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Establishment of Correlation between In Vitro Enzyme Binding Potency and In Vivo Pharmacologic Activity: Application to Liver Glycogen Phosphorylase a Inhibitors.

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ABBREVIATIONS: $fu_{GPa\text{-}buffer}$, the free fraction of an inhibitor in the GPa solution when dialyzed again the buffer; **$fu_{GPa\text{-}liver}$,** the free fraction of an inhibitor in the GPa

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solution when dialyzed against the liver homogenate; **fu_{liver}**, the free fraction of an inhibitor in a diluted liver homogenate; **uFu_{liver}**, the extrapolated free fraction (see Equation 11) of an inhibitor in an undiluted liver homogenate; **GPa**, glycogen phosphorylase a; **$[GPa]_b$**, the GPa concentration bound to an inhibitor; **$[GPa]_f$**, the free GPa concentration; **$[GPa]_t$**, the total GPa concentration; **$[I]_{b\text{-}GPa}$**, the concentration of an inhibitor bound to GPa; **$[I]_f$**, the free concentration of an inhibitor; **$[I]_{t\text{-}GPa}$**, the total concentration of an inhibitor in the GPa chamber of the equilibrium dialysis; **$[I]_{t\text{-}GPa\text{-}liver}$**, the total concentration of an inhibitor in the GPa chamber of the equilibrium dialysis against liver homogenate; **$[I]_{t\text{-}liver}$**, the total concentration of an inhibitor in the liver homogenate; **K_d**, dissociation constant; **$Kd_{GPa\text{-}liver}$**, the dissociation constant of a GPa inhibitor to GPa in presence of liver homogenate; **LC/MS/MS**, liquid chromatography with tandem mass spectrometry; **MED,** the minimum efficacious dose.

Recommended Section: Absorption, Distribution, Metabolism, and Excretion Behavioral Pharmacology.

Abstract:

In drug discovery, establishing a correlation between in vitro potency and in vivo activity is critical for the validation of the selected target and developing confidence in the in vitro screening strategy. The present study developed a competition equilibrium dialysis assay using a 96-well dialysis technique to determine the intrinsic K_d for 13 inhibitors of human liver glycogen phosphorylase a (GPa) in the presence of liver homogenate to mimic the physiological environment. The results provided evidence that binding of an inhibitor to GPa was affected by extra cofactors present in the liver homogenate. A good correlation was demonstrated between the in vitro K_d determined under liver homogenate environment and free liver concentration of an inhibitor at the minimum efficacious dose in diabetic ob/ob mice. This study revealed important elements (such as endogenous cofactors missing from the in vitro assay, free concentration at the target tissue) that contributed to a better understanding of the linkage between in vitro and in vivo activity. The approach developed here may be applied to many drugs in pharmacology studies in which the correlation between in vitro and in vivo activities for the target tissue (such as solid tumors, brain and liver) is critical.

Introduction:

In the drug discovery process within the pharmaceutical industry, initial lead compounds are usually identified from high-throughput in vitro biological screens, for example, an inhibition assay against a target enzyme. It is often hoped that the in vitro potency (such as IC_{50}) can be used to predict the in vivo pharmacological activity (such as EC_{50}). However, discovery scientists often face a question: why doesn't an in vitro potent inhibitor work in vivo or only work at a much higher in vivo concentration? Establishment of a correlation between in vitro potency and in vivo activity is crucial for validation of the target enzyme and for achieving confidence in an in vitro screening strategy.

According to the fundamental free ligand hypothesis, the average free efficacious concentration at the steady-state in vivo should correlate with the intrinsic (unbound) potency determined from an in vitro assay. This hypothesis has been supported by many researchers (Wagner et al., 1965; Wagner, 1976; DeGuchi et al., 1992; Wright et al., 1996). In practice, however, this relationship is often obscured or confounded due to a variety of factors. For example, non-physiological conditions and/or involvement of non-specific binding within in vitro systems may yield an inaccurate estimate of the true intrinsic potency. In addition, complex pharmacokinetic/pharmacodynamic relationships arising from indirect effects or target site disequilibrium may result in the inappropriate determination of in vivo potency.

