

## Short Communication

# Calorie Restriction Increases P-Glycoprotein and Decreases Intestinal Absorption of Digoxin in Mice

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### ABSTRACT

There is wide variation in how patients respond to therapeutics. Factors that contribute to pharmacokinetic variations include disease, genetics, drugs, age, and diet. The purpose of this study was to determine the effect of calorie restriction on the expression of *Abcb1a* in the intestine and whether calorie restriction can alter the absorption of an *Abcb1a* substrate (i.e., digoxin) in mice. Ten-week-old C57BL/6 mice were given either an ad libitum diet or a 25% calorie-restricted diet for 3 weeks. To determine digoxin absorption, mice were administered [<sup>3</sup>H]-labeled digoxin by oral gavage. Blood and intestine with contents were collected at 1, 2, 4, and 12 hours after digoxin administration. Concentrations of [<sup>3</sup>H]-digoxin in plasma and tissues were determined by liquid scintillation. Calorie restriction decreased plasma digoxin concentrations (about 60%) at 1, 2, and 4 hours after

administration. Additionally, digoxin concentrations in the small intestine of calorie-restricted mice were elevated at 4 and 12 hours after administration. Furthermore, calorie restriction increased *Abcb1a* transcripts in the duodenum (4.5-fold) and jejunum (12.5-fold). To confirm a role of *Abcb1a* in the altered digoxin pharmacokinetics induced by calorie restriction, the experiment was repeated in *Abcb1a/b*-null mice 4 hours after drug administration. No difference in intestine or plasma digoxin concentrations were observed between ad libitum-fed and calorie-restricted *Abcb1a/b*-null mice. Thus, these findings support the hypothesis that calorie restriction increases intestinal *Abcb1a* expression, leading to decreased absorption of digoxin in mice. Because *Abcb1a* transports a wide variety of therapeutics, these results may be of important clinical significance.

### Introduction

The absorption of a number therapeutics can be affected by the expression and activity of drug transporters in the intestine. The first drug transporter described was P-glycoprotein (P-gp), also referred to as MDR1 or ABCB1 (Juliano and Ling, 1976; Ueda et al., 1986), which is encoded by the *Abcb1a* and *Abcb1b* genes in mice (Schinkel et al., 1994). P-gp is an ATP-dependent efflux pump that actively transports xenobiotics from cells (Gottesman and Pastan, 1993). P-gp transports a wide range of structurally different therapeutics including colchicine, tacrolimus, quinidine, chemotherapeutic drugs (such as etoposide, doxorubicin, and vinblastine), cardiac glycosides (such as digoxin), glucocorticoids (such as dexamethasone), and human immunodeficiency virus type 1 antiretroviral therapy drugs (Aller et al., 2009). Overexpression of *Abcb1a* and *-1b* can confer drug resistance by increasing efflux of these drugs from cells (Dhir et al., 1990; Raymond et al., 1990) and is a well-established cause of resistance to cancer chemotherapeutic drugs. *Abcb1a* is mainly expressed in the gastrointestinal tract, testis, and capillaries within the brain (Cui et al., 2009). In humans, the *ABCB1* gene encodes P-gp, which has a similar tissue distribution pattern as in mice (Thiebaut et al., 1987, 1989; Cordon-Cardo et al., 1989). Thus, it is thought that P-gp functions to protect the body, limiting xenobiotic intestinal absorption and distribution to the central nervous system and germ cells. In enterocytes, P-gp is located

on the apical surface where it transports xenobiotics back into the intestinal lumen, resulting in limited xenobiotic absorption (Croop et al., 1989; Takano et al., 2006). Because P-gp is known to transport a variety of therapeutics, altering its intestinal expression or activity may alter the bioavailability of a large range of therapeutics.

There is wide variation in the response of humans to therapeutics, both beneficially and adversely. A factor that is often proposed to contribute to this variation is diet (Won et al., 2010; Boullata and Hudson, 2012; Ötles and Senturk, 2014). Until recently, knowledge regarding food-drug interactions was primarily based on anecdotal accounts. However, recent studies have now proven examples of foods that can alter the pharmacokinetics of drugs. For example grapefruit juice inhibits cytochrome P450 (CYP) 3A4 (CYP3A4), which results in increased bioavailability of drugs such as saquinavir, cyclosporine, and felodipine (Seden et al., 2010). Phytochemicals in grapefruit juice (such as bergamottin and quercetin) and green tea catechins inhibit the cellular efflux of P-gp substrates in vitro (Zhou et al., 2004). Furthermore, the amount of protein in the diet can influence drug metabolism and glomerular filtration. For example, in human volunteers, a protein-restricted diet resulted in a 70% decrease in the clearance of oxipurinol and uric acid (Berlinger et al., 1985).

