In Vivo Probes of Drug Transport: Commonly Used Probe Drugs to Assess Function of Intestinal P-glycoprotein (ABCB1) in Humans

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Contents

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Abstract Intestinal P-glycoprotein (P-gp, ABCB1) may significantly influence drug absorption and elimination. Its expression and function is highly variable, regio-selective and influenced by genetic polymorphisms, drug interactions and intestinal diseases. An in vivo probe drug for intestinal P-gp should a registered, safe and well tolerated nonmetabolized selective substrate with low protein binding for which P-gp is rate-limiting during absorption. Other P-gp dependent processes should be of minor influence. The mechanism(s) and kinetics of intestinal uptake must be identified and quantified. Moreover, the release properties of the dosage form should be known. So far, the cardiac glycoside digoxin and the ß$_1$-selective blocker talinolol have been used in mechanistic clinical studies, because they meet most of these criteria. Digoxin and talinolol are suitable in vivo probe drugs for intestinal P-gp under the precondition, that they are used as tools in carefully designed pharmacokinetic studies with adequate biometrically planning of the sample size and that several limitations are considered in interpreting and discussion of the study results.

Keywords P-glycoprotein · ABCB1 · Digoxin · Talinolol · Intestinal absorption

1 Introduction

1.1 Expression, Function and Variability of Intestinal P-glycoprotein in Man

There is substantial evidence that human gut mucosa acts as an invasion barrier for numerous small hydrophobic amphophilic xenobiotic compounds by means of both biotransformation and active secretion, the latter being governed by adenosine triphosphate (ATP)-binding cassette (ABC)-type transporters. The most important ABC transport protein is P-glycoprotein, a 170 kD protein. It was originally identified as an important element in the modulation of the resistance against chemotherapy of cancer. P-glycoprotein acts as an efflux-transporter localized at the apical membranes of various cells with excretory function, like enterocytes, hepatic canalicular cells, proximal tubular cells in kidney, endothelial cells of brain capillaries, or syncytiotrophoblasts in the placenta. It is a major part of the physiological functions of organ barriers (e.g., intestinal absorption barrier, blood–brain barrier, placenta barrier) and excretory organs (liver, kidney, intestine) (Cordon-Cardo et al. 1990; Sugawara et al. 1988; Thiebaut et al. 1987). The potential role for intestinal P-glycoprotein in limiting drug absorption and thereby increasing prehepatic elimination as well as its meaning for drug elimination has been addressed by numerous groups (Chan et al. 2004; Ho and Kim 2005; Murakami and Takano 2008; Takano et al. 2006).

Intestinal expression of P-glycoprotein in humans is highly variable and regioselective. *ABCB1*mRNA and P-glycoprotein content in the duodenum vary in

healthy white subjects by a factor of three to ten (Bernsdorf et al. 2006; Greiner et al. 1999; Lown et al. 1997; Oswald et al. 2006; Schwarz et al. 2007; Siegmund et al. 2002b). However, the expression level is low in the duodenum and proximal jejunum and in the ascending colon. Increasingly higher expression has been observed along more distal regions (distal jejunum, ileum) (Englund et al. 2006; Mouly and Paine 2003; Seithel et al. 2006; Thorn et al. 2005; Zimmermann et al. 2005). The basal expression and function of intestinal P-glycoprotein does not seem to be related to age and gender (Larsen et al. 2007; Schwartz 2003).

There is contradictory information whether the expression of intestinal P-glycoprotein in noninduced subjects is influenced by *ABCB1*-gene polymorphisms. For the first time, Hoffmeyer et al. observed in 21 healthy white subjects, that duodenal P-glycoprotein-content (Western blot analysis) tends to be lower in carriers of the synonymous C3435T-polymorphism in exon 26. The difference observed between carriers of 3435CC and 3435TT was approximately twofold (Hoffmeyer et al. 2000). This phenotype of C3435T was recently confirmed by Larsen et al. (2007). Furthermore, it was found that *ABCB1*mRNA expression was significantly decreased among subjects carrying at least one variant allele for C1236T in exon 12 and G2677GT in exon 21 whereas C3435T was without marked influence (Schwarz et al. 2007). In contradiction to these results, significant correlation between the *ABCB1* genotype and the duodenal *ABCB1*mRNA and P-glycoprotein content could not be observed in a larger group of 37 healthy white subjects. (Siegmund et al. 2002b) Genetic influence on intestinal P-glycoprotein expression was also not found in Japanese recipients of living-donor liver transplantation (Goto et al. 2002; Hosohata et al. 2009). In a small group of 13 healthy Japanese subjects, the variant T allele introduced even higher expression of *ABCB1*mRNA (not significant) (Moriya et al. 2002; Nakamura et al. 2002). It is important to recognize that there is a need for phenotyping P-glycoprotein function, because synonymous single-nucleotide polymorphisms that do not alter coding sequences and expression levels may affect functions of the transport protein by the timing of cotranslational folding and insertion into the membrane, thereby altering the structure of substrate and inhibitor interaction sites (Kimchi-Sarfaty et al. 2007; Schaefer et al. 2006). In conclusion, P-glycoprotein expression and function cannot be predicted by genotyping.

The expression of intestinal P-glycoprotein is highly influenced by drug interactions. Like CYP3A4, P-glycoprotein is regulated by the nuclear pregnane-X receptor (PXR) and the constitutive androstane receptor (CAR) (Burk et al. 2005; Geick et al. 2001). Therefore, expression of intestinal P-glycoprotein increases by about 1.5-fold to 8.3-fold after induction with rifampicin, 3.5-fold (mRNA-level) after carbamazepine, and 1.6-fold after St John's wort (SJW) (Giessmann et al. 2004a, b; Greiner et al. 1999; Oswald et al. 2006; Schwarz et al. 2007; Westphal et al. 2000b). The magnitude of induction by rifampicin and SJW seems to be influenced by the *ABCB1* gene polymorphism (Hoffmeyer et al. 2000; Schwarz et al. 2007).

Many substances were identified as inhibitors of intestinal P-glycoprotein function; e.g. verapamil, quinidine, macrolide antibiotics, valspodar (PSC833),

HIV-protease inhibitors, immunosuppressants or constituents of grapefruit juice (Ho and Kim 2005; Marzolini et al. 2004; Takano et al. 2006).

It is currently unknown whether intestinal P-glycoprotein is influenced by pathological factors. There is some evidence that intestinal P-glycoproteinis is increased in patients with active Crohn's disease to an extent that is twice the content in healthy subjects and decreased in patients with persistent diarrhea (Buchman et al. 2005; Lemahieu et al. 2004). In healthy subjects with experimental subclinical hypothyreosis, duodenal *ABCB1*mRNA expression and immunoreactive P-glycoprotein increased 1.4-fold and 3.8-fold, respectively, after treatment with levothyroxine (200 μg for 17 days) (Siegmund et al. 2002a).

In the enterocytes, P-glycoprotein is embedded in a very complex environment equipped with many other apical and basolateral efflux carriers of the ABC-superfamily and with uptake transporters of the organic anion transporting polypeptide (OATP), organic cation transporter (OCT), and peptide transporter (PEPT) families (Ho and Kim 2005). Little is known so far about the interplay between efflux and uptake carriers to mediate unidirectional transcellular fluxes, which is the precondition for substance absorption and intestinal secretion. It should be recognized that there is ample experimental evidence for the existence of complex adaptation processes in multidrug transporter expression in the intestine (and in other organs) in case of bowel surgery or genetic deficiency of transporters (Cisler and Buchman 2005; Drozdowski and Thomson 2006; Glaeser and Fromm 2008; Johnson et al. 2006; Klaassen and Lu 2008; Oswald et al. 2006b; Severijnen et al. 2004; Weale et al. 2005).

1.2 Criteria for an In Vivo Probe Drug of Intestinal P-glycoprotein

An in vivo probe drug for intestinal P-glycoprotein should be suitable to measure the following variability in the efflux transport function:

- Basal variability in noninduced subjects
- Regio-selective differences along the small intestine
- Influence of ABCB1 gene polymorphisms, gender, age and other physiological conditions
- Influence of potential inducers and inhibitors of P-glycoprotein
- Influence of pathological factors (e.g. gastrointestinal disease, systemic inflammations and auto-immune states, hormonal influences, etc.)
- Adaptation processes to bowel surgery and genetic deficiencies of drug metabolizing enzymes and other multidrug transporter proteins.

In selection of a probe drug for intestinal P-glycoprotein, it is important to know that many substrates of P-glycoprotein also interact with other transport proteins such as ABCC1, ABCC2, ABCC3, or ABCG2 and many are subjected to drug

biotransformation with only a few exceptions (Chan et al. 2004; Kim et al. 1999; Murakami and Takano 2008; Schuetz et al. 1996; Tran et al. 2002; Wacher et al. 1995). Furthermore, it must be considered that inducers of P-glycoprotein also may influence other transport proteins and drug metabolizing enzymes and that most of the inhibitors of P-glycoprotein are also inhibitors of drug metabolizing enzymes (Ho and Kim 2005; Urquhart et al. 2007). Due to the overlapping properties of drug transporters and metabolizing enzymes (substrate selectivity, inhibition, induction), only a few drugs with minor metabolism are candidates for the determination of intestinal P-glycoprotein function in man. In the US Food and Drug Administration (FDA) draft guidance for drug interaction studies (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf), digoxin, loperamide, quinidine, vinblastine and talinolol are listed as acceptable in vitro P-glycoprotein substrates. These examples were selected according to the criteria (1) selectivity, (2) low to moderate membrane permeability, (3) minor metabolism, (4) commercially availability, and (5) suitability as an in vivo probe drug. However, the applicability of a candidate to be an in vivo probe drug in man is dependent on several additional conditions. From our point of view, a probe drug for the quantification of intestinal P-glycoprotein in human beings should meet the following requirements:

- The drug is a selective substrate for P-glycoprotein
- Intestinal efflux via P-glycoprotein is the rate-limiting process in absorption
- Other P-glycoprotein dependent pharmacokinetic processes (e.g. organ distribution, hepatic excretion, renal excretion) are of minor influence
- The mechanism(s) and kinetics of intestinal uptake are identified and quantified
- The release properties of the dosage form are known (e.g. site of disintegration) considering regio-selective expression of P-glycoprotein and variability of intestinal transit
- The drug is not significantly metabolized and has low plasma protein binding
- The drug is safe and well tolerated by healthy subjects
- The drug is registered for use in man and is available for intravenous and oral administration
- The drug is rapidly absorbed and has a short half-life to avoid long clinical study periods and sequence bias
- Validated assays are available to quantify the drug (and metabolites) in blood, urine and feces as recommended for studies on bioavailability, bioequivalence and pharmacokinetics (Viswanathan et al. 2007).

Unfortunately, none of the candidates mentioned above meet all of these criteria. Loperamide influences intestinal motility and quinidine and vinblastine are not suitable for routine clinical studies in healthy subjects because of safety concerns. So far, the cardiac glycoside digoxin and the $ß_1$-selective blocker talinolol have been widely used in many mechanistic clinical studies, because they are nearly not metabolized and are well tolerated. Therefore, this review will focus on suitability and limitations of digoxin and talinolol to measure function of intestinal P-glycoprotein in man.

2 Digoxin

2.1 Safety, Physicochemical Properties and Pharmacokinetics

Digoxin is a secondary glycoside from the common foxglove (*Digitalis lanata*) being used for treatment of heart failure and cardiac arrhythmias. It has a narrow therapeutic window and even small elevations of the plasma levels may cause severe side effects. Common adverse reactions may just occur at therapeutic levels of 0.5–2.0 ng/ml such as cardiac arrhythmias, nausea and vomiting and central nervous effects (Bauman et al. 2006; Piergies et al. 1994; Tuncok et al. 1997).