Several of these variables stated above confounded the initial establishment of a correlation between in vitro potency and in vivo activity for human liver glycogen phosphorylase a (GPa) inhibitors. Inhibition of liver GPa blocks the glycogenolysis pathway and therefore leads to reduction of hepatic glucose production (Martin et al., 1998; Treadway et al., 2001). This approach may have potential to be useful for the treatment of type 2 diabetes mellitus. In this study, thirteen reversible GPa inhibitors (GPa inhibitors) were tested against the human liver GPa and displayed IC_{50}'s in the range of 23 to 460 nM. All these GPa inhibitors except one showed blood glucose lowering following an acute oral dose in fed diabetic ob/ob mice. However, no obvious correlation was observed between in vitro GPa enzyme IC_{50} values and any in vivo measurements such as the minimum efficacious dose (MED), or plasma or liver concentrations at the MED. One hypothesis to explain the poor correlation is that the IC_{50} values were determined under non-physiological conditions due to missing or insufficient amounts of important cofactors or modulators (such as AMP, ATP, etc. in this case) present in vivo, which have been shown to affect the binding affinity of a GPa inhibitor to GPa (Monanu and Madsen, 1987; Ercan-Fang et al., 2002a; Ercan-Fang et al., 2002b; Ercan-Fang et al., 2005). As such, the initial focus of this work was to determine whether a good correlation could be revealed by obtaining GPa inhibitor binding affinity to GPa under more physiological conditions.

Theoretically, the in vivo activity should be driven by the amount of liver GPa bound to a GPa inhibitor, which is determined by the intrinsic dissociation constant (K_d) and the free GPa inhibitor concentration at the target site (liver). Accurate measurement of K_d under

physiological conditions can be complicated and time consuming (Romer and Bickel, 1979; Wright et al., 1996). In the present study, a robust method was developed to determine the intrinsic K_d of thirteen GPa inhibitors to purified human liver GPa in the presence of liver tissue homogenate using a previously validated 96-well equilibrium dialysis apparatus (Cory Kalvass and Maurer, 2002; Banker et al., 2003). These conditions should mimic those in vivo better than the conventional assay (Martin et al., 1998) because most, if not all, cofactors are present in the liver homogenate. Good in vitro-in vivo correlation was found between the K_d determined in vitro in the presence of liver homogenate and the in vivo free liver concentration of a GPa inhibitor at the MED. The MED was used because the study design was aimed to detect in vivo active GPa inhibitors from a large panel of in vitro potent GPa inhibitors while minimizing the utilization of experimental animals. Determination of ED_{50} or ED_{90} requires many more experiments and thus is usually conducted only on the lead compound(s). Because very few GPa inhibitors had ED_{50} or ED_{90} determinations, the correlation with these parameters was not investigated in the present study.

Methods:

Materials – All GPa inhibitors were obtained from Pfizer's proprietary sample bank (Groton, CT); and their identities by chemical names, including stereochemistry, and cLogP (calculated log octanol-to-water partition coeffficient) are given in Table 1. References to the preparation and characterization of these compounds are listed in Table 1, except for compounds **6, 9** and **13**. Compounds **6, 9,** and **13** are previously unreported, but are close analogs which were prepared by closely analogous procedures. All these compounds are neutral and not ionizable at the physiological pH. Human liver GPa was prepared as previously described (Martin et al., 1998). Solvents and other reagents were obtained from commercial sources and were of reagent grade or better.

Phosphorylase Enzyme Inhibition Assay (GP_a IC_{50}) – The assay for GP_a IC_{50} was performed as previously described (Martin et al., 1998). Briefly, human liver GPa (85 ng, ~0.85 pmol) activity was measured in the direction of glycogen synthesis by the release of phosphate from glucose-1-phosphate at 22°C in 100 μL of buffer containing 50 mM Hepes (pH 7.2), 100 mM KCl, 2.5 mM EGTA, 2.5 mM $MgCl_2$, 0.5 mM glucose-1-phosphare and 1 mg/mL glycogen. Phosphate was measured at 620 nm, 20 min after the addition of 150 μL of 1 M HCl containing 10 mg/mL ammonium molybdate and 0.38 mg/mL malachite green. Test compounds were added to the assay in 5 μL of 14% DMSO.