There is a lack of knowledge regarding the effect of decreased calorie consumption on the pharmacokinetics of drugs. However, Renaud et al. (2014) previously reported that in mice a calorie-restricted diet had a profound effect on gene expression in liver. More specifically, Renaud et al. (2014) found that calorie restriction substantially increased hepatic *Abcb1a* transcripts. These results raised the question of whether calorie restriction alters the expression of *Abcb1a* in the intestine, where this transporter is known to have a major impact on xenobiotic absorption. In this study, we reveal that calorie restriction causes decreased absorption of digoxin—likely through a mechanism of calorie restriction-induced expression of P-gp. These results support the hypothesis that diet is an important regulator of drug absorption.

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**ABBREVIATIONS:** CYP, cytochrome P450; P-gp, P-glycoprotein; qPCR, quantitative polymerase chain reaction.

## Materials and Methods

**Chemicals.** Digoxin (0.25 mg/ml) was purchased from Westward Pharm Corp (Eatontown, NJ), RNA-Bee RNA Isolation Reagent was purchased from Tel-Test Inc. (Friendswood, TX), the High Capacity Reverse Transcriptase kit and SYBR Green were purchased from Applied Biosystems (Foster City, CA), [<sup>3</sup>H]-digoxin and Ultima Gold counting fluid were purchased from PerkinElmer (Shelton, CT), and bovine serum albumin was purchased from Amresco (Solon, OH). Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Animals.** C57BL/6 male mice, eight weeks of age, were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Abcb1a/b-null mice were purchased from Taconic Inc. (Hudson, NY) and were back-crossed into the C57BL/6 background (>99% congenicity). Mice were bred and housed in a light-, temperature-, and humidity-controlled environment in an Association for Assessment and Accreditation of Laboratory Animal Care (Frederick, MD) accredited animal housing facility at the University of Kansas Medical Center. All studies were approved by the University of Kansas Medical Center's Institutional Animal Care and Use Committee.

**Calorie Restriction.** After 2-week acclimatization, mice were housed individually and given Laboratory Rodent Chow 8604 (Harlan, Madison, WI), either ad libitum or with 75% of the feed consumed by ad libitum feeding (calorie restriction). The average ad libitum daily consumption of feed was 4 g per mouse (determined using the average daily intake from 10 mice). Thus, mice in the calorie restriction group were given approximately 2.7–3.0 g of feed per day. Mice remained on these diets for 3 weeks, after which they were either used for RNA studies or digoxin absorption studies.

**Tissue Collection for RNA Studies.** In the morning (8:00–10:00 AM) mice were euthanized with pentobarbital and their small intestines were collected. Fecal matter was flushed from the small intestines using a saline solution. Small intestines were divided into three equal parts (duodenum, jejunum, and ileum), immediately frozen in liquid nitrogen, and then stored at –80°C until further analysis.

**RNA Extraction.** RNA was isolated from frozen intestine using RNA Isolation Reagent RNA-Bee following the manufacturer's protocol (Tel-Test Inc.). Using a NanoDrop1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE), RNA concentrations were quantified at a wavelength of 260 nm.

**Messenger RNA Quantification [Reverse Transcription Quantitative Polymerase Chain Reaction (qPCR)].** To perform real-time polymerase chain reaction, we first reverse transcribed RNA to cDNA using an Applied Biosystems High Capacity Reverse Transcriptase kit. In brief, equal volumes of 2X reverse transcriptase, 50 ng/ $\mu$ l RNA, and random primers were mixed and placed in a Mastercycler (Eppendorf, Hauppauge, NY) under the following conditions: 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes. With the resulting cDNA, qPCR was performed as detailed subsequently. The primers for qPCR of Abcb1a (GenBank Accession No. NM\_011076) were designed using Primer-BLAST ([www.ncbi.nlm.nih.gov/tools/primer-blast](http://www.ncbi.nlm.nih.gov/tools/primer-blast)) (forward, GCGACTCCGATACATGGTTT; reverse, ACCCTGTAGCCCTT-CACT) and were synthesized by Integrated DNA Technologies (Coralville, IA). For qPCR, the following were contained per reaction in a 384-well plate (Applied Biosystems): 2.5  $\mu$ l of 3  $\mu$ M forward and reverse primer mix, 5  $\mu$ l of Applied Biosystems SYBR Green PCR master mix, 0.5  $\mu$ l RNase-free H<sub>2</sub>O, and 2  $\mu$ l of 2 ng/ $\mu$ l cDNA. Fluorescence was quantified with an Applied Biosystems 7300 Real Time PCR System under the following conditions: 50°C for 2 minutes and 95°C for 10 minutes (95°C for 15 seconds, 60°C for 1 minute)  $\times$  40 cycles. To ensure primer specificity, melt curves were performed for every reaction. To determine the relative mRNA expression, the comparative  $\Delta\Delta C_t$  method was applied using 18S as a reference transcript. Values from calorie-restricted mice were normalized to values from mice fed ad libitum.