Digoxin (molecular weight 780.95 Da) provides low water solubility (0.065 g/l) and intermediate, pH-independent lipid solubility (logP = 1.67). It is a class II compound with low solubility but high permeability according to the biopharmaceutics classification system (BCS) (Amidon et al. 1995; Wu and Benet 2005). Other authors classified digoxin as a class I and even class IV compound, considering obviously the standard dose of 0.25 mg being soluble in 240 ml water (class I) or to support the conception that so far unknown intestinal uptake transporters are involved in absorption of class IV compounds (Lindenberg et al. 2004; Shugarts and Benet 2009).

Digoxin is rapidly absorbed from the gastrointestinal tract; peak plasma concentrations are reached within 3 h. Digoxin solution is nearly completely absorbed whereas the bioavailability of elixirs and immediate release tablets is incomplete and accounts for 60–80% and 70–85%, respectively. The drug is widely distributed (volume of distribution, 5–8 l/kg); major distribution compartments are skeletal muscles (65%), liver (13%) and heart (4%). Approximately 25% are bound to plasma proteins. Digoxin has a long terminal half-life of 1.5–2 days (Aronson 1980; Iisalo 1977). Approximately 10% of the dose are metabolized by hydrolysis, oxidation and conjugation; major metabolites are 3β-digoxigenin, 3-keto-digoxigenin and their glucuronides and sulfate conjugates (Hinderling and Hartmann 1991; Watson et al. 1973). Sixty to eighty percent of intravenous digoxin are excreted into urine unchanged, predominantly by glomerular filtration (Aronson 1980; Iisalo 1977). Renal handling of digoxin also involves tubular secretion and tubular reabsorption (Doherty et al. 1969; Rengelshausen et al. 2003; Steiness 1974). Approximately 20–40% of an intravenous and oral dose are eliminated by hepatic and intestinal secretion (Table 1) (Caldwell and Cline 1976; Hinderling and Hartmann 1991; Sumner and Russell 1976).

2.2 Affinity to P-glycoprotein In Vitro and in Animal Studies

The first in vitro evidence that digoxin might be a substrate of ABCB1 came from Tanigawara et al. (1992). In this study, it was demonstrated that the transepithelial transport of digoxin (100 nM) in *MDR1*-transfected LLC-PK1 cells, a porcine

Table 1 Major physicochemical and pharmacokinetic characteristics of digoxin and talinolol (Aronson 1980; Atkinson and Begg 1988; Caldwell and Cline 1976; Doherty et al. 1969; Drescher et al. 2003; Giessmann et al. 2004a; Greiner et al. 1999; Hinderling and Hartmann 1991; Iisalo 1977; Kurata et al. 2002; Rengelshausen et al. 2003; Schwarz et al. 2007; Siegmund et al. 2002a; Steiness 1974; Sumner and Russell 1976; Watson et al. 1973; Westphal et al. 2000a, b)

	Digoxin	Talinolol
pKa	13.5	9.16
logP (pH 7.4)	1.67	1.08
Water solubility (g/l)	0.065	5.1
Bioavailability (%)	60–80	55–70
Distribution volume (l/kg)	5–8	3–6
Plasma protein binding (%)	20–30	50–70
Renal clearance (ml/min)	140–160	150–190
Nonrenal clearance (ml/min)	80–100	120–180
Metabolite excretion (%)	5–15	<1
Half-life (h)	30–50	10–17

kidney tubular cell line, was nearly eightfold higher in the basal-to-apical (b → a) direction compared to the apical-to-basal (a → b) direction and could be inhibited by the known ABCB1 inhibitors vinblastine, quinidine, and verapamil (all 20 μM). The same group found that also cyclosporine inhibits (1–10 μM) the secretory net transport of digoxin (55 nM), whereas digoxin (100 μM) did not influence the transport of cyclosporine (85.5 nM) (Okamura et al. 1993). Ito et al. found that the digoxin transport (200 nM) across renal tubular cells is a saturable (K_m = 14.1± 1.6 μM), energy dependent process, because 2,4-dinitrophenol (1 mM) and sodium azide (10 mM) significantly reduced the b → a flux (Ito et al. 1993a). Moreover, inhibitors of P-glycoprotein (quinidine, verapamil and vincristine) significantly increased the a → b flux of digoxin by 132–175% and inhibited the b → a secretion by 49–55%. Schinkel et al. later on confirmed the affinity of digoxin to P-glycoprotein using the LLC-PK1 cells transfected with human and murine *ABCB1/Abcb1a* (Schinkel et al. 1995). The transepithelial transfer of radiolabeled digoxin (2 μM) was considerably higher in the secretory direction than in the absorptive direction without reaching saturation (K_m > 2 μM). Several other researches confirmed that digoxin is a high affinity substrate for P-glycoprotein in studies with Caco-2, LLC-PK1 and *MDR1*-MDCKII cells. The efflux ratios (apparent permeability P_{app} b → a/a → b) ranged in Caco-2 cells from 4 to 8 and in *ABCB1*-transfected cells from 26 to 52 (Table 2) (Keogh and Kunta 2006; Neuhoff et al. 2003; Pauli-Magnus et al. 2001; Rautio et al. 2006; Shirasaka et al. 2006; Taub et al. 2005).

Digoxin seems not to be an inhibitor of P-glycoprotein. Although being highly transported across monolayers of MDR1-transfected cells with high efflux ratio, digoxin showed no effects on b → a permeability of calcein-AM, vinblastine, colchicin, prazosin, and cyclosporine (Okamura et al. 1993; Rautio et al. 2006).

In parallel to the first in vitro experiments using cell models, it was demonstrated in dogs that cyclosporine and quinidine influence the renal excretion of digoxin. After intravenous administration of cyclosporine in cremophor EL (0.5–3.5 μM) as

Table 2 In vitro studies in which was shown that digoxin is a substrate of P-glycoprotein

Cell model	Apical-to-basal (a → b) and basal-to-apical (b → a) transport via cell monolayer	References
Caco-2	In native cells (0.1 μM digoxin): a → b: $1.35 \pm 0.13 \times 10^{-6}$ cm/s, b → a: $5.73 \pm 0.19 \times 10^{-6}$ cm/s, ratio: 4.24 In induced cells (10 nM vincristine): a → b: $0.82 \pm 0.13 \times 10^{-6}$ cm/s, b → a: $8.27 \pm 0.20 \times 10^{-6}$ cm/s, ratio: 10.1	Shirasaka et al. (2006)
L-MDR1, Caco-2	b → a net transport (% of added digoxin, 5 μM): 16.0 ± 4.4% in Caco-2, 11.7 ± 3.6% in L-MDR1 and 3.3 ± 1.1% in LLC-PK1 cells. No b → a/a → b differences in the presence of PSC833 (1 μM)	Pauli-Magnus et al. (2001)
L-MDR1	[^{3}H]digoxin (100 nM), b → a/a → b ratio: 8, inhibited by vinblastine, quinidine or verapamil (all 20 μM)	Tanigawara et al. (1992)
L-MDR1	[^{3}H]digoxin (55 nM), b → a > a → b (no efflux ratio given), inhibited by cyclosporine (1, 5, 10 μM)	Okamura et al. (1993)
L-MDR1	[^{3}H]digoxin (2 μM), substantially higher b → a versus a → b, $K_m > 2$ μM	Schinkel et al. (1995)
MDCKII	[3H]digoxin (20 nM), a → b: $0.79 \pm 0.53 \times 10^{-6}$ cm/s, b → a: $4.07 \pm 0.25 \times 10^{-6}$ cm/s, ratio: 5.2 (2.0 in presence of 200 μM verapamil)	Taub et al. (2005)
LLC-PK1	[^{3}H]digoxin (200 μM), b → a: 254.5 ± 4.2, a → b: 188.2 ± 22.9 fmol/cm^2/h, ratio 3.8 (normalized to mannitol) Saturable b → a transport: K_m 14.1 ± 1.6 μM, V_{max}: 184.5 ± 38.0 pmol/cm^2/h In presence of quinidine (20 μM), verapamil (20 μM), vincristine (20 μM): b → a (10 nM digoxin) decrease by −51%, −45%, −59%; a → b, increase by 75%, 32%, 46%	Ito et al. (1993a)
MDR1-MDCKII	b → a/a → b ratio for digoxin (0.05–10 μM): 33.6 ± 3.2 (1.28 ± 0.13 in presence of 2 μM elacridar) IC_{50} for b → a inhibition: elacridar = 0.18 μM, itraconazole = 0.95 μM, quinidine = 9.4 μM, verapamil = 8.1 μM	Keogh and Kunta (2006)
MDR1-MDCKII	Digoxin (43 nM), P_{app} a → b 3.07 ± 0.30 nm/s, P_{app} b → a: 159 ± 17 nm/s, b → a/a → b ratio: 51.8	Rautio et al. (2006)
MDR1-MDCKII	[3H]digoxin (20 nM), a → b: $0.32 \pm 0.10 \times 10^{-6}$ cm/s, b → a: $8.15 \pm 0.91 \times 10^{-6}$ cm/s, ratio: 25.5 (1.3 in presence of 200 μM verapamil)	Taub et al. (2005)

well as after infusion of quinidine (37.5 μg/kg min), the urinary recovery of digoxin was reduced by about 50% without change of renal blood flow or urine flow (both $p < 0.001$). The authors concluded that there is a luminally localized secretory system for digoxin in the kidneys (de Lannoy et al. 1992). In a landmark study, Schinkel et al. observed that digoxin concentrated 35.3-fold in brain tissue of *Abcb1a*(−/−) knockout mice 4 h after intravenous injection of 1 mg/kg

compared to wild-type animals (Schinkel et al. 1995). In another study with digoxin (0.2 mg/kg), the same group found that genetic deficiency of P-glycoprotein decreased fecal excretion of digoxin significantly by 75% and 48% but increased renal elimination by 120% and 240% after intravenous and oral administration, respectively (Mayer et al. 1996). Wild-type mice with a cannulated gallbladder showed a substantial intestinal secretion of intravenously given digoxin (16.4% of dose); biliary and intestinal excretion in *Abcb1a* knockout mice accounted for only 66% and 13.4%, respectively, of the rates in wild-type animals. Digoxin significantly accumulated 66-fold in brain tissue of the deficient mice (after 8 h). Maximum brain levels were measured 72 h after bolus injection; at that time, the drug was not detectable in plasma anymore. The results obtained with *Abcb1a*(−/−) mice were confirmed later on by the same group in studies using *Abcb1a*/*b*(−/−) knockout mice (Schinkel et al. 1997). Finally, Kawahara et al. compared pharmacokinetics of digoxin in *Abcb1a*(−/−) and wild-type mice. In deficient animals, $AUC_{0–24h}$ was increased nearly threefold and mean residence time 1.3-fold whereas renal and biliary clearance was reduced to one third of the values in wild-type animals (Kawahara et al. 1999).

Mayer et al. measured the influence of the specific P-glycooprotein inhibitor PSC833 (valspodar) on pharmacokinetics of digoxin in wild-type and *Abcb1a*/*b*(−/−) mice (Mayer et al. 1997). In *Abcb1a*/*b*(−/−) mice, the brain-to-plasma ratio was increased 27.8-fold and 10-fold 4 h and 24 h, respectively, after oral and intravenous administration of digoxin (0.05 mg/kg). The fecal excretion was reduced nearly by half whereas urinary excretion was doubled. Concomitant oral administration of PSC833 (50 mg/kg) caused pharmacokinetic changes in disposition of intravenous digoxin in wild-type mice which were similar to the changes in untreated knockout mice. In the *Abcb1a*/*b*(−/−) mice, presence of PSC833 unexpectedly increased urinary excretion and reduced fecal excretion of digoxin; biliary excretion was also significantly lowered. Similar unexpected evidence came from Fromm et al. who studied the effects of quinidine on digoxin disposition in wild-type and *Abcb1a*(−/−) mice. In wild-type animals, coadministration of quinidine (100 mg/kg) increased the concentrations of digoxin (0.5 mg intravenously) in plasma, brain, liver, kidney and intestine 1.4- to 2-fold. Quinidine comedication, however, also increased the plasma and tissue levels by 110–180% in *Abcb1a*(−/−) animals but reduced concentration of digoxin in brain tissue significantly (Fromm et al. 1999). These findings suggest that PSC833 and quinidine are very likely to be also inhibitors of other – currently unknown – excretory and/or uptake systems that are important for the distribution and elimination of digoxin.

