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# Investigation of oral bioavailability and brain distribution of the Ind(8)-Val conjugate of indinavir in rodents

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## Abstract

Protease inhibitors are successfully used for the treatment of acquired immune deficiency syndrome (AIDS) although their biopharmaceutical characteristics are not optimal. Prodrugs have therefore been synthesized to increase protease inhibitor bioavailability and brain distribution. Among several compounds tested, a valine derivative of indinavir (Ind(8)-Val) showed promising characteristics using an in-vitro Caco-2 cell model. The objective of this study was to further investigate this compound using in-situ and in-vivo approaches. The pharmacokinetics of indinavir (Ind) and Ind(8)-Val were investigated in rats after intravenous and oral administration. Free indinavir resulting from in-vivo hydrolysis of Ind(8)-Val could not be detected in the plasma of rats receiving Ind(8)-Val. Furthermore Ind(8)-Val bioavailability was only 32% on average compared with 76% for indinavir, and effective permeability coefficients determined with a single-pass intestinal perfusion method were close to $25 \times 10^6$ cm s$^{-1}$ for the two compounds. Brain-to-plasma concentration ratios in the post equilibrium phase after intravenous administration to mice were $9.7 \pm 8.1\%$ for indinavir and $2.5 \pm 2.7\%$ for Ind(8)-Val. In conclusion, the promising biopharmaceutical characteristics of Ind(8)-Val suggested from previous in-vitro experiments with the Caco-2 cell model were not confirmed by in-situ and in-vivo experiments.

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## Introduction

The discovery of protease inhibitors has been a major advance in the treatment of acquired immune deficiency syndrome (AIDS) (Lewis 1997). The first potent human immunodeficiency virus (HIV) protease inhibitor (L-687,908) had very low water solubility, precluding its use as a therapeutic agent (Vacca et al 1991). Polar groups have been added to molecule structures, leading to a new compound (L-689,502) with an improved, but still low, oral bioavailability (5% in dogs) (Thompson et al 1992).

Efforts to improve protease inhibitor water solubility and oral bioavailability were continued and indinavir was synthesized (Dorsey et al 1994) in 1992 (Figure 1). Protease inhibitor bioavailability, however, remains incomplete in particular because of their affinity for the P-glycoprotein (P-gp) efflux transport system (Kim et al 1998; Choo et al 2000). P-gp is physiologically expressed at the intestinal barrier and at the blood–brain barrier (BBB) and transports structurally unrelated compounds out of epithelial cells of the small and large intestine (Thiebaut et al 1987) and out of the brain capillary endothelial cells (Cordon-Cardo et al 1989), thus limiting both oral absorption and brain distribution of HIV protease inhibitors. Therefore, despite the major therapeutic advances provided by the protease inhibitors, HIV cannot be eradicated within viral sanctuaries such as the brain (Chun & Fauci 1999; Pomerantz 2002), resulting in the maintenance of low-level active replication of the virus (Kolson et al 1998) and in neurological disorders such as the AIDS dementia complex.

The high structural diversity of P-gp substrates makes the design of protease inhibitors with low or no affinity for P-gp difficult. Therefore drug permeation through the intestinal and the blood–brain barrier may be improved by targeting influx carrier-mediated transport systems (Tsuji & Tamai 1999). The prodrug approach has gained

