the incorporation of regional variations in permeability, mucosal volumes, metabolic capacity and more (Agoram et al., 2001; Darwich et al., 2010). PBPK modelling of drug bioavailability allows the estimation of the gut-wall extraction and further implementation to accommodate mechanistic prediction of drug-drug interactions (DDIs) in the small intestine, including: Reversible inhibition, mechanism-based inhibition (MBI) and enzyme induction (Fahmi et al., 2009; Rowland Yeo et al., 2010).

Dynamic modelling of intestinal metabolism requires knowledge of the level of enzyme in the gut-wall where the amount at steady state is a product of the rate of synthesis and degradation ($k_{\text{deg}}$). Enzyme turnover plays an important role in model-based predictions of MBIs and enzyme induction in the gut, where the reliance upon $k_{\text{deg}}$ becomes especially apparent for drugs exhibiting high small intestinal metabolism and for substrates of cytochrome P450 (CYP) 3A (Yang et al., 2008). Current PBPK models utilise a lumped first-order enzyme turnover rate to describe the combined turnover of enzyme and enterocytes. Lumping $k_{\text{deg}}$ of the enzymes and the enterocytes does not consider the difference in dynamics between these two where steady state levels of the enzyme will be disrupted following MBI whereas the enterocyte steady state levels may not. Widely utilised values of $k_{\text{deg}}$ in the area of PBPK tend to be informed via indirect measures, such as clinical MBI.
studies or grapefruit juice studies, where estimates $k_{\text{deg}}$ can vary up to four-fold (0.02-0.08 h$^{-1}$) (Takanaga et al., 2000a; Takanaga et al., 2000b; Yang et al., 2008).

In order to give an indication of the impact of the lumped $k_{\text{deg}}$ on predictions of the level of MBI simulations where carried out using the Simcyp Simulator v13 (Simcyp Ltd, Sheffield, UK) minimal PBPK model. Predictions of level of MBI were performed in the gut-wall following five 60 mg doses of oral diltiazem (dose interval of 8h), as per Rowland Yeo et al. (2010), varying $k_{\text{deg}}$ between 0.01 and 0.05h$^{-1}$ for CYP3A4 in the gut wall, resulting in around 20-30% variation in the activity of CYP3A4 at 1 and 24h post diltiazem dosing (Figure 1). Thus illustrating the importance of a well-characterised $k_{\text{deg}}$ parameter.

Where earlier research has characterised the indirect and in vitro enzyme turnover utilising meta-analysis no similar efforts have been made to determine enterocyte turnover (Takanaga et al., 2000a; Takanaga et al., 2000b; Yang et al., 2008). Physiologically, the enterocytes are produced through cell division of progenitor stem cells in the crypt at the base of the villi in the intestinal tissue. Matured enterocytes will migrate up the crypt-villous axis where the turnover will be governed by apoptosis or shedding into the gut lumen at the tip of the villi. The time from enterocyte generation to shedding has been reported to occur within days after proliferation (Wilson and Potten, 2004; Malato et al., 2011).

Having reliable estimates of enterocyte turnover may not only impact predictions in the area of DDIs but may also further aid the predictions of oral absorption of nanoparticle drug delivery systems subject to trapping in the enterocytes, where intestinal shedding may act to limit their absorption (Ensign et al., 2012).
The aim of the current study was to conduct a comprehensive literature review and meta-analysis of published studies in order to assess the enterocyte turnover in pre-clinical species and human and to identify the most commonly utilised methods for determining enterocyte turnover. Further simulation-based sensitivity analysis was to be employed in order to test the sensitivity of the level of enzyme turnover on the level of MBI.
Material and Methods

A comprehensive literature search was performed in order to identify published data on direct determination of gastrointestinal (GI) enterocyte turnover, lifespan, cell cycle or migration rate in healthy adults in preclinical species (including: rat, mouse, pig, guinea pig, rabbit and hamster) as well as methods utilised to determine the turnover using PubMed (1950 to November 2013). The search for human data was extended to include enterocyte turnover in patient populations due to the sparcity of data. Additional sources were identified through publication citations for all species.

Data was analysed using descriptive statistics, calculating weighted arithmetic means (WX). Where data allowed, the geometric mean (GM) and standard deviation (GSD) of the enterocyte turnover was calculated. Statistical analysis was carried out using Welch’s t test ($P<0.05$) with post-hoc Dunn-Šidák correction where applicable in Matlab® R2010a (Mathworks, Natick, USA) (Supplemental Methods).
Results

Overall, a total of 85 studies where identified reporting enterocyte turnover in 1,602 subjects in six different species. In 84% of the studies determination of enterocyte turnover was carried out using isotope labelling, including $^3$H-thymidine and BrdUrd, the remaining 16% were carried out using mitotic arrest methods and biopsy analysis. The outcome of the data analysis of enterocyte turnover follows below.