**Digoxin Absorption Experiments.** The protocol followed for these experiments was adapted from an earlier study (Mayer et al., 1996). Each mouse was given 0.2 mg/kg digoxin by oral gavage, labeled with [<sup>3</sup>H]-digoxin (1  $\mu$ Ci/30g b.wt.). Wild-type mice were euthanized 1, 2, 4, or 12 hours after digoxin administration, and Abcb1a/b-null mice were euthanized 4 hours after digoxin administration. Blood was collected by orbital bleeding into heparinized tubes and centrifuged for 10 minutes at 2000g to isolate plasma. Plasma (100  $\mu$ l) was transferred to 4 ml of Ultima Gold high counting efficiency scintillation cocktail. The entire intestine from stomach to rectum (including fecal matter) was also collected, weighed, and homogenized in 4% (w/v) bovine serum albumin. Thus, all intestine samples included the fecal matter. Henceforth, we will refer to the

intestine + contents samples as intestine. Two-hundred  $\mu$ l of intestine homogenate was added to 4 ml Ultima Gold high counting efficiency scintillation cocktail. Radioactivity was quantified by liquid scintillation counting using a Packard Tri-Carb 2100TR Liquid Scintillation Analyzer (Packard Instrument Company, Meriden, CT). Concentrations of digoxin were calculated from a standard curve.

**Statistics.** Statistical differences between ad libitum and calorie restriction were determined using an unpaired, two-tailed Student's *t* test. For the RNA expression data (Fig. 1), the individual values were log transformed to obtain the normal distribution before performing the *t* test. Comparisons resulting in  $P < 0.05$  were considered statistically significant. All data are presented as the mean + S.E.M.

## Results

**Calorie Restriction Increases Abcb1a mRNA Expression in the Small Intestine.** The effect of calorie restriction on mRNA expression of Abcb1a in the small intestines of mice was assessed by reverse transcription qPCR (Fig. 1). Calorie restriction significantly ( $P < 0.05$ ) increased expression of Abcb1a mRNA in the duodenum (4.5-fold) and jejunum (12.5-fold) compared with mice fed the ad libitum diet (control). Expression of Abcb1a was also increased in the ileum; however, it was not statistically different from the controls.

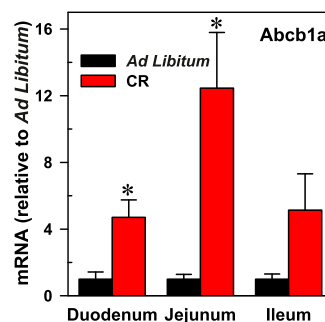
**Calorie Restriction Alters Absorption of Digoxin in Mice.** To determine whether calorie restriction alters absorption of drugs transported by Abcb1a in the intestine, mice were given [<sup>3</sup>H]-labeled digoxin and euthanized 1, 2, 4, and 12 hours after administration. The time courses of the intestine-digoxin and plasma-digoxin concentrations are presented Fig. 2. The intestine of calorie-restricted mice contained 1.5-fold more digoxin at 4 and 12 hours than the intestine of ad libitum fed mice. In contrast to the intestine, the plasma concentrations of digoxin were 61%, 69%, and 57% lower in the calorie-restricted mice than in the control mice at 1, 2, and 4 hours, respectively.

**Calorie Restriction Does Not Alter Digoxin Absorption in Abcb1a/b-Null Mice.** In mice fed ad libitum, compared with wild-type mice, Abcb1a/b-null mice had lower digoxin concentrations in their intestines (974 versus 265 ng digoxin/gram intestine) and higher digoxin concentrations in plasma (0.035 versus 0.100 mean ng digoxin/ $\mu$ l plasma) 4 hours after digoxin administration (Figs. 2 and 3). This result was as predicted because Abcb1a-null mice do not have the capacity to efflux digoxin via P-gp, thus resulting in increased digoxin absorption.