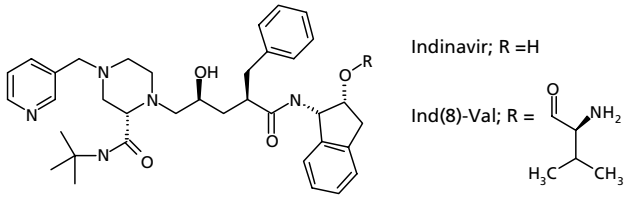


**Figure 1** Chemical structures of indinavir and Ind(8)-Val.

attention as a technique for improving drug therapy in the early 1970s, and since then numerous prodrugs have been designed and developed to improve oral bioavailability or tissue distribution of poorly membrane-permeable compounds (Stella et al 1985; Borchardt 1999; Han & Amidon 2000; Vierling & Greiner 2003). Human intestinal peptide transporters are targets of choice for the delivery of numerous drugs, such as beta-lactams and thrombin inhibitors (Dantzig et al 1994; Walter et al 1995), as well as protease inhibitors. Recently, several candidate prodrugs of various protease inhibitors have been synthesized (Farèse-Di Giorgio et al 2000; Rouquayrol et al 2001; Gaucher et al 2004) and preliminary assessment of their biopharmaceutical characteristics has been carried out using the Caco-2 intestinal barrier model (Rouquayrol et al 2002). Among many tested compounds, permeation characteristics of Ind(8)-Val (Figure 1) showed a dramatic improvement compared with the parent compound (Figure 1) and this was therefore selected as a candidate for further in-situ and in-vivo investigations in an attempt to characterize both its oral bioavailability and CNS distribution. This paper compares the pharmacokinetics of indinavir and Ind(8)-Val after intravenous and oral administration, as well as their absorption characteristics using an in-situ jejunum perfusion model in rats, and brain uptake after intravenous administration to mice.

## Materials and Methods

### Materials

Indinavir was extracted from Crixivan tablets and Ind(8)-Val was synthesized as previously described (Farèse-Di Giorgio et al 2000; Gaucher et al 2004). Amprenavir was kindly given by GSK (Hertfordshire, UK). Solvents were of HPLC grade and all other chemicals were of analytical grade. Highly purified water was produced using a MilliQ gradient Plus Millipore system (St Quentin-en-Yvelines, France). Analysis Oasis MCX Solid Phase-Extraction (SPE) cartridges were supplied by Waters (St Quentin-en-Yvelines, France).

### Animals

Male Sprague-Dawley rats, 220–271 g, and male Swiss mice, 25–28 g (Déprés Breeding Laboratories, Saint Doulchard, France) were housed in a light- (12-h light–dark cycle) and temperature-controlled environment for at least 5 days before experiments, with free access to water and food. Food was withdrawn 12 h before experiments or 24 h before in-situ rat jejunum perfusion studies. Studies were approved by the Animal Ethic Committee of the Faculty of Pharmacy (BHE/2001/12/AE).

### Solutions

For intravenous administration, indinavir or Ind(8)-Val (4 mM) solutions were prepared in 0.9% (m/v) saline containing 2% dimethyl sulfoxide (DMSO) or 10% ethanol (v/v), respectively. For oral administration, indinavir or Ind(8)-Val (8 mM) were dissolved in a 0.5% (m/v) carboxymethylcellulose aqueous solution containing 2% DMSO or 10% ethanol, respectively. Both intravenous and oral solutions were adjusted to pH 3 with 1 M HCl.

The 20 $\mu$M indinavir or Ind(8)-Val solutions for jejunal perfusion were prepared in perfusion medium (composition in mM: 135 NaCl, 5 KCl, 1 $CaCl_2$, 5 glucose and 5 mannitol in 10 mM MES (2[*N*-morpholino]ethansulfonic-acid) buffer, pH 6.5).

### Pharmacokinetic studies in rats

Rats equipped with femoral artery and femoral vein catheters for drug administration and blood collection, respectively (Chenel et al 2003), were divided in four groups. Groups 1 and 2 (n = 4) received 30-min intravenous infusions (4 mL $h^{-1}$) of indinavir and Ind(8)-Val (31.25 $\mu$mol $kg^{-1}$), respectively. Groups 3 and 4 (n = 6) received a single oral dose (62.5 $\mu$mol $kg^{-1}$) of indinavir or of Ind(8)-Val by gastric tubing. Blood samples (250 $\mu$L) were collected in heparinized vials for indinavir or Ind(8)-Val HPLC determination, and replaced with an equal volume of 0.9% (m/v) saline.

### In-situ rat jejunum perfusion

The procedure was adapted from Li et al (2002). Rats anaesthetized with thiopental sodium (60 mg $kg^{-1}$) were laid on a 37°C heating pad. After a midline longitudinal incision was made, a 10-cm jejunum segment at 15 cm downward from the ligament of Trietz was selected for inlet and outlet cannulation with glass cannulas. The inlet tubing was connected to a 20-mL syringe placed in an infusion pump (Harvard Apparatus, Les Ulis, France). After rinsing with the perfusion medium for 30 min (0.2 mL $min^{-1}$), drug solutions (20 $\mu$M) were perfused at a 0.05 mL $min^{-1}$ flow rate for 120 min to reach steady state. Intestinal effluent samples were then collected every 10 min, weighed for volume estimation and centrifuged at 10 000 *g* for 10 min. Supernates were immediately analysed by HPLC. After drug perfusions, a 31.25 $\mu$g $mL^{-1}$ phenol red solution, a virtually non-absorbable marker, was perfused in perfusion medium to check for the integrity of the intestinal epithelium. Phenol red concentration was determined by spectrophotometry at 556 nm.