In vivo acute Plasma Glucose Lowering in the ob/ob Mouse – The acute hypoglycemic activity of a GPa inhibitor was determined using seven to eight week old male C57BL/6J-ob/ob mice (Jackson Laboratory, Bar Harbor, ME) housed five mice per cage under standard animal care practices. After a one-week acclimation period, the animals were weighed and 25 μL of blood was collected from the retro-orbital sinus prior to treatment. The blood sample was immediately diluted with 100 μL saline containing 0.025% sodium heparin, and held on ice for bioanalysis. Animals were assigned to treatment groups such that each group (n = 5 mice) had a similar mean for baseline plasma glucose concentration, and then animals were dosed orally with the vehicle alone or with a GPa inhibitor at dose ranging from 1 to 25 mg/kg. The vehicle consisted of 10% DMSO/0.1% Pluronic® P105 Block Copolymer Surfactant (BASF Corporation, Parsippany, NJ) in 0.1% saline without pH adjustment or neat polyethylene glycol 400 (Sigma, St. Louis, MO). Mice were then bled from the retro-orbital sinus one and three h post dose for determination of plasma glucose levels and exposure. Mice were terminated following the last bleeding and liver samples were collected for exposure. Freshly collected blood samples were centrifuged for two minutes at 10,000 x g at room temperature. The supernatant was analyzed for glucose by the Abbott VP™ (Abbott Laboratories, Diagnostics Division, Irving, TX) and VP Super System® Autoanalyzer (Abbott Laboratories), using the A-Gent™ Glucose-UV Test reagent system (Abbott Laboratories). Alternatively, plasma glucose was measured using a Roche/Hitachi 912 Clinical Chemistry Analyzer (Roche Diagnostics Corp., Indianapolis, IN). Hypoglycemic activity of the test compounds was determined by statistical analysis (unpaired t-test) of the mean plasma glucose concentration between the test compound

group and vehicle-treated group. The MED was determined by the lowest dose at which significant plasma glucose lowering was observed in the test compound group. Aliquots of plasma samples were also analyzed for exposure. All animal procedures involved the humane care and use of animals, were performed within an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility, and were approved by the Pfizer Global Research and Development-Groton/New London Laboratories Institutional Animal Care and Use Committee.

Liver and Plasma Sample Preparation for Concentration Determination – The liver tissues from studied animals were homogenated by adding ~0.2 g of liver tissue to ~1 mL of the ice-cold buffer (1:5 dilution), composed of 50 mM BES (n,n-bis(2-hydroxyethyl)2-amino-ethanesulfonic acid), 4 mM NaCl, 1 mM EDTA, 0.5 mM DTT (dithiothreitol) and 0.02% sodium azide, in a glass tube sitting on ice followed by sonication via a probe homogenizer (twice for 10-15 seconds each time at maximum speed). Ten μL of liver homogenate (0.2 g/mL) was precipitated using 200 μL of methanol-acetonitrile (1:1). The calibration curve was prepared by mixing 10 μL of a control tissue homogenate (0.2 g/mL), 10 μL of a standard in methanol-acetonitrile and 190 μL of methanol-acetonitrile. The standard samples (9 concentrations per standard curve, dilution factor = 5) were processed as the unknowns. The plasma samples from studied animals were diluted 5-fold with saline upon bleeding. Fifty μL of the plasma sample was precipitated by adding 100 μL of methanol-acetonitrile. The calibration curve was prepared by mixing 10 μL of control plasma, 40 μL of saline and 10 μL of a standard in methanol-acetonitrile followed by precipitation with 90 μL of methanol-acetonitrile. The standard samples (9

concentrations per standard curve) were processed exactly as the unknowns. All samples were then vortexed, centrifuged, and the supernatant were analyzed by a liquid chromatography with tandem mass spectrometry (LC/MS/MS) assay. The calibration curve was obtained by fitting linear least squares regression.