2.3 Evidence from Mechanistic Clinical Studies

Convincing evidence for digoxin to be secreted by intestinal P-glycoprotein was obtained in mechanistic clinical studies using an intestinal multilumen perfusion catheter (Drescher et al. 2003; Glaeser et al. 2002; Igel et al. 2007). The authors

confirmed that intestinal P-glycoprotein is involved in digoxin elimination after intravenous administration from the blood into the gut lumen and prevents systemic exposure with luminally administered digoxin (eight male healthy subjects). After intravenous administration of 1.0 mg digoxin, 0.45 ± 0.24% of the dose appeared in the perfusate of a jejunal segment. Assuming the overall length of the small intestine of 3–5 m and that P-glycoprotein expression is constant along the small intestine, the authors calculated an average digoxin elimination of more than 11% within 3 h. Perfusion with quinidine reduced the intestinal digoxin secretion by 50%. The concentration of quinidine in the segment (104 ± 43.4 μM) was nearly 50-fold higher than the IC_{50} for inhibition of digoxin transport in Caco-2 cells (2.2 μM) (Wandel et al. 1999). After pretreatment of the healthy subjects with rifampicin (600 mg daily, 10 days), secretion of digoxin into the perfusion segment in the proximal jejunum increased by about 80%. Nonrenal clearance increased by 88% and the renal excretion decreased by 18% whereas renal clearance and half-life remained unchanged by transporter induction with rifampicin. 53% of the $AUC_{0–96h}$ were predicted by P-glycoprotein expression in shed enterocytes (Drescher et al. 2003).

Oral bioavailability of 0.5 mg digoxin was studied in seven healthy male subjects using the same technique; about 22% of the dose were absorbed into the systemic circulation via the 20-cm jejunal perfusion segment. In the presence of quinidine (116 ± 69 μM), bioavailability increased by about 150% and the amount excreted into urine by 250%. 1.26% of the absorbed digoxin dose were secreted back into the adjacent segment (Igel et al. 2007).

2.4 Digoxin Disposition and Induction of Intestinal P-glycoprotein

Greiner et al. (1999) used for the first time digoxin as a probe drug for intestinal P-glycoprotein in man. In order to induce P-glycoprotein they treated eight healthy male subjects with 600 mg rifampicin once daily for 10 days (Greiner et al. 1999). Rifampicin induced duodenal P-glycoprotein by 1.4-fold (immunohistochemistry) and 3.5-fold (Western blot), respectively. This up-regulation of P-glycoprotein was associated with 3.2-fold increase of the nonrenal clearance of digoxin and decrease of bioavailability by 21%. Urinary excretion of digoxin after oral and intravenous dosing lowered by 31% and 17%, respectively, whereas renal clearances and half-lives remained unchanged. More than 50% of the $AUC_{0–144h}$ were predicted by intestinal P glycoprotein expression. A borderline correlation was also observed between plasma AUC of digoxin and intestinal CYP3A4 levels. Basal, noninduced P-glycoprotein content correlated not to AUC of digoxin. However, this clinical study confirmed convincingly that intestinal protein content of P-glycoprotein predicts absorption of digoxin in man, and in turn, that digoxin is a suitable probe drug for the function of intestinal P-glycoprotein.

Similar indirect evidence for the value of digoxin as a probe drug for intestinal P-glycoprotein function was obtained in drug interaction studies with SJW (Gurley et al. 2008; Johne et al. 1999; Mueller et al. 2004). Ingredients of SJW act like rifampicin as ligands of the nuclear PXR and induce intestinal P-glycoprotein in man (Dresser et al. 2003; Durr et al. 2000; Fromm et al. 2000; Geick et al. 2001; Giessmann et al. 2004b; Greiner et al. 1999; Luo et al. 2002; Moore et al. 2000; Schwarz et al. 2007; Wentworth et al. 2000; Westphal et al. 2000b). In a single-blind, placebo-controlled, parallel-group study, 25 healthy subjects were treated with 0.25 mg digoxin for 15 days (Johne et al. 1999). After loading for 5 days, SJW ($N = 13$) or placebo ($N = 12$) were coadministered for 10 days. Single dose SJW was without any effect on digoxin disposition. After multiple-dose comedication, $AUC_{0–24h}$, C_{max} and C_{trough} decreased by 20–26% whereas half-lives remained unchanged. Interestingly, the digoxin trough levels decreased in dependence on the duration of SJW treatment; a significant decrease from the placebo group occurred after 7 days. The pharmacokinetic changes resulted most likely from increasingly reduced absorption of digoxin following induction of intestinal P-glycoprotein. In another placebo-controlled, parallel-group study in 96 healthy subjects, it was shown later, that the interaction of SJW with digoxin varies with the SJW preparation and seems to be correlated to the dose, particularly to the ingredient hyperforin (Mueller et al. 2004). The chronic effects of rifampicin (300 mg, twice daily, 7 days) and SJW (300 mg three times daily) on digoxin pharmacokinetics (0.25 mg) were confirmed by the results of a recent study in 18 healthy subjects. The $AUC_{0–24h}$ and C_{max} of digoxin decreased by about 25% and about 37%, respectively, after both kinds of induction. Half-lives also remained unchanged in this study (Gurley et al. 2008).

2.5 *Digoxin Disposition and Inhibition of Intestinal P-glycoprotein*

In the US Food and Drug Administration (FDA) draft guidance for drug interaction studies, digoxin is the recommended probe drug for the investigation of potential substrates/inhibitors or inducers of P-glycoprotein (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf). The suitability of digoxin to evaluate interactions with intestinal P-glycoprotein has been confirmed in numerous clinical trials. Some of the most important studies are addressed in the following (Table 3).

The influence of verapamil (240 mg/day, t.i.d.), an unspecific and moderate modulator of P-glycoprotein, on the steady-state pharmacokinetics of digoxin (0.25 mg twice daily, 2 weeks) was evaluated by Rodin et al. in ten healthy subjects (Rodin et al. 1988). Verapamil increased the AUC of digoxin by 50% and the C_{max} by 40%. Renal clearance remained unchanged. These results are explained by increase of digoxin bioavailability as caused by inhibition of intestinal P-glycoprotein, given that the volume of distribution was not influenced by

Table 3 Single dose (SD) and multiple dose (MD) drug interaction studies with digoxin and talinolol after oral (po) and/or intravenous (iv) administration

Inhibitor	Design	AUC ratio	C_{max} ratio	CL_R ratio	IC_{50} (μM)	Peak plasma concentration (μM)	Gut concentration (μM)	Reference(s)
Studies with digoxin								
Talinolol	SD, po	1.23*	1.45*	1.04	198	0.73	1,100	Collett et al. (2005) and Westphal et al. (2000a)
Itraconazole	SD, po	1.52*	1.34	0.80*	1.3	0.53	1,134	Jalava et al. (1997) and Keogh and Kunta (2006)
Clarithromycin	SD, po	1.64*	1.83*	0.83	~300	1.63	1,337	Rengelshausen et al. (2003) and Wakasugi et al. (1998)
Clarithromycin	SD, iv	1.19	–	–	~300	1.63	1,337	Rengelshausen et al. (2003) and Wakasugi et al. (1998)
Clarithromycin	SD, po	1.47*	1.75*	–	~300	1.63	1,337	Gurley et al. 2008, Rengelshausen et al. (2003) and Wakasugi et al. (1998)
Grapefruit juice	SD, po	1.10*	1.23	1.0	–	–	–	Becquemont et al. (2001)
Ritonavir	SD, po	1.86*	–	0.65*	28.2	19.6	1,665	Ding et al. (2004) and Keogh and Kunta (2006)
Verapamil	MD, po	1.50*	1.44*	1.04	10/5.9	0.13	704	Fenner et al. (2009), Keogh and Kunta (2006) and Rodin et al. (1988)
Verapamil	MD, po	–	1.50*	1.13	10/5.9	0.13	704	Fenner et al. (2009), Hedman et al. (1991) and Keogh and Kunta (2006)
Quinidine	MD, po	1.77*	1.75*	–	14.1/21	8.4	–	Fenner et al. (2009), Keogh and Kunta (2006) and Pedersen et al. (1983)
Quinidine	MD, po	–	1.55	0.71*	14.1/21	4.5	3,083	Fenner et al. (2009), Hedman et al. (1990) and Keogh and Kunta (2006)
Valspodar[a]	MD, po	1.74*	1.74*	0.35*	0.1	1.56	1,317	Kovarik et al. (1999) and Song et al. (1999)

Valspodar[b]	MD, po	3.05*	2.44*	0.25*	0.1	1.49	659	Kovarik et al. (1999) and Song et al. (1999)
Carvedilol[c]	MD, po	1.56*	1.38*	–	4	65.2	61.5	Baris et al. (2006), Fenner et al. (2009) and Tenero et al. (2000)
Carvedilol[d]	MD, po	1.24	1.0	–	4	65.2	61.5	Baris et al. (2006), Fenner et al. (2009) and Tenero et al. (2000)
Carvedilol	SD, po	1.19*	1.60*	–	4	265	246	De Mey et al. (1990), Fenner et al. (2009) and Tenero et al. (2000)
Carvedilol	SD, iv	0.96	1.05	–	4	265	246	De Mey et al. (1990), Fenner et al. (2009) and Tenero et al. (2000)
Studies with talinolol								
Erythromycin	SD, po	1.52*	1.26*	–	>100	17.1	10,900	Josefsson et al. (1982), Keogh and Kunta (2006) and Schwarz et al. (2000)
TPGS[e]	SD, po	1.20*	1.36*	–	–	–	–	Bogman et al. (2005)
silymarin	MD, po	1.30*	1.27*	–	–	–	–	Han et al. (2009)
Ginkgo biloba extract	MD, po	1.22*	1.26*	–	–	–	–	Fan et al. (2009b)
Schisandra chinensis extract	MD, po	1.52*	1.51*	–	–	–	–	Fan et al. (2009a)

The ratios for AUC, C_{max} and CL_R indicate the pharmacokinetic changes in the presence of the potential P-glycoprotein inhibitor. Furthermore, IC_{50} values and peak plasma concentrations from the studies or from literature and apparent gut concentrations (I_2, dose/250 mL administration volume) of the inhibitors are given

*p at least <0.05 compared to control

[a]Valspodar was given single-dose orally (400 mg)

[b]Valspodar was given repeatedly twice daily (a 200 mg)

[c]Study was performed in males ($N = 12$)

[d]Study was performed in females ($N = 12$)

[e]Tocopheryl polyethylene glycol 1000 succinate (0.04%)

verapamil. This conclusion is supported by the results of a clinical investigation in six patients with chronic atrial fibrillation receiving oral digoxin (0.25–0.5 mg/day) and verapamil (240 mg/day) for at least 4 weeks (Hedman et al. 1991). Verapamil significantly elevated steady-state plasma concentrations of digoxin by 44%, whereas renal clearance was not influenced. Hepatic and/or intestinal secretion of digoxin as assessed by duodenal-marker-perfusion technique was reduced by 43% in the presence of verapamil, which corroborates the conception that this effect is caused by inhibition of intestinal P-glycoprotein. Inhibition of intestinal P-glycoprotein also seems to be the reason for an increase in digoxin bioavailability by 18% after comedicaton of 100 mg talinolol as it was observed in ten healthy subjects (Westphal et al. 2000a).