Methods for determining the turnover of enterocytes

Various in vivo and in vitro methods were identified for the determination of enterocyte turnover. The GI epithelia differ from other physiological cell lines as it is a highly organised tissue, allowing the study of cell migration within the tissue (Wilson and Potten, 2004).

Isotope labelling methods

The most commonly used method for measuring enterocyte turnover was the ‘pulse-chase’ method, where DNA of a population of cells is labelled with an isotopic nucleoside, such as $^3$H-thymidine, or a synthetic analogue, such as bromodeoxyuridine (BrdUrd). Following administration of the isotopic nucleotide the label will incorporate into the DNA during the synthetic phase (S-phase). The cell line can be monitored post labelling using autoradiography, allowing quantitative determination of the different phases (Quastler and Sherman, 1959; Creamer, 1967; Scragg and Johnson, 1980).

With the knowledge of the duration of the S-phase ($T_S$) and the labelling index of the cell population (LI), the turnover of a cell line can be determined in vitro or in vivo, where turnover time ($T_\alpha$) is equal to the ratio of $T_S$ and LI (Equation 1) (Scragg and Johnson, 1980).
The enterocyte turnover can be determined by estimating migration time of cells from the villous-crypt junction to the tip of the villi if their height has been determined. The migration time is therefore equal to the enterocyte lifespan or turnover (Leblond and Stevens, 1948).

*Mitotic arrest methods*

The mitotic arrest method involves stathmokinetic agents (such as: colchine, colcemid, vinblastine or vincristine) arresting cells in the metaphase entering mitosis. This will result in an accumulation of mitotic figures, allowing the determination of number of cells in metaphase by histological examination. Mitotic arrest can be used *in vitro or in vivo* by administering the blocking agent via intravenous or intraperitoneal injection (Scragg and Johnson, 1980). Cell production rate can be determined from the relation between the number of cells in metaphase against time. The relation can be used to estimate the cell cycle times defined as the invert relation of the rate of cells entering mitosis (MIL/t) (Equation 2).

\[
T_c = \frac{1}{MIL/t}
\]

Equation 2

Additionally, the villous transit time can be determined as a ratio between the villous population and the cell influx per villous (Equation 3) (Al-Nafussi and Wright, 1982).

\[
\text{Transit time} = \frac{\text{Villous population}}{\text{Cell influx/villus}}
\]
Equation 3

Cytophotometric methods
Cytophotometric methods involve staining of the cell line DNA using dyes, such as ethidium bromide, binding specifically to DNA and fluoresce with an intensity proportional to the amount of bound DNA. This allows the different cell cycle states to be quantitatively determined along with the proliferative index of a population (Scragg and Johnson, 1980).

Enterocyte turnover in pre-clinical species
The literature search of enterocyte turnover in healthy adult subjects of pre-clinical species identified a total of 68 studies (n subjects=1,337). In the rat, 24 studies consisting of 501 subjects were identified (Supplemental Table 1); where the WX GI enterocyte turnover was determined to be 2.37 days. Segmental turnover of the small intestinal epithelia were within close proximity of each other, with WX of 1.89, 2.22 and 1.40 days, in the duodenum, jejunum and ileum respectively. The colon and stomach appeared to display longer turnover as compared to the small intestine with WX of 2.76 and 2.84 days respectively. Reported standard deviations were limited to two radiographic studies of the duodenum and jejunum. The weighted GM and GSD of these reports produced an enterocyte turnover of 2.76 (±1.68) days (n rats=14). Due to the scarcity in reported variance statistical verification of any regional differences was limited, albeit reported turnover in the duodenum (1.20±0.20 days, n=4) and jejunum (3.95±0.54 days, n=10) displayed a statistically significant difference (P<0.05) (Figure 2) (Leblond and Stevens, 1948; Stevens and Leblond, 1953; Bertalanffy, 1960; Loran and Althausen, 1960; Messier, 1960; Bertalanffy and Lau, 1962; Koldovsky et al., 1966; Altmann and Enesco, 1967; Shambaugh et al., 1967; Tutton and Barkla, 1976; Sunter et al., 1978; Sunter et al., 1979; Menge et al., 1982; Holt et al., 1983; King et al.,
In the mouse, a total of 31 studies consisting of 651 healthy adult mice were identified reporting the enterocyte turnover in the small intestinal epithelia (Supplemental Table 2). The weighted GM enterocyte turnover for the GI tract was 2.81 (±1.14, n=169 mice) days. The duodenum, jejunum, ileum and colon displayed enterocyte turnovers of 2.83 (±1.06, n=40), 2.97 (±1.05, n=35), 2.56 (±1.05, n=35) and 2.67 (±2.04, n=38) days respectively. All small intestinal segments differed at a statistically significant level from each other (P<0.05), whereas colonic turnover was not significantly different compared to that of the small intestine due to high combined variability (CV=76%; P>0.05). Gastric epithelial turnover displayed an WX of 3.05 (n=19) days, lacking reported variance (Figure 3) (Leblond and Messier, 1958; Walker and Leblond, 1958; Quastler and Sherman, 1959; Creamer et al., 1961; Fry et al., 1961; Lesher et al., 1961; Fry et al., 1962; Lipkin and Quastler, 1962; Thrasher, 1967; Grey, 1968; Merzel and Leblond, 1969; Aluwihare, 1971; Chang and Leblond, 1971; Bottomley and Cooper, 1973; Kovacs and Potten, 1973; Cheng and Leblond, 1974; Potten et al., 1974; Chang and Nadler, 1975; Richards, 1977; de Rodriguez et al., 1979; Tsubouchi, 1981; Baril et al., 1982; Cheng and Bjerknes, 1983; Smith et al., 1984; Lee, 1985; Thompson et al., 1990; Ferraris et al., 1992; Karam and Leblond, 1993a; Karam and Leblond, 1993b; Nowacki, 1993).