### In-vivo brain diffusion in mice

Indinavir or Ind(8)-Val solutions were administered to mice (21.5 $\mu$mol $kg^{-1}$, n = 6) via the caudal vein. Blood

samples were collected by cardiac puncture 30 min after administration, then mice were sacrificed and brains were collected and rinsed with 0.9% (m/v) saline. The left and right parts of the brains, including cerebellum, were separated, weighed and stored at −20°C until HPLC determination. The left and right parts were used to assess Ind(8)-Val and indinavir brain concentrations, respectively.

### HPLC analysis

*Plasma sample preparation*

Indinavir was determined after extraction from plasma as described previously (Wu et al 1997; van Heeswijk et al 1998). Fifty microlitres (10 $\mu$M) of amprenavir (internal standard), 50 $\mu$L of a 5 M sodium hydroxide solution, 50 $\mu$L of methanol and 1 mL of ethyl acetate were added to 50 $\mu$L of defrosted plasma. The mixture was immediately shaken (30 s) and then centrifuged (3000 *g*, 10 min). Organic layers were rapidly collected and evaporated under nitrogen at 40°C. Residues were re-dissolved in 100 $\mu$L HPLC mobile phase for HPLC analysis. Ind(8)-Val was indirectly determined after conversion into indinavir by alkaline hydrolysis. Fifty microlitres (10 $\mu$M) of amprenavir, 50 $\mu$L of a 5 M sodium hydroxide solution and 50 $\mu$L of methanol were added to 50 $\mu$L of defrosted plasma. The mixture was left for 1 h at room temperature to allow complete hydrolysis of Ind(8)-Val into indinavir. Then 1 mL of ethyl acetate was added and the same extraction procedure was applied as described above.

*Brain sample preparation*

For indinavir extraction, 125 $\mu$L of 0.9% (m/v) saline, 50 $\mu$L of 10 $\mu$M amprenavir solution and 1 mL of 0.1 M HCl were added to 250 mg defrosted brain samples. After grinding the mixture for 2 min, tubes were centrifuged (3000 *g*, 10 min), supernates were then collected and centrifuged again for 5 min at 12 500 *g*. The second supernate was transferred onto SPE cartridges conditioned with 1 mL of methanol followed by 1 mL of water. Cartridges were then successively washed with 1 mL of a 5% methanol–2% ammonium hydroxide aqueous solution and 1 mL of a 20% methanol–2% ammonium hydroxide aqueous solution. Analytes were then eluted with 1 mL of methanol and collected. After methanol evaporation under nitrogen at 40°C, residues were re-dissolved in 150 $\mu$L of mobile phase for HPLC analysis.

For Ind(8)-Val determination, the extraction procedure slightly differed. After the first centrifugation, 0.5 mL of 5 M NaOH was added to the supernate and Ind(8)-Val hydrolysis into indinavir was allowed to occur for 1 h at room temperature. Samples were then centrifuged for 5 min at 12 500 *g*. Supernates (1 mL) were neutralized with 0.5 mL of 5 M HCl before transfer onto SPE as described above.

*HPLC procedure*

For in-vivo studies, HPLC analysis was carried out using a Kromasil $C_{18}$ analytical column (5 $\mu$m, 150 × 3 mm i.d.; Varian, Paris, France). The mobile phase was constituted with an acetonitrile 15 mM sodium acetate–15 mM 1-pentanesulfonic acid aqueous solution mixture (41:59 v/v), adjusted to pH 6 with acetic acid, and run at a 1 mL min$^{-1}$ flow rate. In these conditions retention times for indinavir and Ind(8)-Val were 3.7 min and 4.4 min, respectively. UV wavelength for detection was set at 210 nm. Injection volumes were 40 and 60 $\mu$L for plasma and brain samples, respectively. Plasma calibration curves were linear from 0.19 $\mu$M (limit of quantification, LOQ) to 25 $\mu$M, and brain calibration curves from 0.037 (LOQ) to 1.2 $\mu$mol kg$^{-1}$ brain. Coefficients of variation were always less than 10% even at the LOQ.