However, there are many other clinical studies, in which the increase of the digoxin AUC in presence of a P-glycoprotein substrate/inhibitor was more than 43%, e.g. after administration of PSC833 (valspodar), itraconazole or clarithromycin (Gurley et al. 2008; Jalava et al. 1997; Kovarik et al. 1999; Rengelshausen et al. 2003). Increase of AUC-values by 43% is the maximum elevation that can be solely explained by complete blockade of the presystemic elimination of digoxin via intestinal P-glycoprotein efflux assuming that the bioavailability of commonly used immediate release digoxin tablets is 70%. This upper limit of the "AUC-calibration range" ensures that inhibitory potency of clinical relevance can be reliably measured with digoxin if taken in mind that the world-widely accepted equivalence range for primary outcome characteristics for decisions in drug interaction studies is 0.80–1.25 (http://www.emea.europa.eu/pdfs/human/ewp/056095en.pdf, www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070124.pdf). If AUC-values are elevated by more than about 40% in presence of an inhibitor, influences on distribution or elimination routes for digoxin, e.g. by the intestine or kidneys, have to be taken into account.

Many efforts have been spent to predict pharmacokinetic interactions between digoxin and potential substrates and/or inhibitors of P-glycoprotein in order to facilitate the decision whether a clinical trial is needed. The FDA draft guidance document recommends that a drug interaction study should be performed if the ratio of maximum systemic inhibitor concentration at steady state [I] over inhibitory potency measures (inhibitor constant, K_i or concentration to inhibit 50% P-glycoprotein activity, IC_{50}) is above 0.1. As recently mentioned by Fenner et al., it appears that the FDA guidance has applied the ratio introduced by Rowland et al., who showed that for a single route of clearance, the increase in the AUC in the presence of an inhibitor can be represented as $1 + [I]/K_i$. When $[I]/K_i$ is 0.1, the predicted increase in AUC ratio will be 10% (Fenner et al. 2009; Rowland et al. 1973). However, there are many doubts whether the inhibitor concentrations (K_i/IC_{50}) for interaction with P-glycoprotein in tumor cells or transfected cells are similar to the concentrations necessary for inhibition of a transporter embedded in the apical membrane of highly differentiated human cells (enterocytes, tubular cells in kidneys and liver). Furthermore, it is unknown whether plasma levels of an inhibitor are identical with the concentrations on P-glycoprotein receptor site in the opposite, apical cell membrane.

To predict interactions with P-glycoprotein that occur during absorption, the ratio $[I_2]/IC_{50} > 10$ has been recommended with $[I_2]$ being the inhibitor gut concentrations (dose divided by administration volume). However, the real concentrations in the apical membrane after oral administration of the inhibitor are dependent on many factors, which are not to predict easily (disintegration and dissolution rate of the test and reference drug, availability of luminal water, rate of intestinal water absorption, etc.). Fenner et al. recently reevaluated 123 digoxin drug interaction trials. Taking AUC-ratios and C_{max}-ratios as measures for systemic exposure of digoxin at steady-state and the in vitro characteristics of $[I]/IC_{50}$ and $[I_2]/IC_{50}$, it appeared that $[I]/IC_{50} > 0.1$ is predictive for a P-glycoprotein related interaction with digoxin and that, however, to a limited extent, $[I_2]/IC_{50} < 10$ is predictive for lack of interactions. Nevertheless, the authors observed a high number of false-negative predictions with $[I]/IC_{50} < 1$ and false-positive predictions with $[I_2]/IC_{50} > 10$ (Fenner et al. 2009).

2.6 *Regioselective Absorption of Digoxin*

Intestinal absorption of digoxin seems to be dependent on the intestinal site of disintegration of the digoxin dosage form and dissolution of the drug substance. This is in agreement with the regio-selective expression of intestinal P-glycoprotein; with the transporter being higher expressed in distal regions (Englund et al. 2006; Wilding et al. 1992; Zimmermann et al. 2005). Concomitant administration of the anticholinergic drug propantheline (10 mg t.i.d., 10 days) which is known to inhibit intestinal transit causes as much as a threefold increase in serum concentrations in patients on maintenance therapy with a slowly dissolving brand of digoxin tablets (Manninen et al. 1973b). In contrast, metoclopramide (10 mg t.i.d., 10 days), which increases gastrointestinal transit rate produced a marked decrease in digoxin concentrations (Manninen et al. 1973a). Propantheline had no effect when digoxin was administered in solution (eight healthy subjects) (Manninen et al. 1973a).

2.7 *Digoxin as a Probe Drug for Genetic Polymorphisms of P-glycoprotein*

The database on the influence of *ABCB1* gene polymorphisms on digoxin disposition in man is currently as contradictory as the data on genetic variability of intestinal P-glycoprotein expression (Table 4). Unfortunately, most of the pharmacogenetic studies with digoxin were not performed in a prospective manner with adequate primary endpoints and sufficient statistical power. The study samples commonly comprised the control groups from previous drug interactions studies.

Table 4 Gene polymorphisms of ABCB1 and changes in the pharmacokinetics of digoxin and talinolol

Ethnic group	Genotype/ haplotype number of carriers	Dose	Changes observed	References
Digoxin				
Caucasians	C3435T 7 CC, 7 TT	0.25 mg tablet steady-state	↑ 38% C_{max} for TT	Hoffmeyer et al. (2000)
Caucasians	C3435T 8 CC, 8 CT, 8 TT	0.5 mg, oral steady-state	↑ 21% $AUC_{0–4h}$, ↔ $AUC_{0–24h}$, ↑ 23% C_{max}, ↑ 36% C_{trough}, ↑ 20% A_e for TT	Johne et al. (2002)
25 Caucasians, 6 Africans, 1 Asian	C3435T 14CC, 8CT, 10TT		↑ 20% $AUC_{0–48h}$ for TT	Verstuyft et al. (2003)
Caucasians	C3435T 12CC, 25CT, 13TT	1.0 mg tablet single dose	No difference of $AUC_{0–4h}$	Gerloff et al. (2002)
25 Caucasians, 6 Africans, 1 Asian	G2677/A 12GG, 11GT, 7TT, 1GA, 1AA	0.5 mg tablet single dose	No difference of $AUC_{0–48h}$	Verstuyft et al. (2003)
Caucasians	G2677/A 12GG, 21GT, 7TT, 7GA, 3AA	1.0 mg tablet single dose	No difference of $AUC_{0–4h}$	Gerloff et al. (2002)
Japanese	C3435T 5CC, 4CT, 6TT	0.25 mg tablet single dose	↓ 20% $AUC_{0–4h}$, ↓ 35% $AUC_{0–24h}$ (ns)	Sakaeda et al. (2001)
Japanese	C3435T 5CC, 6TT	0.25 mg tablet 0.25 mg/5 ml solution	Tablet: not difference Solution sprinckled over duodenal mucosa: ↓ 25% $AUC_{0–4h}$, ↓ 55% C_{max}	Morita et al. (2003)
Japanese	G2677T/A/ C3435T 5GC/GC, 5GC/ TT, 5TT/TT	0.5 mg, oral-iv, single dose	↑ 30% F, ↑ 25% AUC_{iv}, ↑ 70% AUC_{po}, ↓ 32–40% CL_R, ↓ 32–40% CL_{NR} for TT/TT	Kurata et al. (2002)
Chinese	C1236T/G2677T/ A/C3435T 7TTT/TTT, 5TGC/CGC	0.25 mg, oral, single dose	↑ 45% $AUC_{0–4h}$, ↑ 80% C_{max} for TTT/TTT	Xu et al. (2008)
Caucasians	C1236T/G2677T/ A/C3435T 38 CGT, 33 TTT	0.57 mg (DDD)	↑ C_{trough} by 0.18–0.21 ng/ml per additional T allele; 4.3-fold higher risk for digoxin levels >2.0 mg/ml for TTT	Aarnoudse et al. (2008)
Talinolol				
Caucasians	C3435T 13CC, 29 CT, 13 TT	30 mg iv, 100 mg po, single dose	No difference for TT	Siegmund et al. (2002b)

(*continued*)

Table 4 (continued)

Ethnic group	Genotype/ haplotype number of carriers	Dose	Changes observed	References
Caucasians	G2677T/A 19GG, 22GT, 9TT, 2GA, 3TA	30 mg iv, 100 mg po, single dose	↑ AUC in TT/TA	Siegmund et al. (2002b)
Chinese	C3435T 6CC, 6CT, 6TT	100 mg po, single dose	No difference for TT	Han et al. (2009)
Caucasians	C1236T/G2677T/A/C3435T 5TTT, 4CGC	30 mg iv, 50 mg po, single dose	↑ Inductive response in TTT	Schwarz et al. (2007)

ns not significant, *DDD* defined daily dose, *po* per os, *iv* intravenous

Primary pharmacokinetic parameters that are suitable to describe intestinal P-glycoprotein activity are bioavailability and nonrenal clearance or intestinal (fecal) clearance of intravenous digoxin. Instead, week surrogate parameters were commonly measured as $AUC_{0–4h}$, C_{max}, C_{trough} (at steady state) or t_{max}. A pharmacogenetic study on digoxin pharmacokinetics in healthy subjects with adequate sample size is still missing.

The *ABCB1* 3435T allele seems to be associated with higher digoxin bioavailability and higher plasma exposure as shown in retrospective studies with small sample size (5–14 per group) and digoxin doses of 0.5 and 1.0 mg (single doses) and 0.25 mg (steady-state) (Johne et al. 2002; Kurata et al. 2002; Verstuyft et al. 2003). These findings are in agreement with probably lower expression of intestinal P-glycoprotein in carriers with the 3435T allele (Hoffmeyer et al. 2000). Xu et al. observed markedly higher digoxin plasma levels in subjects with the TTT–TTT in comparison to the TGC–CGC haplotype in subjects comprising the *ABCB1* C1236T, G2677T/A and C3435T single nucleotide polymorphisms (Xu et al. 2008). This observation agrees well with the haplotype dependent expression of intestinal P-glycoprotein as described by Schwarz et al. (2007).

In a prospective population-based cohort study (Rotterdam study), digoxin serum levels and DNA to genotype *ABCB1* C1236T, G2677T/A and C3435T were available from 195 participants (Aarnoudse et al. 2008). All *ABCB1* variants were significantly associated with increased serum digoxin concentration by 0.18–0.21 ng/ml per additional *T* allele. Up to 11.5% of the variability in digoxin concentrations is explained by the *TTT* haplotype. It was concluded from the obtained data, that *TTT* carriers of the combined C1236T-G2677T/A-C3435T are also at higher risk for toxic digoxin serum concentrations above 2.0 ng/ml (OR 4.3; 95% CI 1.4–13.4).

These results were contradicted by Gerloff et al. (2002), Sakaeda et al. (2001) and Kurzawski et al. (2007). Gerloff et al. (1.0 mg digoxin per os) observed no influence of *ABCB1* G2677T/A and C3435T on digoxin plasma concentrations (Gerloff et al. 2002). Sakaeda et al. (0.25 mg digoxin per os) measured even

lower plasma levels in carriers of 3435TT. (Sakaeda et al. 2001) Kurzawa et al. found no influence of *ABCB1* G2677T/A and C3435T on steady-state digoxin plasma levels (0.25 mg digoxin) in small groups of Polish patients (Kurzawski et al. 2007).

2.8 Limitations of Digoxin

Pharmacokinetic studies with digoxin require sampling (plasma, urine, feces) for at least 7 days because of the long terminal half-life of digoxin. Therefore, controlled studies with digoxin are of long duration and of high risk for dropping out and sequence effects. Furthermore, analytical problems are likely because of the small range between the average C_{max}-values of 1–5 ng/ml after single oral doses of 0.25–1.0 mg and the limit of quantification of 0.1–0.2 ng/ml provided by immunological assays; e.g. (Greiner et al. 1999; Gurley et al. 2000a). As a consequence, it is likely that the terminal elimination rate of digoxin has been underestimated in many pharmacokinetic studies, particularly after single administration of low doses. Analytical problems may be also the reason for the lack of data on fecal excretion of digoxin in clinical studies. However, the availability of highly sensitive mass-spectrometric assays will overcome these limitations in future studies (Hashimoto et al. 2008; Kirby et al. 2008; Ni et al. 2008).