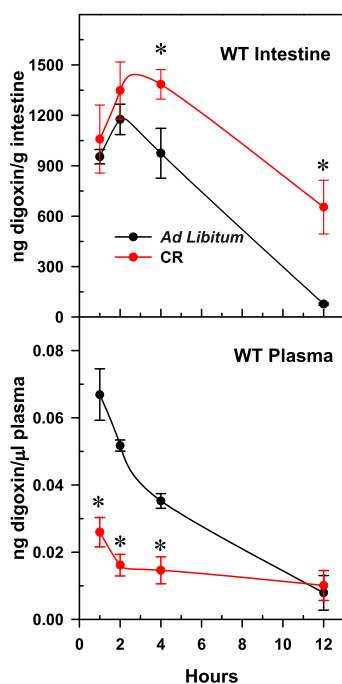
No statistical differences in the concentrations of digoxin in intestine or plasma were observed between ad libitum fed and calorie-restricted Abcb1a/b-null mice (Fig. 3). Thus, these results support the findings that decreased digoxin absorption in calorie-restricted wild-type mice is likely due to increased intestinal P-gp expression.

## Discussion

In clinical practice, a challenging problem in drug therapy is individual variations in patient response. Environmental, genetic, and pathophysiologic



**Fig. 1.** Mean mRNA of Abcb1a ( $\pm$  S.E.M.) in mice on an ad libitum diet or calorie restriction (CR). Asterisks (\*) denote data values statistically significant from control (ad libitum) ( $P < 0.05$  as determined by a Student's *t* test,  $n = 3$ ).

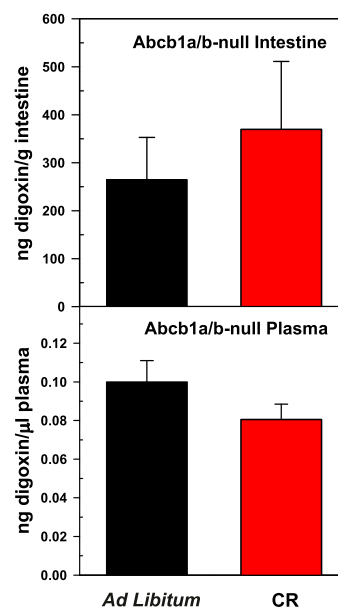


**Fig. 2.** Mean concentrations of digoxin ( $\pm$  S.E.M.) in intestine and plasma over time in mice fed an ad libitum diet or calorie restriction. Asterisks (\*) denote data values statistically significant from the same time point ad libitum control ( $P < 0.05$  as determined by Student's  $t$  test,  $n = 4$  or 5).

factors are known contributors to variations in drug responses and adverse effects; however, little attention has been given to the impact of food and diet on drug disposition. Thus, the results in this study help further our knowledge of how diet impacts drug absorption. Specifically, the current work revealed that calorie restriction can increase the expression of intestinal *Abcb1a* and decrease drug absorption. Because *Abcb1a* transports a wide variety of therapeutics, these results in mice beg the question of whether this phenomenon might also occur in humans.

The gastrointestinal tract functions to digest and absorb nutrients from the diet. However, this organ is also exposed to ingested xenobiotics, and thus also functions as a defense barrier, expressing many metabolic enzymes and efflux transporters. Modulation of efflux transporter expression or function in the intestinal tract can lead to altered systemic and local xenobiotic concentrations (Murakami and Takano, 2008; Huang et al., 2010). The best characterized efflux transporter is P-gp. It is located apically on enterocytes and transports substrates back into the intestinal lumen, resulting in lowered systemic drug concentrations. Increased intestinal P-gp function or expression can markedly affect drug pharmacokinetics, leading to decreased therapeutic effect. In humans, intestinal *MDR1* mRNA is inversely correlated with oral tacrolimus concentrations (Masuda et al., 2004), cyclosporine pharmacokinetics (Masuda and Inui, 2006), and talinolol pharmacokinetics (Bernsdorf et al., 2006). Additionally, xenobiotics that alter the function of P-gp can cause drug-drug interactions; for example, St. John's Wort induces intestinal P-gp, leading to decreased talinolol area under the curve in human subjects (Schwarz et al., 2007). There are many known therapeutics that inhibit P-gp, leading to drug-drug interactions including cyclosporine A, ketoconazole, quinidine, ritonavir, verapamil, and reserpine, to name a few (<http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm>). However, little is known about how nutrients in the diet or the amount of calories consumed affect P-gp activity or expression.