Enterocyte turnover in human

The literature search for human enterocyte turnover data identified 17 studies, with a total sample size of 265 individuals (Supplemental Table 3), reporting the turnover of human GI
epithelial cells in the form of mean and standard deviation (n=86), mean only (n=153) and ranges (n=26). The majority of turnover values were from colonic, rectal (n=157), duodenal (n=60) and gastric epithelial cells (n=36), whereas jejunum and ileum was limited to a sample size of 3 and 9, respectively. A weighted GM turnover of 3.48 (±1.55) days was obtained for the whole GI epithelia (n=86). Analysis of independent GI segments identified a weighted GM of 1.50 (±2.90, n=3) days for the duodenum, 2.83 (±1.60, n=30) days for the stomach and 4.12 (±1.32, n=53) days for the colorectal region. The stomach displayed a statistically faster turnover as compared to the colorectal region utilising post-hoc test (P<0.05) (Figure 4) (Bertalanffy and Nagy, 1961; Cole and Mc, 1961; Deschner et al., 1963; Lipkin et al., 1963a; Lipkin et al., 1963b; Macdonald et al., 1964; Shorter et al., 1964; Shorter et al., 1966; Bell et al., 1967; Lipkin, 1969; Bleiberg et al., 1970; Weinstein, 1974; Bleiberg and Galand, 1976; Wright et al., 1977; Potten et al., 1992; Patel et al., 1993; Bullen et al., 2006).

**Summary results on enterocyte turnover**

Data on GI enterocyte turnover was further identified for healthy adult rabbits, guinea pigs and hamsters, albeit being sparse. The WX enterocyte turnover in rabbit, guinea pig and hamster corresponded to 3.40 (n=20 rabbits), 2.08 (n=68) and 1.37 (n=29) days respectively. Statistical analysis identified mouse enterocyte turnover to be significantly shorter as compared to human (P<0.05) (Figure 5) (Sawicki et al., 1968; Sawicki and Rowinski, 1970; Rowinski and Sawicki, 1972; Hattori and Fujita, 1976; Cremaschi et al., 1982; Cremaschi et al., 1984; Smith et al., 1984; Cremaschi et al., 1986; Grant and Specian, 2001).

**Discussion**

Turnover of the GI epithelia was established based on a large sample size in rats, mice and human, and based on sparse data in rabbits, guinea pig and hamster. Statistical analysis
indicated a shorter enterocyte turnover in preclinical species as compared to human, where the mouse displayed a significantly shorter turnover time. The limitation of data did however not allow a conclusive analysis for the remaining species where human turnover was mainly derived from disease populations.

Statistical analysis of regional differences in enterocyte turnover indicated the ileum to display the fastest turnover, followed by the duodenum and jejunum in mouse. A similar trend was indicated in rat although a majority of the data lacked variance and could therefore not be determined statistically. Human data on GI epithelial turnover identified the colorectal cell renewal to be slower as compared to the stomach, whereas data from the small intestinal regions were too sparse in order to observe any regional differences. Additional indications of regional differences in enterocyte turnover include observations from neonatal pig, where the distal small intestine displayed a slower turnover (10.2±1.5 days, n=16) as compared to the proximal region (4.7±0.4 days, n=16) (Fan et al., 2001). In summary, evidence for regional differences in enterocyte turnover in man is inconclusive which would favour the utilisation of a single parameter value of 3.48 (±1.55) days for the intestine. Generation of further human enterocyte turnover data may allow the utilisation of regional differences in enterocyte turnover, giving a more true depiction of the enzyme renewal along the small intestine. This may be of importance in the prediction of MBIs where the perpetrator or victim drug display a short absorption window and an overall average enterocyte turnover is not representative of the intestinal segment in question.