Digoxin has low water solubility and high permeability and belong to the Class 2 drugs of the BCS system if the low therapeutic dose and the existence of intestinal uptake transporters for digoxin are ignored (Amidon et al. 1995; Lindenberg et al. 2004; Shugarts and Benet 2009; Wu and Benet 2005). According to the current hypothetical conception, high permeability of digoxin will allow rapid transfer into apical gut membranes but its low solubility in water will limit concentration in enterocytes, thereby preventing saturation of the efflux transporter (Wu and Benet 2005). Therefore, the suitability of digoxin to be a probe drug of P-glycoprotein must be highly influenced by the properties of the dosage form. Digoxin solution administered as soft gelatin capsules seems therefore inapplicable for mechanistic studies because its bioavailability is close to 100%, i.e., the uptake of digoxin solution into the systemic circulation by which mechanisms, however, it is mediated cannot be rate limited by the function of intestinal P-glycoprotein. Morita et al. have recently shown, that digoxin is better and more rapidly absorbed when the dose of 0.25 mg is sprinkled in 5 ml solution directly over the surface of the duodenum using an endoscope compared to swallowing a conventional tablet with 200 ml tap water (Morita et al. 2003). Obviously, intestinal P-glycoprotein is a major variable in digoxin absorption only when it appears in the apical membrane in low concentrations at which the balance between the rate of uptake can be measurably limited by the P-glycoprotein mediated efflux. However, the real concentration of digoxin at the place of absorption is unknown because it is dependent on several luminal factors as disintegration/dissolution of the dosage form, availability of water for dissolution, gastric emptying or small intestinal transit time.

So far, it is unknown what is behind high permeability of digoxin; simple nonionic diffusion or coordinate interplay of apical uptake transporters with basolateral efflux carriers, which function is dependent on expression level and drug affinity. For other organs, uptake carriers for digoxin are already discovered. Digoxin is an unique substrate of the human uptake transporter OATP1B3 (Kullak-Ublick et al. 2001; Noe et al. 1997). Therefore, variability of cellular uptake may influence the suitability of digoxin to be a probe for intestinal P-glycoprotein. For instance, digoxin uptake in rat hepatocytes and Oatp2-expressing *Xenopus oocytes* significantly decreases in the presence of amiodarone (10 μM). The same concentration of the inhibitor had only a slight inhibitory effect on the P-glycoprotein-mediated digoxin transfer via LLC-PK_1 cell monolayers (Kodawara et al. 2002). Therefore, the known increase of digoxin plasma levels after comedication of amiodarone in man may result, at least in part, from competition with the hepatic uptake of digoxin (Holt et al. 1983; Nademanee et al. 1984). For the strong OATP-inhibitor rifampicin, it was shown, that it reduces hepatic uptake and exposure of digoxin to hepatic biotransformation in rats (perfusion models), whereas quinidine increases liver cell exposure by inhibition of the canalicular P-glycoprotein (Lau et al. 2004; Shitara et al. 2002; Weiss et al. 2008). Single dose administration of rifampicin in rats may even mask the inductive effects of dexamethasone on digoxin disposition (Lam et al. 2006). However, digoxin is extensively metabolized by cytochrome P4503A in rats (>70% of an i.p. dose) (Harrison and Gibaldi 1976; Schmoldt and Ahsendorf 1980; Shitara et al. 2002). Contrary to the situation in rats, there is no convincing information from clinical studies in man, whether inhibitors of intestinal P-glycoprotein may influence hepatic uptake of digoxin. Triscari et al. investigated the interaction of digoxin (0.2 mg) and of the OATP1B1 substrate pravastatin (20 mg) at steady-state (9 days) in 18 healthy male subjects. In the presence of pravastatin, AUC, C_{max}, t_{max} and urinary excretion of digoxin were unchanged (Triscari et al. 1993). Therefore, OATP1B1-mediated hepatic uptake seems to be a minor variable in disposition of digoxin. However, some recent evidence point to an insert variant allele of the OATP1B3 gene to be associated with higher digoxin plasma concentrations in Japanese patients with terminal renal failure (Tsujimoto et al. 2008). Future clinical studies should focus on whether drug induced variability in hepatic uptake of digoxin is really a negligible limitation in drug interaction studies with digoxin in healthy subjects, even though the drug is nearly unmetabolized and only a small dose portion enters the entero-hepatic circle (less than 10%).

Another problem arises in multiple-dose studies with digoxin, because continuous exposure to the drug might induce P-glycoprotein expression in concentration-dependent manner as shown in Caco-2 cells (Takara et al. 2002a, b). Taken into mind that the magnitude of P-glycoprotein induction seems to be lower in carriers of variant ABCB1 alleles, the genotype related differences at steady-state as described by Hoffmeyer et al., Johne et al., and Aarnoudse et al. may have been overestimated or they result entirely from genotype-related extent of transporter up-regulation (Aarnoudse et al. 2008; Hoffmeyer et al. 2000; Johne et al. 2002).

Distribution volume is another critical P-glycoprotein-related variable in digoxin disposition. Carriers of the *ABCB1* 3435TT genotype have a lower apparent

distribution volume for digoxin compared to carriers of the 3435CC allele as recently demonstrated using nonlinear mixed-effect model simulations. That means, that the genotype related AUC differences in the study of Verstuyft et al. resulted entirely from changes in distribution volume rather than from difference in digoxin absorption (Comets et al. 2007; Verstuyft et al. 2003). It is also to consider that the half-life depends on the distribution volume and is not a suitable measure for intestinal P-glycoprotein function. Therefore, subtle pharmacokinetic phenotyping with digoxin needs a study arm with intravenous digoxin to exclude P-glycoprotein-related variations in distribution volume.

The major elimination route for digoxin from the systemic circulation is glomerular filtration. Up to 50% of the excretion of digoxin accounts to renal tubular secretion as the glomerular filtation rate in healthy subjects is approximately 120 ml/min compared to the renal clearance of 140–160 ml/min and considering the unbound fraction in plasma being 70–80%. Two active transporter in renal tubular cells were identified so far; the apical efflux carrier P-glycoprotein and the recently discovered organic anion transporter OATP4C1, which serves as a basolateral uptake transporter for cardiac glycosides (digoxin $k_m = 7.7$ μM; ouabain $k_m = 0.38$ μM) at the proximal tubule cells in the human kidney (de Lannoy and Silverman 1992; Ito et al. 1993b; Mikkaichi et al. 2004; Tanigawara et al. 1992). Renal P-glycoprotein can be influenced by the known P-glycoprotein inhibitors quinidine, verapamil, vinca alkaloids, cyclosporine, clarithromycin or ritonavir as shown by in vitro studies using kidney epithelial cell lines and by various animal models (de Lannoy et al. 1992; Hori et al. 1993; Ito et al. 1993b; Okamura et al. 1993; Tanigawara et al. 1992; Wakasugi et al. 1998). It is unknown so far, whether P-glycoprotein inhibitors modulate the basolateral transporter OATP4C1.

Inhibition of renal P-glycoprotein has to be assumed if the AUC-values of conventional digoxin tablets increase by more than 43% after comedication of inhibitors. Digoxin AUC increases after clarithromycin (250 mg, b.i.d., 3 days) by 64%, after intraconazole (200 mg once daily, 5 days) by 67%, and after valspodar (PSC833) by 74% (400 mg, single dose) and 200% (200 mg twice daily, 4 days), respectively (Jalava et al. 1997; Kovarik et al. 1999; Rengelshausen et al. 2003). In order to differentiate, whether interaction with intestinal or renal P-glycoprotein or interaction with both has caused higher digoxin systemic exposure, a careful pharmacokinetic evaluation is required which includes determination of bioavailability, renal and nonrenal clearance and the amount excreted into urine. For example, in presence of itraconazole, the amount of digoxin excreted into the urine significantly increased by 22% although renal clearance significantly decreased by 20% (Jalava et al. 1997). Itraconazole obviously influenced both intestinal and renal P-glycoprotein. Therefore, inhibition of intestinal P-glycoprotein is the reason for higher AUC-values only if changes of renal clearance are excluded by the interaction study. It is also important to recognize that many drugs given concomitantly with digoxin may influence renal blood flow and renal digoxin clearance independent of whether they interact with P-glycoprotein as suggested for nitropusside, hydralazine or captopril (Cleland et al. 1986; Cogan et al. 1981; Mujais et al. 1984).

Another, more or less theoretical limitation for digoxin as a probe drug for intestinal P-glycoprotein results from its biotransformation because about 15% of an oral dose are metabolized by sugar cleavage and glucuronidation, a significant part by presystemic processes (Hinderling and Hartmann 1991; Lacarelle et al. 1991). A borderline correlation was observed between plasma AUC of oral digoxin and intestinal CYP3A content ($r = -0.54$, $p < 0.05$) in the interaction study with rifampicin mentioned above (Greiner et al. 1999). This correlation may have reflected coregulation of intestinal P-glycoprotein and CYP3A4 by rifampicin (Urquhart et al. 2007). So far, there is no evidence from literature that metabolism of digoxin is significantly influenced by inducers and/or inhibitors of P-glycoprotein in man.

It has been reported that digoxin undergoes colonic bacterial degradation by *Eubacterium lentum*. This may result in increased digoxin exposure in case of comedication with antimicrobial drugs (Dobkin et al. 1982; Lindenbaum et al. 1981). It seems however unlikely that eradication of *Eubacterium lentum* may influence bioavailability of immediate release digoxin, because the germ is rarely found in the jejunum and ileum of healthy subjects (Simon and Gorbach 1984).

3 Talinolol

3.1 Safety, Physicochemical Properties and Pharmacokinetics

Talinolol (Cordanum®) is a selective postsynaptic ß$_1$-adrenoceptor antagonist without partial agonistic activity which was launched on the German market for treatment of arterial hypertension and coronary heart disease. The oral standard dose of 100 mg is safe and well tolerated. There are no reports on drug related adverse events being probably or likely related to the study medication in single-dose and repeated-dose studies in healthy subjects applying doses of 50–100 mg talinolol (Giessmann et al. 2004a; Siegmund et al. 2002a; Siegmund et al. 2003; Westphal et al. 2000a, b). Even single doses of 400 mg were well tolerated (De Mey et al. 1995).

Talinolol is highly soluble in water (pH 7.4: 1.24 g/l; pH 7.0: 4.5 g/l; 37°C) and has a pH-dependent partition coefficient (pH 7.4: logP 1.08; 37°C). Accordingly, it belongs to the class III (high solubility, low permeability) of the BCS classification system (Amidon et al. 1995; Le Petit 1985). The drug is erratically and incompletely absorbed from the gastrointestinal tract. The absolute bioavailability is about 55–70%. It is widely distributed (V_d = 3–6 l/kg) and 50–70% are bound to plasma proteins (Giessmann et al. 2004a; Siegmund et al. 2002a; Trausch et al. 1995; Westphal et al. 2000a, b). A characteristic finding in talinolol pharmacokinetics after oral administration is the double-peak phenomenon which is probably caused by an intestinal "storage pathway" beyond the gut lumen (Weitschies et al. 2005). After repeated-dose administration of 100 mg talinolol (5–7 days), the

minimum (trough) serum concentrations are approximately 45–60 ng/ml, maximum (peak) concentrations are about 300 ng/ml and the AUC_{0-24h} are in the range of 3,000 ng h/ml at steady-state (Giessmann et al. 2004a; Westphal et al. 2000b). Talinolol is nearly not metabolized (Giessmann et al. 2004a; Siegmund et al. 2002a; Westphal et al. 2000a, b). Small amounts (below 1% of the dose) of 4-*trans* and 3-*cis* hydroxytalinolol are generated by CYP450 dependent hydroxylation of the cyclohexyl ring; 2-*trans* and 3-*trans* isomers have in some cases also been detected. Hydroxylation of the phenyl ring, degradation of the side chains or conjugation of talinolol and the metabolites do not occur (Oertel et al. 1994; Schupke et al. 1996). Significant chiral differences in the metabolism were not observed (Wetterich et al. 1996; Zschiesche et al. 2002). Pretreatment with rifampicin significantly increases the metabolic clearance of talinolol in healthy subjects; however, metabolism still accounts for less than 1% of the total body clearance. The elimination half-life of talinolol is between 10 and 17 h (Giessmann et al. 2004a; Siegmund et al. 2002a, b; Trausch et al. 1995; Westphal et al. 2000a, b). 3.7–25% (median: 9.3%) of intravenous talinolol (30 mg) is excreted into the bile (six cholecystectomized patients) with slight preference of the S(−)-enantiomer. The biliary concentrations are up to 90-fold above the serum levels (Terhaag et al. 1989). After intravenous administration (30 mg, 18 healthy subjects), about 43% of the dose are excreted with the urine, and about 22% is recovered in the feces. After oral administration (100 mg), about 30% are excreted with the urine and another 30% appear in the feces (Bernsdorf et al. 2006). Renal clearance is about 150–190 ml/min which is higher than the product of nonprotein bound talinolol ($f_u \sim 0.4$) and filtration rate in healthy subjects (~120 ml/min) (Bernsdorf et al. 2006; Giessmann et al. 2004a; Siegmund et al. 2002a; Westphal et al. 1996, 2000b). Therefore, active tubular secretion must contribute to the renal elimination. There is a small intrasubject variability but high intersubject variability of all talinolol pharmacokinetic characteristics as shown in a bioavailability study with four bioequivalent tablet formulations (Siegmund et al. 2003).