Equilibrium Dialysis A (see Figure 1a) – A validated two-chamber 96-well equilibrium dialysis apparatus was used (Banker et al., 2003). The buffer used in dialysis and liver homogenate was the same as described above. The two chambers (150 μL each) were separated by a preconditioned Spectra-Por Number 2 membrane with molecular weight cutoff of 12-14 kDa. One chamber contained purified human liver GPa at 0.2 mg/mL (~2 μM, m.w. = ~97 kD for the monomeric form (Treadway et al., 2001)), and the other contained an ob/ob mouse liver homogenate at 0.2 g/mL (prepared as described above). To facilitate rapid equilibrium, the compound was added to both chambers at a concentration of 250 ng/mL (~0.5 μM). The sealed apparatus was incubated in a 37°C water bath with a gentle shaking at 60 rpm. Equilibrium was achieved between 4-7 h without detectable degradation for tested compounds (recovery ranged 80-110%). At the end of dialysis, 90 μL of GPa sample and 10 μL of liver homogenate sample were taken from the dialysis apparatus to separate HPLC vials containing 100 μL of methanol-acetonitrile (1:1). Ten μL of control liver homogenate (0.2 g/mL) and 90 μL of unfortified GPa solution (0.2 mg/mL) were added to the GPa sample vial and the liver homogenate sample vial, respectively, to yield an identical matrix. All samples were then vortexed, centrifuged, and the supernatant was analyzed by an LC/MS/MS assay to

determine the total concentration of the inhibitor in the GPa chamber ($[I]_{t\text{-}GPa}$) and the total concentration of the inhibitor in the liver homogenate chamber ($[I]_{t\text{-}liver}$).

Equilibrium Dialysis B (see Figure 1b) – The assay was carried out as described above in the Equilibrium Dialysis A except that the GPa solution was replaced by the buffer. The compound was added to the liver homogenate chamber at 500 ng/mL (~1 μM). Equilibrium was achieved by 7 h of incubation. After dialysis, 10 μL of the liver homogenate sample and 90 μL of the buffer sample were taken from the dialysis apparatus to separate HPLC vials containing 100 μL of methanol-acetonitrile (1:1). Ninety μL of unfortified buffer and 10 μL of control liver homogenate (0.2 g/mL) were added to the liver homogenate sample vial and the buffer sample vial, respectively, to yield an identical matrix. All samples were then vortexed, centrifuged, and the supernatant was analyzed by an LC/MS/MS assay. The concentrations in the buffer chamber (equal to the free concentration, ($[I]_f$)) and in the liver homogenate chamber (equal to the total concentration, $[I]_{t\text{-}liver}$) were determined, respectively.

Equilibrium Dialysis C (see Figure 1c) – The assay was carried out as described above in the Equilibrium Dialysis A except that the liver homogenate was replaced by the buffer. The compound was added to the GPa chamber at 500 ng/mL (~1 μM). Equilibrium was achieved by 7 h of incubation. After dialysis, 10 μL of the GPa sample and 90 μL of the buffer sample were taken from the dialysis apparatus to separate HPLC vials containing 100 μL of methanol-acetonitrile (1:1). Ninety μL of unfortified buffer and 10 μL of unfortified GPa solution (0.2 mg/mL) were added to the GPa sample vial and the buffer

sample vial, respectively, to yield an identical matrix. All samples were then vortexed, centrifuged, and the supernatant was analyzed by an LC/MS/MS assay. The concentrations in the buffer chamber ($[I]_f$) and in the GPa chamber ($[I]_{t\text{-}GPa}$) were thus determined, respectively.

Plasma protein binding – The assay was carried out as previously described using the two-chamber 96-well equilibrium dialysis apparatus (Tracey et al., 2004). Briefly, fresh plasma samples from ob/ob mice were spiked with the test compound to achieve a concentration of 500 ng/mL. An aliquot of a fortified plasma sample (150 µL, n=4-6) was loaded into one chamber and dialyzed against 150 µL of sodium phosphate buffer (pH 7.4) in another chamber at 37°C for 5 h. At the end of the dialysis period, 20 µL of the dialyzed plasma and 90 µL of the buffer were transferred to HPLC vials containing 100 µL of methanol-acetonitrile (1:1). Control buffer (90 µL) was added to the vial containing the plasma sample, and 10 µL of control plasma was added to the vial containing the buffer sample. All samples were then vortexed, centrifuged, and the supernatant was analyzed by an LC/MS/MS assay. The plasma free fraction was estimated by the ratio of drug concentration in the buffer chamber to the drug concentration in the plasma chamber.