Several factors have been identified to alter the turnover of the enterocytes, and may therefore account for variability seen in the data set, these factors include the age of the subjects, where several publications have reported slower enterocyte turnover in neonatal or
infant pigs, guinea pigs, rats, mice and hamsters as compared to adults (Creamer et al., 1961; Koldovský et al., 1966; Grey, 1968; Rundell and Lecce, 1972; Al-Nafussi and Wright, 1982; Holt et al., 1983; Cremaschi et al., 1986; Fan et al., 2001; Leaphart et al., 2008). In addition, altered enterocyte turnover has been reported in rats subject to numerous environmental changes and disease states, including: Small intestinal resection, dietary changes, diabetes and irradiation (Menge et al., 1982; Menge et al., 1983; Cheeseman, 1986; Thomson et al., 1994). It can therefore be concluded that the enterocyte turnover is highly sensitive to numerous environmental factors.

In addition, the utilised method for determining enzyme turnover may significantly impact the produced value. Turnover based on mitotic figures may greatly underestimate the true turnover of the cell line as in particular the enterocytes are thought to be mainly eliminated via shedding into the gut lumen, in such case radiolabelling, such as H-thymidine labelling, is a more reliable candidate for determining turnover. Radiolabelling does however impose some ethical issues as a radioactive compound is utilised, thus little data from healthy volunteers in humans. In situ or ex vivo determination of enterocyte turnover may not be representative of the turnover in vivo as the conditions seen in vivo are difficult to meet in vitro (Messier and Leblond, 1960; Bertalanffy and Nagy, 1961).

The quality of data posed the perhaps most significant limitation in this study, with a large number of publications only reporting mean data or ranges of enterocyte turnover, this was especially true for the human data where labelling studies tended to be performed in colorectal cancer patients, which may influence the turnover. The enterocyte turnover in mouse, rat and human could however be established in a reasonable large data set.
The human enterocyte turnover, as determined in this analysis (approximately 3.48 days), suggests the GI epithelia to display a comparable rate of renewal to that of the CYP enzymes as determined in vitro, where Yang, and co-workers, found reported CYP turnover to vary between 0.96 and 4.33 days. The turnover of the enzyme and the enterocytes exhibit hierarchical dependencies where the individual impact of these processes on enzyme recovery following DDIs is not completely straightforward to compare. The rate of turnover of the enzymes will follow a first-order rate following mechanism-based inhibition whereas the enterocytes can be assumed to be renewed at a zero-order rate as steady-state levels of the enterocyte population will remain undisrupted following enzyme inhibition. Exploring the impact of nesting enzyme and enterocyte turnover for the prediction of DDIs and MBI requires a more sophisticated model and may be considered particularly well suited for the utilisation of PBPK modelling and simulation (Bell et al., 1967; Yang et al., 2008; van Leeuwen et al., 2009).

This is the most extensive analysis of enterocyte turnover in multiple species, including human to the authors’ knowledge. The incorporation of enterocyte turnover data in PBPK modelling and simulation may be of assistance in improving the predictions of DDIs where substrates are subject to small intestinal metabolism through the incorporation of independent enterocyte and enzyme turnover in the gut-wall, thus allowing a more mechanistic description of the recovery of enzyme following MBI, induction or disease states where enzyme or enterocyte turnover are altered independently.

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Authorship contributions


Performed data analysis: A.S.D.

Wrote or contributed to the writing of the manuscript: A.S.D., D.M.A., A.R.H.
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Legends for Figures

Figure 1. Activity of CYP3A4 over time ($A_{\text{CYP3A}}$) following 5 doses of 60 mg diltiazem administered every 8 hours the first 40 hours changing enzyme turnover ($k_{\text{deg}}$).

Figure 2. Reported means and standard deviations (SD; n rats=14) and mean only data (n=248) of small intestinal enterocyte turnover in rat and the combined weighted geometric mean and SD (GSD) based on dataset of mean and SD.

Figure 3. Reported means and standard deviations (SD; n=113 mice) and mean only data (n=260) of small intestinal enterocyte turnover in the mouse and the combined weighted geometric mean and SD (GSD) based on dataset of mean and SD.

Figure 4. Reported means and standard deviations (SD; n individuals=86), mean only data (n=153), and ranges (n=26) of human gastrointestinal epithelial turnover and the combined weighted geometric mean and geometric SD (GSD; n=86) based on dataset of mean and SD.

Figure 5. Reported enterocyte turnover in days across species, ranked approximately according to body weight, in: Human (n individuals=86), rabbit (n =20), guinea pig (n=Not clear), rat (n=287), hamster (n=8) and mouse (n=131).
Figure 5

Turnover (days)

Species

Human

Rabbit

Guinea pig

Rat

Hamster

Mouse

(Chart showing turnover times for different species.)