For in-situ perfusion experiments, Ind(8)-Val analysis procedure was carried out with a Kromasil $C_{18}$ (5 $\mu$m, 250 × 3 mm i.d.) analytical column, an acetonitrile–water mixture (37:63 v/v) at a 1.5 mL min$^{-1}$ flow rate, as the mobile phase and a 20-$\mu$L injection volume. Retention times for indinavir and Ind(8)-Val were 17.9 min and 22.5 min, respectively. Calibration curves were linear over the range 1.56 (LOQ) to 25 $\mu$M.

### Pharmacokinetic analysis

Non-compartmental pharmacokinetic analysis was carried out with the software WinNonLin (version 1.1; SCI software, Cary, NC). Observed peak concentrations ($C_{max}$) and time to peak ($t_{max}$) are reported. Half-life (t½), total area under the plasma drug concentration–time curve (AUC), plasma clearance (CL) and steady-state volumes of distribution (Vdss) of indinavir and Ind(8)-Val were calculated according to standard procedures (Chenel et al 2003). The averaged mean bioavailability (F) was estimated from the mean AUC values estimated after oral ($AUC_{PO}$) and intravenous ($AUC_{IV}$) administrations after correction for dose:

$$F = [(AUC_{PO}/AUC_{IV}) \cdot (Dose_{IV}/Dose_{PO})] \cdot 100 \quad (1)$$

The jejunal effective permeability ($P_{eff}$) was calculated at steady state according to Li et al (2002) as follows:

$$P_{eff} = (Q/2\pi rL) \cdot \ln(C_{in}/C'_{out}) \quad (2)$$

where Q is the perfusion flow rate (mL s$^{-1}$), r (cm) is the radius of the jejunal segment equal to 0.2 cm, L (cm) is the length of the perfused intestinal segments determined at the end of the experiment (L = 10.5 ± 1.2 cm), $C_{in}$ is the solute inlet concentration and $C'_{out}$ is the solute outlet concentration corrected as follows:

$$C'_{out} = (C_{out} \cdot V)/(Q \cdot \Delta t) \quad (3)$$

where V is the volume of effluent, $\Delta t$ is the collection interval and $C_{out}$ is the drug concentration in the exiting perfusate. For each experiment, $P_{eff}$ was obtained from the arithmetic mean of three determinations made from three consecutive 10 min effluent samples at steady state. Four experiments were conducted for each compound.

Results are presented as means ± s.d., except for $t_{max}$ (median and range). Friedman's test followed by Dunn's test was used to compare half-lives determined in the four groups of

rats. Other comparisons ($P_{eff}$) were made with Mann–Whitney *U*-tests. Significance level was set at $P < 0.05$.

## Results

### Pharmacokinetics in rats

Pharmacokinetic parameters are presented in Table 1. Following intravenous or oral administration of Ind(8)-Val to rats, no indinavir could be detected in the plasma. On average, Ind(8)-Val clearance was about half that of indinavir and its volume of distribution at steady state was about twice as high. As a consequence, Ind(8)-Val elimination half-life was several-fold higher than that of indinavir (Figure 2). For both compounds, elimination half-lives estimated after oral and intravenous administration were not statistically different, suggesting that absorption was not the rate limiting step for elimination. Yet after oral administration, peak concentrations of Ind(8)-Val were considerably reduced (5-fold on average) compared with those of indinavir and time to peak was delayed (Figure 3). The systemic availability of indinavir and Ind(8)-Val was estimated to be about 76% and 32%, respectively.