3.2 Affinity to P-glycoprotein In Vitro and in Animal Studies

Talinolol shares the typical physicochemical properties and in vitro findings of common P-glycoprotein substrates (Tables 1 and 5). In transport experiments using Caco-2 cell monolayers, the transport rates of talinolol were 2-fold to 26-fold higher in the b → a as compared to the a → b direction. The differences are significantly decreased or abolished in the presence of strong inhibitors of P-glycoprotein as verapamil, LY335984, constituents of grapefruit juice or surfactants (Bogman et al. 2005; de Castro et al. 2007; El Ela et al. 2004; Hayeshi et al. 2008; Ingels et al. 2004; Ofer et al. 2005). Permeability in both directions is not stereoselective (Wetterich et al. 1996). The affinity of talinolol to P-glycoprotein was also confirmed in monolayer studies using Caco-2 and LLC-PK1 cells transfected with human *ABCB1*. In Caco-2 cells, the b → a permeability for R(+)

Table 5 In-vitro studies with talinolol and comparative in vitro studies with digoxin and talinolol to show affinity to P-glycoprotein

Cell model	Apical-to-basal (a → b) and basal-to-apical (b → a) transport via cell monolayer		References
Caco-2	S(−) talinolol (200 μM)	a → b: 181 ± 118 ng/cm^2 h, b → a: 1,736 ± 314 ng/cm^2 h, ratio: 9.6	Wetterich et al. (1996)
	R(+) talinolol (200 μM)	a → b: 200 ± 109 ng/cm^2 h, b → a: 1,737 ± 352 ng/cm^2 h, ratio: 8.7	
	Presence of verapamil (0.5 mM)	S(−) talinolol, a → b: 457 ± 0.5 ng/cm^2 h, b → a: 645 ± 17.6 ng/cm^2 h, ratio: 1.41 R(+) talinolol, a → b: 459 ± 14.9 ng/cm^2 h, b → a: 639 ± 58.8 ng/cm^2 h, ratio: 1.39	
Caco-2	rac. Talinolol (1 mM)	a → b: 0.17 × 10^{-6} cm/s, b → a: 6.1 × 10^{-6} cm/s, ratio: 36 a → b: 1.93 × 10^{-6} cm/s (in presence of 0.5 mM verapamil) a → b: 0.66 × 10^{-6} cm/s (in presence of 50% grapefruit juice)	Spahn-Langguth and Langguth (2001)
Caco-2	rac. Talinolol (100 μM)	a → b: 1.9 × 10^{-7} cm/s, b → a: 14 × 10^{-7} cm/s, ratio: 7.4	de Castro et al. (2007)
	IC_{50} for inhibition b → a	Verapamil 28 μM, grapefruit juice 0.6%, naringin 2,409 μM, naringenin 236 μM, dihydroxybergmottin 34 μM, epoxybergamottin 0.7 μM	
Caco-2	Efflux ratios for talinolol (30 μM) between 2.05 and 25.6 (bach 1) and 1.03 and 13.05 (bach 2) from 10 laboratories		Hayeshi et al. (2008)
ABCB1-Caco-2	S(−) talinolol (250 μM)	a → b: 23 ± 8.9 pM/cm^2 h, b → a: 1,282 ± 73 pM/cm^2 h, ratio: 55	Doppenschmitt et al. (1999)
	R(+) talinolol (250 μM)	a → b: 18.1 ± 8.4 pM/cm^2 h, b → a: 1,253 ± 60 pM/cm^2 h, ratio: 69	
ABCB1-LLC-PK1	rac. Talinolol (10 mM)	a → b: 1.09 × 10^{-7} cm/s, b → a: 5.33 × 10^{-7} cm/s, ratio: 4.89 a → b: 1.57 × 10^{-7} cm/s, b → a: 4.08 × 10^{-7} cm/s, ratio: 2.60 (with 10 μM cyclosporine)	Shirasaka et al. (2010)
Caco-2	Digoxin (2 nM)	a → b: 4.8 ± 0.2 × 10^{-6} cm/s, b → a: 40.4 ± 2.6 × 10^{-6} cm/s, ratio: 8.4	Neuhoff et al. (2003)
	Talinolol (10 μM)	a → b: 4.1 ± 0.4 × 10^{-6} cm/s, b → a: 24.8 ± 1.9 × 10^{-6} cm/s, ratio: 6.0	
Caco-2	Digoxin	a → b: K_m = 1,150 ± 179 μM, J = 718 ± 2.38 pM/min, efflux activity 10.5±1.70 × 10^{-6} cm/s b → a: K_m = 177 ± 9.2 μM, J = 434 ± 97.4 pM/min, efflux activity 40.9 ± 11.0 × 10^{-6} cm/s	Troutman and Thakker (2003)
	Talinolol	a → b: K_m = 414 ± 60.4 μM, J = 212 ± 19.7 pM/min, efflux activity 8.62 ± 0.5 × 10^{-6} cm/s b → a: K_m = 103 ± 5.1 μM, J = 200 ± 20.5 pM/min, efflux activity 32.5 ± 5.1 × 10^{-6} cm/s	

talinolol and S(−) talinolol was about 55-fold to 70-fold higher than the a → b permeability (Doppenschmitt et al. 1999). In LLC-PK1 cells, the b → a permeability was 4.9-fold higher than the a → b permeability; the ratio decreased to 2.60 in presence of strong P-glycoprotein inhibitor cyclosporine A (10 μM) (Shirasaka et al. 2009). In 2003, Neuhoff et al. compared the transport properties of talinolol, quinidine and digoxin across Caco-2 monolayers in dependence of the pH (Neuhoff et al. 2003). The transport characteristics of talinolol and digoxin at pH 7.4 were very similar: Talinolol, P_{app} (a → b) 4.13 ± 0.4 × 10^{-6} cm/s, P_{app} (b → a) 24.8 ± 1.9 × 10^{-6} cm/s, efflux ratio 6.0; digoxin, P_{app} (a → b) 4.8 ± 0.2 × 10^{-6} cm/s, P_{app} P_{app} (b → a) 40.4 ± 2.6 × 10^{-6} cm/s, efflux ratio 8.4. In another comparative in vitro study with Caco-2 monolayers including digoxin and talinolol, the apparent K_m, flux rates (J) and the intrinsic ABCB1-mediated efflux activity for the absorptive and secretory transport was determined. The K_m-values for digoxin and talinolol in the a → b direction and b → a direction were in the same order of magnitude (Troutman and Thakker 2003).

Talinolol seems to be a week inhibitor of P-glycoprotein. It increased digoxin a → b transport (10 μM) about twofold with IC_{50} of 198 ± 11 μM in Caco-2 cells (Collett et al. 2005). In vinblastine-induced Caco-2 cells using a radioligand assay with verapamil, the IC_{50} value for racemic talinolol was 2,271 ± 335 μM compared to 2.11 ± 0.47 μM for racemic verapamil (Neuhoff et al. 2000). In another study, the IC_{50} for talinolol was 1,064 ± 105 μM compared to 303 ± 26 μM for quinidine, 34 ± 1.2 μM for vinblastine and 2.5 ± 0.3 μM for verapamil (Doppenschmitt et al. 1999). There seems to be also a binding site with higher affinity for talinolol (Doppenschmitt et al. 1998; Doppenschmitt et al. 1999).

The following observations support the hypothesis that talinolol is a substrate of intestinal P-glycoprotein in animals:

1. In everted sacs of rat ileum, the serosal-to-mucosal permeability of talinolol exceeds the mucosal-to-serosal permeability. The differences are abolished after incubation with verapamil (Spahn-Langguth et al. 1998).
2. Transporter induction with rifampicin results in lower intestinal permeability of talinolol in the duodenum, jejunum and colon of rats (in situ perfusion) (Hanafy et al. 2001). In the contrary, the small instinal permeability of talinolol (100 μM) is fivefold increased in the presence of verapamil (150 μM) both in Wistar rats and in NMR1 mice (Mols et al. 2009). Furthermore, bioavailability of oral talinolol in rats (10 mg/kg) is significantly increased after comedication of grapefruit juice or some of its constituents like the P-glycoprotein inhibitors narigin or bergamottin (de Castro et al. 2007, 2008; Spahn-Langguth and Langguth 2001).
3. In *Abcb1a/1b*(−/−) knock-out mice, the apparent permeability in perfused intestinal tissue is sevenfold increased for talinolol but not for atenolol or metoprolol (Mols et al. 2009). The plasma levels of talinolol in knock-out mice after oral administration exceeded nearly threefold the levels in wildtype mice (Schwarz et al. 2001).

3.3 Evidence from Mechanistic Clinical Studies

Absorption of talinolol in doses between 25 mg and 50 mg is controlled by a capacity-limited process, as shown in a dose-escalation study with 25, 50, 100 and 400 mg talinolol in 12 healthy subjects. Total body clearance of talinolol decreased in the range 25 mg > 50 mg > 100 mg = 400 mg while terminal half-life remained unchanged. It was hypothesized by the authors that P-glycoprotein mediated efflux transport was the underlying intestinal process that saturates in subtherapeutic doses of talinolol (Wetterich et al. 1996). The disposition of talinolol in single oral doses above 100 mg is not dose-dependent as confirmed by a bioequivalence study with doses of 100 mg and 200 mg (Siegmund et al. 2003). Accordingly, for pharmacokinetic studies in healthy subjects, immediate release talinolol in doses of 50–100 mg seems to be the adequate study medication under standard conditions (overnight fasting, upright position, 240 ml water) to provide substrate concentrations on the receptor site which just saturates the efflux transporter.

Gramatté and coworkers have been the first proving active intestinal secretion of talinolol in man using an intestinal steady-state perfusion method (triple lumen tubing technique) (Gramatte et al. 1996; Gramatte and Oertel 1999). The appearance of talinolol in the upper jejunum (110–130 cm beyond the teeth) was measured after intravenous infusion of 25 mg within 140 min in six healthy subjects (Gramatte and Oertel 1999). During all intestinal perfusions, the mean intraluminal talinolol concentrations were 2.4-fold to 7.5-fold (median, 5.5-fold) higher than the corresponding serum concentrations; i.e., talinolol was secreted into the gut lumen against a steep concentration gradient. The intestinal secretion rate ranged from 1.94 to 6.62 μg/min per 30 cm jejunum. The luminal concentration could not be explained by base-trapping; the expected concentration gradient at the measured intraluminal pH 7.14 is 1.9. Therefore, intestinal secretion of talinolol must have been caused by an active process that is susceptible to inhibition by R-verapamil, a strong inhibitor of P-glycoprotein (Haussermann et al. 1991; Noviello et al. 1997). This was concluded from the finding that the intestinal secretion rates decreased by 44–71% when R-verapamil (565 μM) was perfused into the jejunal lumen by the tube system. The intestinal secretion of talinolol was not influenced by fluid secretion (solvent drag); talinolol was secreted even at high fluid absorption rates.