LC/MS/MS Assay – Two Shimadzu LC-10ADVP binary pumps with a 10 µL static mixer and a PE-Sciex API3000 triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Ontario, Canada) were used in all experiments. An aliquot of sample (5 µL) was injected onto a Phenomenex 40 x 2 mm 5 µm C18 column maintained at 37°C with a run time of

~3 min. The analyte was eluted at 0.5 mL/min flow rate with a linear gradient program consisting of methanol (pump A, 5-95% ramping) and 10 mM ammonium acetate (pump B, 95-5% ramping). The column effluent was analyzed using a Turbo Ionspray source at 500°C of API3000. Compounds were measured using multiple reaction monitoring (MRM) with positive ionization and retention time between 1 and 4 min.

Theoretical:

1. Equilibrium dialysis (Wright et al., 1996):

In the equilibrium dialysis assay A, the ratio of the fortified GPa concentration to the liver homogenate concentration was 1:1000 (w/w). Under this ratio, the amount of endogenous mouse GPa in the liver homogenate chamber was approximately equal to the amount of fortified human GPa in the GPa chamber (~0.1% of whole wet liver tissue (Treadway, et al. unpublished data)). In addition, small cofactors (smaller than 12 kDa) released from the liver tissue can pass through the dialysis membrane into the GPa chamber. At equilibrium, the $[I]_f$ should be same in both chambers. Therefore, the $[I]_{t\text{-}liver}$ in the liver homogenate chamber times the free fraction of the inhibitor in the liver homogenate (fu_{liver}) should be equal to the total inhibitor concentration ($[I]_{t\text{-}GPa\text{-}liver}$) in the GPa chamber times the free fraction of the inhibitor in the GPa solution ($fu_{GPa\text{-}liver}$, see Equation 1):

$$[I]_{t\text{-}GPa\text{-}liver} \times fu_{GPa\text{-}liver} = [I]_{t\text{-}liver} \times fu_{liver} \qquad (1)$$

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The $[I]_{t\text{-}GPa\text{-}liver}$ and $[I]_{t\text{-}liver}$ were directly measured from samples collected from the equilibrium dialysis assay A as described above. The fu_{liver} was estimated from the equilibrium dialysis assay B by the ratio of the inhibitor concentration in the buffer chamber to the inhibitor concentration in the liver homogenate chamber (see Equation 2). Therefore, the $fu_{GPa\text{-}liver}$ can be estimated using Equation 3.

$$fu_{liver} = \frac{[I]_f}{[I]_{t-liver}} \qquad (2)$$

$$fu_{GPa\text{-}liver} = \frac{[I]_{t-liver}}{[I]_{t-GPa-liver}} \times fu_{liver} \qquad (3)$$

$$fu_{GPa\text{-}buffer} = \frac{[I]_f}{[I]_{t-GPa}} \qquad (4)$$

In the equilibrium dialysis assay C, the inhibitor is bound to the GPa in the buffer without presence of cofactors released from the liver homogenate. The free fraction of an inhibitor, $fu_{GPa\text{-}buffer}$, is estimated by the ratio of the inhibitor concentration in the buffer chamber to the inhibitor concentration in the GPa chamber (see Equation 4).