Factors in the diet currently suspected to affect P-gp activity include certain flavonoids. In vitro studies have shown that the flavonoids morin, biochanin A, silymarin, and phloretin all have the ability to



**Fig. 3.** Mean concentrations of digoxin ( $\pm$  S.E.M.) 4 hours after administration in intestine and plasma of *Abcb1a/b*-null mice fed an ad libitum diet or calorie restriction (CR) ( $n = 5$ ).

increase intracellular daunomycin (a P-gp substrate) concentrations in human P-gp positive cells but not in P-gp negative cells. Furthermore, the increase in daunomycin accumulation by these flavonoids is dependent on both flavonoid concentration and P-gp expression, suggesting that these flavonoids inhibit P-gp activity (Zhang and Morris, 2003). Thus, one would anticipate that these flavonoids would increase the absorption of drugs; however, in vivo, biochanin A failed to alter the pharmacokinetics of orally administered P-gp substrates (Zhang et al., 2010). The authors conclude that the disconnect between the in vitro and in vivo data may be due to poor bioavailability and rapid clearance of biochanin A in vivo. It remains to be determined whether flavonoids have the ability to inhibit P-gp in vivo.

Grapefruit juice and orange juice are known to inhibit CYP3A4 and are also suspected to alter P-gp activity (reviewed in Won et al., 2010). Grapefruit juice is well known to increase plasma concentrations of drugs by decreasing CYP3A4, and possibly also by inhibiting P-gp and organic anion-transporting polypeptide activity. Thus, considering that many P-gp substrates are also CYP3A substrates, it is difficult to distinguish the contribution of each factor in grapefruit juice-induced altered drug pharmacokinetics.

The molecular mechanism of how calorie restriction causes an induction of P-gp remains unknown at the present time. In vitro studies using cells derived from intestinal tissues have indicated the pregnane-X receptor as a key player in drug-induced P-gp expression (Maier et al., 2007; Kim et al., 2015). For example, rifampin induces P-gp via casein kinase 2-mediated phosphorylation of heat shock protein 90 $\beta$ , and subsequent stabilization of the pregnane-X receptor (Kim et al., 2015). Thus, increased activation of the pregnane-X receptor might be a good candidate to investigate as a possible mechanism of calorie restriction-induced P-gp expression.

The present study used the P-gp substrate digoxin as an indicator of P-gp activity. This drug is commonly used to evaluate P-gp function in mice because the pharmacokinetic attributes of digoxin are highly P-gp dependent. Additionally, another advantage of using digoxin is that it is not significantly metabolized in mice (Schinkel et al., 1995, 1997; Mayer et al., 1997; Kawahara et al., 1999). Although it is possible that digoxin is transported by other transporters (Taub et al., 2011), our observation was that calorie-restricted *Abcb1a/b*-null mice did not have

decreased plasma concentration or increased digoxin content in intestine compared with ad libitum *Abcb1a/b*-null mice. This suggests that the altered digoxin concentrations observed in calorie-restricted wild-type mice is most likely due to altered P-gp expression. Additionally, digoxin is almost exclusively excreted by the gut mucosa (Mayer et al., 1996), thus it is not likely that the decrease in plasma digoxin concentrations is due to altered P-gp expression in kidney or liver.

In rats, protein-calorie malnutrition suppresses the hepatic expression of P-gp, causing reduced canalicular excretion of the P-gp substrate daunomycin (Lee et al., 2003). These results are opposite what we observed in intestinal tissue in the present study and in hepatic tissue in the study by Renaud et al. (2014). However, these two studies are difficult to compare due to species and diet differences. The protein-calorie-restricted diet in the study by Lee et al. (2003) was iso-caloric to the control diet but contained only 20% of the protein (qualitative malnutrition). However, in the present study and in a previous study by Renaud et al. (2014), the calorie-restricted mice received 25% less of the entire diet compared with the amount consumed by mice fed ad libitum (quantitative malnutrition). Nevertheless, it is evident that the quantity and the composition of the diet are certainly capable of modulating P-gp expression in both rats and mice.

A calorie-restricted diet can arise out of choice (weight loss strategy) or from complications of an illness or drug therapy. Appetite loss leading to a calorie-restricted diet often occurs in cancer and AIDS patients, which is particularly relevant to this study because P-gp transports many chemotherapeutics as well as human immunodeficiency virus antiviral therapies. Thus, the results from this study showing that calorie restriction can alter the absorption of a P-gp substrate have implications for pharmacokinetic research and clinical practice.

In summary, this paper indicates that a calorie-restricted diet can affect the absorption of P-gp substrates in mice, and is probably due to induced expression of intestinal P-gp. This study further underscores the importance of evaluating the influence of diet on drug disposition. Determining how diet can influence drug pharmacokinetics is essential to improving our understanding of interindividual differences in response to therapeutic agents.

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Wrote or contributed to the writing of the manuscript: Renaud, Klaassen, Csanaky.

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