Active intestinal secretion was also confirmed for talinolol administered directly into the gut lumen in six healthy subjects (steady-state perfusion with triple lumen tubes) (Gramatte et al. 1996). To evaluate regional differences, 625 μg talinolol per minute were infused for 160 min while the test segment of the tube was located between 95 and 115 cm and, in a second study period, between 160 and 235 cm beyond the teeth. In both positions, the talinolol transport rates were in linear correlation to the amount of talinolol perfused per time unit (intestinal perfusion rate). During each distal perfusion, however, there was a shift of this relationship towards higher perfusion rates; that means, to achieve the same talinolol absorption rate in the more distal intestinal regions, a higher perfusion rate was needed.

The authors concluded from the regional differences, that there is site-dependent transepithelial transport of talinolol along the small intestine of humans. Interestingly, in about 20% of the measurements, the amount of talinolol leaving the intestinal test segment was greater than the amount that had entered it. At perfusion rates below 600 µg/min, already absorbed talinolol was back-secreted (negative transport rate values) into the gut lumen against a steep concentrations gradient of about 4,200. Extrapolating the secretion rates in the test segment to the length of the small intestine (3–5 m) and ignoring regio-selective expression of P-glycoprotein, about 30–40 mg talinolol have been secreted per hour from the systemic circulation into the small intestinal tract. Regio-selective absorption of talinolol was also confirmed by the data on systemic availability of talinolol that was simultaneously measured. AUC and C_{max} decreased with increasing distance of the perfusion port from the teeth by up to 85%. The results of both perfusion studies reflected the findings on regio-selective expression of intestinal P-glycoprotein published several years later (Englund et al. 2006; Zimmermann et al. 2005).

3.4 Talinolol Disposition and Induction of Intestinal P-glycoprotein

Further clinical evidence for the applicability of talinolol as a probe drug for intestinal P-glycoprotein came from interaction studies with rifampicin and SJW (Schwarz et al. 2007; Westphal et al. 2000b). The effect of rifampicin induction (600 mg, 9 days) on the pharmacokinetics of talinolol after intravenous (30 mg) and repeated oral administration (100 mg, 14 days) was investigated in eight male healthy volunteers (Westphal et al. 2000b). After up-regulation, higher systemic clearance, shorter half-life and somewhat higher nonrenal clearance (not significant) after intravenous administration were observed. Furthermore, up-regulation of P-glyoprotein was associated with lower bioavailability by 21%. The amount of oral talinolol excreted into urine decreased by 25%. Renal clearance remained unchanged. About 50% of the systemic clearance of intravenous talinolol was predicted by the content of duodenal P-glycoprotein. As the renal clearance of talinolol was not influenced by rifampicin induction, the increase in systemic clearance has to be attributed to the intestinal clearance. The effects of rifampicin were even more pronounced after oral administration. Cmax, AUC and bioavailability were reduced by 38%, 35% and 20%. Despite markedly lowered serum concentrations, the ß-blocking effect as assessed by bicycle exercise was not significantly decreased. The effect of transporter induction with SJW (900 mg, 12 days) on disposition of intravenous (30 mg) and oral talinolol (50 mg) was evaluated in nine male healthy subjects (Schwarz et al. 2007). After SJW, nonrenal clearance of intravenous talinolol increased by 35%. Bioavailability was reduced by 25% and the amount excreted into urine by 44%. Nonrenal clearance tended to increase by 36%. Although there was no correlation between pharmacokinetic parameters of talinolol and intestinal *ABCB1*mRNA levels, the pattern of

pharmacokinetic changes pointed to induction of intestinal P-glycoprotein to be the rationale behind the effects after consumption of SJW.

3.5 Talinolol Disposition and Inhibition of Intestinal P-glycoprotein

The impact of P-glycoprotein inhibition on pharmacokinetics of oral talinolol (50 mg) was investigated in a study with erythromycin (2.0 g) (Eberl et al. 2007; Schwarz et al. 2000). In the presence of erythromycin, the $AUC_{0–24h}$, the C_{max} and the amount of talinolol excreted into urine were increased by 52%, 26% and 36%, respectively. Renal clearance remained unchanged. T_{max} and half-life were reduced by 46% and 16%, respectively, most likely as result of faster talinolol absorption. The higher serum concentrations ($AUC_{0–24h}$) and the higher amount of talinolol in urine are most likely caused by a strong, possibly even complete, inhibition of intestinal P-gp and, consequently, complete absorption of talinolol from gut lumen. The faster absorption of talinolol results obviously from faster gastric emptying as cause by the prokinetic effect of erythromycin (Keshavarzian and Isaac 1993).

A comparable influence on the extent of oral talinolol absorption was observed in nine healthy subjects when talinolol (50 mg) was swallowed with the surfactant D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS), an inhibitor of P-glycoprotein in vitro. In the presence of TPGS, the $AUC_{0–\infty}$ and the C_{max} of talinolol increased (20% and 36%, respectively). The elimination half-life remained unchanged. Quite similar changes in pharmacokinetics of talinolol (increased $AUC_{0–\infty}$ and C_{max}, unchanged half-life) were obtained in studies with healthy Chinese subjects after treatment (14 days) with silymarin (420 mg), a purified extract from the seeds of milk thistle, and with extracts of *Schisandra chenensis* (600 mg) and *Ginkgo biloba* (360 mg) that were shown to inhibit P-glycoprotein in vitro (Fan et al. 2009a, b; Han et al. 2009; Wan et al. 2006; Wang et al. 2005; Zhang and Morris 2003a, b).

3.6 Regioselective Absorption of Talinolol

Regio-selective absorption from the gastrointestinal tract is a characteristic feature of substrates of intestinal P-glycoprotein. Such a behavior was also shown for talinolol (100 mg) prepared in immediate release hard gelatin capsules and enteric-coated sustained release hard gelatin capsules (Weitschies et al. 2005). Enteric-coated capsules are known to disintegrate predominantly in the distal jejunum or the ileum where the expression of P-glycoprotein is increased (Englund et al. 2006; Wilding et al. 1992; Zimmermann et al. 2005). The capsules contained additionally 100 mg paracetamol to assess the intestinal site of capsule disintegration; paracetamol is rapidly and completely absorbed from all parts of the intestine (Naslund et al. 2000). Enteric-

coated paracetamol appeared in serum with a lag-time of about 3 h and reached maximum concentrations after about 4 h; uncoated paracetamol appeared after 1 h. In accordance with the conception that expression of P-glycoprotein is increased along the small intestine, talinolol absorption from enteric-coated capsules was reduced by about 50% compared to absorption from immediate release capsules, whereas the absorption of paracetamol remained unchanged.

3.7 *Talinolol as a Probe Drug for Genetic Polymorphisms of P-glycoprotein*

In 37 healthy Caucasian subject, no impact of nine *ABCB1* polymorphisms including C1236T, G2677T/A, and C3435T on duodenal expression of P-glycoprotein was observed. Despite larger interindividual variability in expression of ABCB1 in the noninduced subjects, the AUC-values of talinolol were significantly higher in subjects with the 2677TT/TA genotype compared to carriers with at least one wild-type allele (Siegmund et al. 2002b). Interestingly, in an interaction study with SJW, the *ABCB1* genotype appeared to be associated with lower basal expression of intestinal P-glycoprotein and lower magnitude of induction by SJW. Subjects with the combined *ABCB1* genotype comprising C1236T, G2677T/A, and C3435T polymorphisms had a lower basal intestinal mRNA content and were less inducible by SJW. Individuals that are heterozygous for the synonymous C1236T in exon 12 showed a significantly lower decrease in the $AUC_{0-\infty}$ of oral talinolol compared to wild-type subjects. Subjects that are heterozygous for the nonsynonymous G2677T/A had a lower decline in bioavailability after SJW compared with wild-type (Schwarz et al. 2007).

3.8 *Limitations of the Application of Talinolol as a Probe Drug*

Soon after publication of convincing evidence on active intestinal secretion of talinolol in man, the same group presented some unexpected pharmacokinetic observations that challenge the conception that talinolol is a selective probe-drug for intestinal P-glycoprotein (Gramatte et al. 1996; Gramatte and Oertel 1999; Schwarz et al. 1999). After concomitant oral administration of talinolol (50 mg) and R-verapamil (120 mg) in nine subjects, a significantly reduced AUC_{0-24h}, a lower renal excretion (both by 24%) was observed, whereas half-live and renal clearance were not changed. The anticipated effect in the presence of R-verapamil was an increase rather than a decrease of talinolol bioavailability because R-verapamil is an inhibitor of P-glycoprotein (Haussermann et al. 1991; Noviello et al. 1997). Interestingly, t_{max} was shorter in the presence of verapamil (1.5 ± 1.0 h vs. 3.2 ± 0.8 h) and one distinct early concentration maximum appeared instead of the characteristic second peak after 3–5 h. Talinolol was

obviously better absorbed in proximal regions of the small intestine, probably by inhibition of intestinal P-glycoprotein in the presence of verapamil. Verapamil was very rapidly absorbed and reached maximum concentrations just at the time when the initial talinolol peak appeared. At that time, the authors could not explain the absence of the expected delayed absorption peak of talinolol after verapamil comedication. However, lower instead of higher talinolol absorption in the presence of verapamil was also measured in *Abcb1a*/*b*(−/−) knock-out mice (Schwarz et al. 2001). As expected, the plasma levels of talinolol in the deficient animals were about threefold higher than in wild-type animals. Surprisingly, coadministration of verapamil (16 mg/kg) resulted in significantly lower talinolol concentrations in both groups and not, as expected after "chemical Abcb1 knock-out", in better absorption and higher plasma levels in wild-type rats, similar to the situation in nonpretreated *Abcb1a*/*b*(−/−) knock-out mice. The authors concluded that inhibition of an intestinal uptake transport may overshadow the effects of verapamil on P-glycoprotein.

Further evidence for the existence of an unknown uptake transporter for talinolol came from a clinical study with talinolol (50 mg) in 24 healthy subjects after single (300 ml) and repeated ingestion (900 ml/day for 6 days) of grapefruit juice. Under both conditions, $AUC_{0-\infty}$, C_{max} and urinary excretion of talinolol were lowered by more than 50% whereas half-life and renal clearance remained unchanged. These findings were contrary to the study hypothesis to which the authors had expected higher bioavailability of talinolol because grapefruit juice and its constituents inhibit the unidirectional talinolol transport in Caco-2 cells and increase talinolol absorption in rats (de Castro et al. 2007, 2008; Spahn-Langguth and Langguth 2001). Otherwise, grapefruit juice is a potent inhibitor of human OATP1A2 (competition assay with fexofenadine uptake in HeLa cells) at concentrations (0.5–5%) that had no effect on P-glycoprotein (competition assay with vinblastine in L-MDR1 and LLC-PK1 cells) (Dresser et al. 2002). One active ingredient of grapefruit juice seems to be narigin that inhibits fexofenadine uptake in HeLa cells transfected with human OATP1A2 nearly as potent as verapamil (IC_{50} 3.6 μM versus 2.6 μM) (Bailey et al. 2007). Because human OATP1A2 seems to be the only intestinal uptake transporter for fexofenadine and grapefruit juice and narigin decrease the $AUC_{0-\infty}$ and C_{max} of fexofenadine in healthy subjects by at least 40%, it can be speculated that OATP1A2 is also an intestinal uptake transporter for talinolol in man (Bailey et al. 2007; Dresser et al. 2002, 2005; Glaeser et al. 2007). With regard to the double-peak phenomenon of talinolol, it should be mentioned that OATP1A2 is highly expressed in the ileum but low in the jejunum (Meier et al. 2007).