**Table 1** Pharmacokinetic parameters of indinavir and Ind(8)-Val after intravenous (n = 4) and oral (n = 6) administration to rats

| | **Indinavir** | | **Ind(8)-Val** | |
|---|---|---|---|---|
| | Intravenous | Oral | Intravenous | Oral |
| Dose ($\mu$mol kg$^{-1}$) | 31.25 | 62.50 | 31.25 | 62.50 |
| AUC ($\mu$M min) | 708 ± 161 | 1079 ± 515 | 1334 ± 447 | 866 ± 287 |
| CL (L h$^{-1}$) | 0.66 ± 0.16 | | 0.37 ± 0.15 | |
| Vdss (L) | 0.30 ± 0.04 | | 0.57 ± 0.20 | |
| t½ (min) | 24.7 ± 9.6[a] | 25.9 ± 15.5[b] | 75.8 ± 6.6[a] | 90.2 ± 16.0[b] |
| $C_{max}$ ($\mu$M) | | 16.2 ± 6.3 | | 3.4 ± 1.5 |
| $t_{max}$ (min) | | 30 ± 15 | | 91 ± 44 |
| F % | | 76% | | 32% |

Data are mean values ± s.d. [a,b]$P < 0.05$; i.v. vs oral t½ not significantly different.

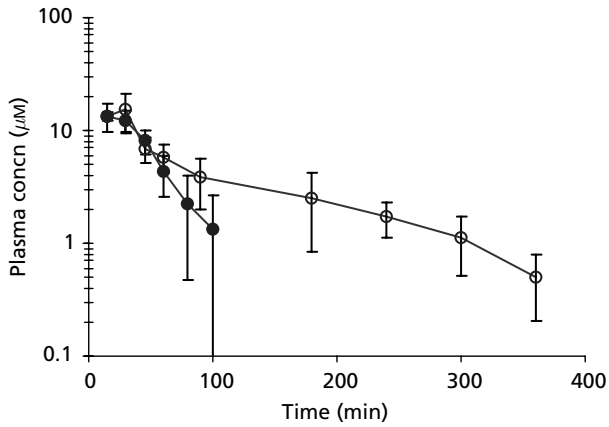


**Figure 2** Mean plasma concentration (±s.d.) of indinavir (●) and Ind(8)-Val (○) in rats (n = 4) after intravenous administration at a dose of 31.25 $\mu$mol kg$^{-1}$.

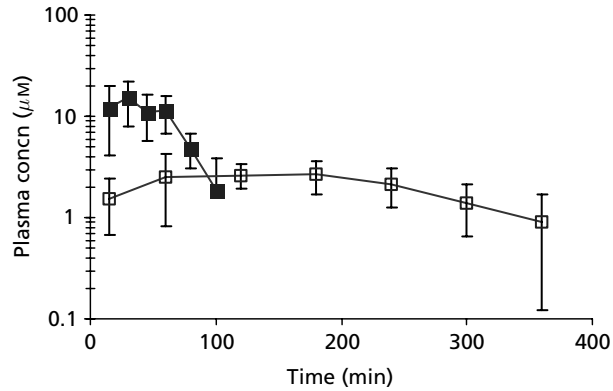


**Figure 3** Mean plasma concentration (±s.d.) of indinavir (■) and Ind (8)-Val (□) in rats (n = 6) after oral administration at a dose of 62.50 $\mu$mol kg$^{-1}$.

### In-situ rat jejunal perfusion

The recovery of phenol red was 94 ± 2.1%, indicating that the intestinal mucosa was not damaged during the experiment. Steady state was reached after 90 min. Ind(8)-Val degradation in perfusion blank medium at 37 °C for 3 h was negligible (3.2 ± 2.9%). During perfusion with Ind(8)-Val, free indinavir was detected in the outlet perfusate at steady state, at concentrations equal, on average, to 15 ± 16% of those of Ind(8)-Val. The $P_{eff}$ of indinavir and Ind(8)-Val was estimated to be 25.8 ± 4.72 × 10$^{6}$ and 24.0 ± 4.43 × 10$^{6}$ cm s$^{-1}$, respectively (not significantly different).

### Brain uptake in mice

Thirty minutes following the intravenous administration of indinavir to mice, mean drug concentrations measured in the plasma and brain were 1.35 ± 1.03 $\mu$M and 0.08 ± 0.04 $\mu$mol kg$^{-1}$, respectively, corresponding to an average brain-to-plasma concentration ratio of 9.7 ± 8.1%. Following administration of Ind(8)-Val, concentrations of the unchanged compound in the plasma and brain were 3.08 ± 0.60 $\mu$M and 0.08 ± 0.07 $\mu$mol kg$^{-1}$, respectively, corresponding to an average brain-to-plasma concentration ratio of 2.5 ± 2.7%. In these samples, indinavir levels were always below the quantification limit of the methods both in plasma and brain.