2. K_d determination:

The K_d of an inhibitor to GPa under the condition of equilibrium dialysis A ($Kd_{GPa\text{-}liver}$) is defined as in Equation 5, where $[GPa]_f$ is the free GPa concentration and $[I]_{b\text{-}GPa}$ is the inhibitor concentration bound to GPa. Because under this condition $[I]_{b\text{-}GPa}$ is equal to $[I]_{t\text{-}GPa\text{-}liver}$ times $(1\text{-}fu_{GPa\text{-}liver})$ and the ratio of $[I]_f$ to $[I]_{t\text{-}GPa\text{-}liver}$ is equal to $fu_{GPa\text{-}liver}$, the $Kd_{GPa\text{-}liver}$ can be derived by Equation 6. The $[GPa]_f$ is derived by Equation 7 where

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$[GPa]_t$ and $[GPa]_b$ are the total and bound GPa concentrations, respectively. The $[GPa]_b$ should be the same as the $[I]_{b\text{-}GPa}$, and therefore, introducing Equation 7 into Equation 6 gives Equation 8. Thus $Kd_{GPa\text{-}liver}$ can be estimated by using Equation 8.

$$Kd_{GPa\text{-}liver} = \frac{[GPa]_f \times [I]_f}{[I]_{b-GPa}} \quad (5)$$

$$Kd_{GPa\text{-}liver} = \frac{fu_{GPa-liver} \times [GPa]_f}{1 - fu_{GPa-liver}} \quad (6)$$

$$[GPa]_f = [GPa]_t - [GPa]_b = [GPa]_t - [I]_{t\text{-}GPa\text{-}liver} \times (1\text{-}fu_{GPa\text{-}liver}) \quad (7)$$

$$Kd_{GPa\text{-}liver} = \frac{fu_{GPa-liver} \times ([GPa]_t - [I]_{t\text{-}GPa\text{-}liver} \times (1 - fu_{GPa\text{-}liver}))}{1 - fu_{GPa-liver}} \quad (8)$$

3. Estimation of the inhibitor concentration bound to GPa ($[I]_{b\text{-}GPa}$):

$$\frac{[I]_{b-GPa}}{[I]_{t-liver}} = \frac{[I]_{t-GPa} - [I]_f}{[I]_{t-liver}} = \frac{[I]_{t-GPa}}{[I]_{t-liver}} - fu_{liver} \quad (9).$$

The $[I]_{b\text{-}GPa}$ in a liver homogenate can be estimated based on Equation 9. Assuming the ratio of $\frac{[I]_{b-GPa}}{[I]_{t-liver}}$ determined from the in vitro dialysis assay is same as in vivo at the MED, then $[I]_{b\text{-}GPa}$ in vivo ($[I]^{invivo}_{b-GPa}$) can be estimated based on the in vitro ratio ($\frac{[I]^{invitro}_{b-GPa}}{[I]^{invitro}_{t-liver}}$) times the total inhibitor concentration in the liver in vivo ($[I]^{invivo}_{t-liver}$, Equation

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10), which can be measured ex vivo after administration of an inhibitor to the animal as described above.

$$[I]_{b-GPa}^{invivo} = \frac{[I]_{b-GPa}^{invitro}}{[I]_{t-liver}^{invitro}} \times [I]_{t-liver}^{invivo} \qquad (10).$$

4. Determination of free fraction of an inhibitor in undiluted liver tissue (uFu_{liver}) and free liver concentration:

The free fraction in an undiluted liver tissue (uFu_{liver}) was estimated using Equation 11 previously described by Kalvass and Mauer (Cory Kalvass and Maurer, 2002). The fu_{liver} was determined as described in equilibrium dialysis B, and D was the dilution factor (D = 5 in the present study). The free liver concentration of a GPa inhibitor in vivo was estimated by the liver concentration times uFu_{liver}.

$$uFu_{liver} = \frac{1/D}{((1/fu_{liver}) - 1) + 1/D} \qquad (11)$$

Results:

In Vitro and In Vivo Activities – The in vitro IC_{50}'s of the panel of GPa inhibitors tested in a purified human liver GPa assay are summarized in Table 2. The MED was determined following a sequential oral administration in diabetic ob/ob mice starting from a dose of 25 mg/kg to a dose approximately 2-fold lower than the MED. The total in vivo liver and plasma concentrations of GPa inhibitors at MED are recorded in Table 2. The plasma concentration listed in Table 2 was either at 1 or 3 h post dose whichever the lower concentration associated with the glucose lowering at the time. The IC_{50} values range from 0.023 μM to 0.46 μM. No correlation was found between in vitro IC_{50} and in vivo activities determined by MED (Table 2), or total plasma or liver concentrations (R^2 values were all less than 0.05, Figure 2). There was no obvious correlation either between the IC_{50} and the free plasma or liver concentrations (R^2 are 0.497 and 0.184, respectively, Figure 3).