This conception is supported by recent in vitro studies using *Xenopus laevis* oocytes expressing rat Oatp1a5 which is the closest equivalent to human OATP1A2. (Shirasaka et al. 2010). Talinolol was taken up into the oocytes by the saturable Oatp1a5 (K_m = 2 mM). Naringin was a strong inhibitor of the uptake (IC_{50} = 12.7 μM). In contrast, the narigin IC_{50} for inhibition of the P-glycoprotein mediated b → a permeability of talinolol in Caco-2 cells was much higher (2 mM) (de Castro et al. 2008). In line with these data, naringin in low concentrations (200 μM) lowered the permeability of talinolol in rat small intestine (in situ closed loop method) by

about 40% (inhibition of Oatp1a5). In the presence of high naringin concentrations (2 mM), permeability increased by 75% (inhibition of P-glycoprotein). The effect of naringin on talinolol (10 mg/kg) absorption in rats was also concentration dependent; 20 μM naringin were without effect, 50 μM naringin lowered the $AUC_{0–6h}$ by 57%, but 200 μM and 2,000 μM naringin increased talinolol absorption by approximately 90% (Shirasaka et al. 2009). It can be concluded from these rat experiments, that intestinal absorption of talinolol is obviously influenced by an uptake transporter of the OATP-family, which is more sensitive to inhibition by constituents of grapefruit juice than the intestinal P-glycoprotein transporter. The species differences in the effects of grapefruit juice on absorption of talinolol in man and rats results most likely from the species differences in the affinity of naringin to OATPs and P-glycoprotein (Shirasaka et al. 2009). Very recently, Bolger et al. have simulated nonlinear talinolol absorption using the ACAT model in the GastroPlus software using data on regio-selective expression of P-glycoprotein and OATP1A2 as variables (Bolger et al. 2009; Tubic et al. 2006).

Further candidates for intestinal talinolol uptake may be member(s) of the multidrug organic cation transporter (OCT) family for which other ß-adrenergic antagonists are substrates and verapamil is an inhibitor (Dudley et al. 2000; Zhang et al. 1998). Talinolol was found to be an inhibitor of the [^{14}C]tetraethylammoniumbromide (TEA) uptake in LLC-PK_1 cells expressing OCT2. The IC_{50} value was 150 μM compared to 18 μM, 38 μM and >5,000 μM for verapamil, naringenin and naringin, respectively. However, little is known on expression and functional meaning of OCTs in the human intestine. Therefore, it is entirely speculative to conclude from the in vitro date in LLC-PK1 cells on the role of OCTs in talinolol absorption and whether the unexpected decrease of talinolol absorption after concomitant administration of verapamil and grapefruit juice have resulted (at least in part) from inhibition of intestinal uptake carriers of the OCT multidrug transporter family.

There is evidence from a pharmacokinetic study in *Abcc2*-deficients rats (GY-TR$^-$) that talinolol is a substrate of the efflux transporter ABCC2 (MRP2) (Bernsdorf et al. 2003). Absorption of talinolol was also shown to be influenced by *ABCC2* gene polymorphisms (Haenisch et al. 2008). However, there was also shown to be a wide overlapping of substrate spectrum of P-glycoprotein and MRP2, intestinal P-glycoprotein and MRP2 are coregulated via the same nuclear receptor signal pathway and most of the P-glycoprotein inhibitors are also inhibitors of MRP2 (Fromm et al. 2000; Giessmann et al. 2004b; Urquhart et al. 2007).

4 Conclusions and Recommendations

4.1 Selectivity for Intestinal P-glycoprotein

Digoxin and talinolol are substrates of P-glycoprotein as confirmed in different cell models (Caco-2, LLC-PK1, L-MDR1, MDR1-MDCKII) and in *Abcb1* knock-out mice. Digoxin and talinolol have similar affinity to P-glycoprotein as shown in

transport studies using Caco-2 cells monolayers. Digoxin seems not to be an inhibitor of P-glycoprotein; talinolol has low inhibitory potency compared to standard inhibitors of P-glycoprotein (verapamil, vinblastine, quinidine and valspodar). Digoxin is also a substrate of the hepatic uptake transporter OATP1B3 and of the basolateral uptake transporter OATP4C1 in the proximal tubule cells in the human kidney. There is evidence, that talinolol is also a substrate of ABCC2, OATPs (e.g. OATP1A2) and OCTs (e.g. OCT2).

Intestinal transfer of digoxin and talinolol in animals is significantly influenced by the function of P-glycoprotein; this was shown by competition studies using in situ intestinal perfusion models. In human beings, digoxin and talinolol are secreted against steep concentrations gradients into the lumen of the small intestine by a mechanism that can be influenced by inhibitors and inducers of P-glycoprotein as experimentally confirmed by intestinal perfusion methods. Intestinal P-glycoprotein is a rate-limiting process in the pharmacokinetics of digoxin and talinolol; approximately 50% of the AUC of oral digoxin and of intravenous talinolol can be predicted by expression of P-glycoprotein in the duodenum. Inhibition of intestinal P-glycoprotein (e.g. verapamil, erythromycin) leads to higher bioavailability whereas up-regulation of the intestinal efflux transporter (e.g. by rifampicin, SJW) results in lower bioavailability and increased intestinal (nonrenal) excretion of digoxin and talinolol.

The function of P-glycoprotein in other organs may influence distribution volume of the probe drugs, as hypothesized at least for digoxin. Major influence on disposition of digoxin, however, comes from P-glycoprotein function in the kidneys as confirmed in drug interactions studies with valspodar, ritonavir, or quinidine. For talinolol, variability of renal P-glycoprotein function might be of minor influence.

Digoxin and talinolol are nearly not metabolized and moderately bound to plasma protein. Therefore, the function of hepatic uptake transporters (e.g. OATP1B1, OATP1B3), hepatic P-glycoprotein and drug metabolizing enzymes and changes in protein binding may not significantly limit the suitability of digoxin and talinolol as a measure of intestinal P-glycoprotein.

4.2 Limitations Resulting from Intestinal Uptake Mechanisms

The net uptake of digoxin and talinolol obviously results from the difference of the intestinal uptake capacity minus the P-glycoprotein mediated efflux capacity. The intestinal uptake mechanism(s) for digoxin and talinolol, however, are not identified so far. For digoxin, there must be a so far unknown low-affinity high-capacity uptake transporter system which dominates the intestinal net absorption at concentrations that can be reached in the apical membrane after administration a single therapeutic dose of digoxin in solution; in that scenario, digoxin is nearly completely absorbed. The intestinal P-glycoprotein transporter can constrain the net uptake by approximately 30%, if the membrane concentrations of the slowly and poorly water soluble digoxin are relatively lower, e.g. after administration of

conventional 0.25 mg tablets. In that alternative scenario, the uptake transporter is activated to a lower extent leading to significant reduction of overall uptake caused by P-glycoprotein to which digoxin has obviously higher affinity than to the uptake transport system. So far, there is no information whether factors that influence P-glycoprotein function (e.g. inhibitors or inducers) may also influence the digoxin uptake mechanism(s).

In case of talinolol, intestinal P-glycoprotein seems to be just saturated by test doses of 50–100 mg in solution or immediate release tablets which bioavailability is about 55–70%; lower doses are of lower bioavailability. There is strong evidence that intestinal uptake of talinolol is mediated by a member(s) of the OAPT-family (e.g. OATP1A2) which is susceptible to inhibition by substances known to be also inhibitors of P-glycoprotein just at common doses (e.g. R-verapamil, constituents of grapefruit juice). Therefore, inhibition of the intestinal uptake transporter(s) leading to lower bioavailability may overshadow the functional outcome of P-glycoprotein inhibition, which is increased bioavailability of talinolol. This major limitation must be definitely considered in planning drug interaction studies with talinolol. However, affinity of talinolol to efflux and uptake carriers may be a useful property in mechanistic studies to evaluate the complex interplay between efflux and uptake transporters in the intestinal tract.

4.3 Safety and Methodological Issues

Single dose studies with digoxin (0.25–0.5 mg, intravenous and per os) and talinolol (30 mg intravenous, 50–100 mg per os) are safe and severe adverse reactions are not expected. Both drugs are commercially available. The experimental conditions in probe drug studies with digoxin and talinolol must be strictly standardized (adequate dosage form, upright position during administration, intake of water and meal, etc.). The discrepancies in study results may origin, at least in part, from the experimental conditions. Investigators have to consider, that the "barrier function" of P-glycoprotein in digoxin absorption depends on the properties of the dosage form, that the expression of intestinal P-glycoprotein is regio-selective ("absorption window" in the proximal jejunum), that the existence of a regio-selective intestinal uptake for digoxin and talinolol cannot be excluded, and that meal and coadministration of drugs (interaction studies) may influence intestinal transit and dissolution of the probe drugs. Repeated-dose studies are not recommended because up-regulation of P-glycoprotein during the study periods cannot be excluded.

Mechanistic clinical studies on function of intestinal P-glycoprotein with digoxin and talinolol as in vivo probe drugs should be biometrically planned according to the international recommendations for bioequivalence studies and drug interaction studies.

To confirm absence of a P-glycoprotein-mediated influence, e.g. by potential inhibitors or inducers, $AUC_{0-\infty}$ and C_{max} may be primary pharmacokinetic characteristics as in other bioequivalence studies using an intrasubject, cross-over

design. The sample size should be at least 20 in studies with digoxin and 12 in studies with talinolol, assuming (1) intrasubject coefficients of variation of the $AUC_{0-\infty}$ of 8–20% for digoxin and 14% talinolol, (2) the standard equivalence range of 0.80–1.25, (3) a significance level of 0.05 (alpha error), and (4) a power of 80% of two one-sided *t*-tests (log-scale) (Siegmund et al. 2003; Steinijans et al. 1995). If the power is set to 90%, 26 and 14 subjects are needed in studies with digoxin and talinolol, respectively (nQuery 5.0, StatSol, Cork, Ireland).

To evaluate P-glycoprotein related differences as caused for instance by drug interactions, genetic polymorphisms, gender, age, or gastrointestinal diseases, a parallel-group study design is needed. In such mechanistic studies, primary study characteristics should be (1) oral bioavailability to measure the intestinal "absorption barrier function" and (2) intestinal clearance after intravenous administration to measure the "excretory function" of intestinal P-glycoprotein. Nonrenal clearance might be used as a surrogate for intestinal clearance. Secondary characteristics to be measured are volume of distribution, renal clearance and half-life to discuss nonintestinal P-glycoprotein related variability and influence by additional factors (e.g. organ perfusion). The mean intersubject coefficients of variation of for oral bioavailability of digoxin and talinolol are 10.7% and 25.3% and for nonrenal clearance are 28.1% and 29.5%, respectively (Drescher et al. 2003; Giessmann et al. 2004a; Greiner et al. 1999; Kurata et al. 2002; Rengelshausen et al. 2003; Schwarz et al. 2007; Siegmund et al. 2002a; Westphal et al. 2000b; Westphal et al. 2000a). To confirm a 20% difference in bioavailability from control with statistical power of 80% and significance level of 0.05 (Mann-Whitney rank-sum test), eight subjects per group are needed in digoxin studies and 30 subjects in talinolol studies. To confirm 20% difference in nonrenal clearance, 36 subjects and 39 subjects, respectively, should be included.

Because of the recommendations for blood sampling to cover at least 80% of the $AUC_{0-\infty}$ by data points (AUC_{0-t}) and to measure cumulative urinary and fecal excretion of the probe drugs completely (a precondition to assess clearance values), sampling periods of at least 7–9 days are necessary in single dose studies with digoxin and of 5 days with talinolol. Further preconditions are ambitious study participants and staff members, in particular to quantify fecal excretion of the probe drugs; for digoxin, highly sensitive mass-spectrometric assays must be developed.

Digoxin and talinolol are suitable in vivo probe drugs for intestinal P-glycoprotein under the precondition, that they are used as tools in carefully designed pharmacokinetic studies with adequate biometrically planning of the sample size and that several limitations are considered in interpreting and discussion of the study results.

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