## Discussion

The major finding of this study was that conjugation of valine to indinavir as in Ind(8)-Val increases neither oral bioavailability nor brain uptake. In fact indinavir was

never detected in rat plasma after Ind(8)-Val administration. Because the elimination half-life of Ind(8)-Val is longer than that of indinavir, independently of the route of administration (Table 1), indinavir is probably formed by hydrolysis of Ind(8)-Val, but this formation step rate limits elimination (Rowland & Tozer 1980).

Although Ind(8)-Val was initially designed as a candidate prodrug, it also presents a high anti-HIV activity on its own (Farèse-Di Giorgio et al 2000). However, in rats, Ind(8)-Val bioavailability (32% on average) is reduced compared with that of indinavir (76%), and its brain distribution in mice is also reduced compared with that of indinavir (mean brain-to-plasma ratio of $2.5 \pm 2.7\%$ and $9.7 \pm 8.1\%$, respectively).

In previous Caco-2 cell experiments, a six-fold increase of Ind(8)-Val apical-to-basolateral transport and a two-fold decrease of its basolateral-to-apical transport were observed by comparison with indinavir (Rouquayrol et al 2002). These data are consistent with the involvement of active transport systems such as the oligopeptide transporters PEPT1 or HPT1, shown to be responsible for an increased bioavailability of aciclovir administered as a valine-conjugated prodrug (Balimane et al 1998; Han et al 1998; Landowski et al 2003), which constituted the rational for Ind(8)-Val development, together with the reduced efficacy of P-gp efflux.

These data, promising in terms of intestinal absorption characteristics (Rouquayrol et al 2002), were not confirmed in-vivo. Various explanations can be proposed. Results obtained with Caco-2 cells may be greatly affected by experimental conditions, such as pH and co-solvents (Yamashita et al 2000). Transport system expression in Caco-2 cells, in particular P-gp, PEPT1 and HPT1 expression, is also known to vary greatly with experimental conditions (Anderle et al 1998; Chu et al 2001; Behrens & Kissel 2003).

Indinavir $P_{eff}$ estimates ($25.8 \pm 4.7 \times 10^6\,cm\,s^{-1}$ at a concentration of $20\,\mu M$) were consistent with values previously reported ($57.8 \pm 13.6 \times 10^6\,cm\,s^{-1}$ at a concentration of $10\,\mu M$) by Li et al (2002).

Although indinavir and Ind(8)-Val had similar in-situ effective permeability, the bioavailability of the prodrug was lower than that of indinavir (Table 1). The intestinal first-pass effect probably contributed to this reduced bioavailability, since indinavir was measured in the outlet effluent during jejunum perfusion studies at concentrations corresponding to about 15% of Ind(8)-Val levels. As Ind(8)-Val was stable in the perfusion liquid, in similar conditions of time and temperature, enzymes are involved in the presystemic degradation observed during the in-situ perfusion experiments. By contrast an extensive hepatic first-pass effect leading to Ind(8)-Val hydrolysis is very unlikely to occur, since indinavir was not detected in plasma after Ind(8)-Val oral administration.

Because Caco-2 results suggested that Ind(8)-Val affinity for P-gp could be reduced compared with that of indinavir, it was interesting to investigate CNS distribution of this conjugate. Thirty minutes post intravenous administration, the brain-to-plasma concentration ratio of indinavir in mice was low ($9.7 \pm 8.1\%$) and in good agreement with previously reported values (Kim et al 1998), but surprisingly this ratio was 3.6 times lower with Ind(8)-Val ($2.5 \pm 2.7\%$). However due to its longer elimination half-life, plasma concentrations of Ind(8)-Val were higher than those of indinavir, resulting in virtually equal concentrations of the two compounds in the brain.

In conclusion, the promising biopharmaceutical characteristics of Ind(8)-Val suggested from previous in-vitro experiments with the Caco-2 cell model were not confirmed by in-situ and in-vivo experiments. Many parameters may have contributed to these apparent discrepancies and no specific explanation could be favoured. Although disappointing, these new results constitute an interesting illustration of the limits of simple in-vitro models to predict more complex in-vivo behaviour.

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