Determination of the dissociation constant of a GPA INHIBITOR to GPa in presence of liver homogenate ($Kd_{GPa\text{-}liver}$) – The $Kd_{GPa\text{-}liver}$ in the liver homogenate environment (equilibrium dialysis A) was estimated based on Equation 8 for all 13 GPA INHIBITORs after obtaining values for $fu_{GPa\text{-}liver}$ (Table 3), $[GPa]_t$ (0.2 mg/mL ≈ 2 μM), fu_{liver}, and $[I]_{t\text{-}liver}$. The estimated $Kd_{GPa\text{-}liver}$ values are present in Table 3 and ranged from 0.05 to 0.92 μM. The relationship between the $Kd_{GPa\text{-}liver}$ and the free liver concentration of a GPa inhibitor in vivo at the MED is illustrated in Figure 4. However, there was no obvious correlation between $fu_{GPa\text{-}liver}$ and $fu_{GPa\text{-}buffer}$ for the thirteen GPa inhibitors (Table 3),

suggesting the binding of a GPa inhibitor to GPa in the presence of liver homogenate differs from the binding in the buffer.

Inhibitor bound to GPa versus non-specific binding to liver tissue – The fu_{liver} was determined for all compounds (data not shown), and the extrapolated uFu_{liver} is listed in Table 3. The measured ratios of total drug concentration in the GPa chamber to that in the liver homogenate chamber ($[I]_{t\text{-}GPa}/[I]_{t\text{-}liver}$) are summarized in Table 3 and ranged from 0.076 to 0.94. The concentration ratio of an inhibitor bound to GPa over total inhibitor concentration in the liver homogenate ($[I]_{b\text{-}GPa}/[I]_{t\text{-}liver}$) estimated from Equation 9 was listed in Table 3. The $[I]_{b\text{-}GPa}$ in vivo in the liver of an ob/ob mouse for all of the GPa inhibitors at their respective MED (estimated by Equation 10) was found to be relatively constant with mean ± CV% of 1.42 ± 37% μM. The relationship between the $[I]_{b\text{-}GPa}/[I]_{t\text{-}liver}$ ratio and the inhibitor concentration in the liver of an ob/ob mouse 3 h post dose at the MED is shown in Figure 5.

Discussion:

The techniques used in the present study combined a published method for distribution dialysis (Bickel et al., 1987; Clausen and Bickel, 1993) and a 96-well equilibrium dialysis apparatus (Banker et al., 2003). These techniques have been used extensively to determine drugs binding to plasma protein, tissue protein or any macro-molecules (Pacifici and Viani, 1992; Cory Kalvass and Maurer, 2002). Many of the drug-binding studies were aimed at predicting drug distribution in vivo (Khalafallah and Jusko, 1984; Barre et al., 1988; Clausen and Bickel, 1993) or to understand pharmacokinetic consequences (Wilkinson, 1983; Fichtl et al., 1991). Similar studies were conducted to understand pharmacokinetics/pharmacodynamics and to predict drug effect based on receptor binding at the target (Proost et al., 1996), or to determine free drug concentration at the target site (Cory Kalvass and Maurer, 2002). However, few publications have revealed a correlation between in vitro enzyme binding potency and in vivo drug activity for multiple compounds in a drug discovery setting.

It was hypothesized that the previous method to determine GPa IC_{50} (Martin et al., 1998) was under non-physiological conditions, and the binding potency of an inhibitor to GPa might be influenced by the assay conditions. A human hepatocyte (SK-HEP1) assay was used to determine glycogenolysis inhibition in the cells for GPa inhibitors. A marked right shift in IC_{50}'s was observed for all GPa inhibitors compared to ones determined from the GPa enzyme assay. No obvious correlation to in vivo potency was established (data not shown). One plausible explanation is that the protein binding of the compounds

in this hepatocyte assay was not determined and this may differ between the two systems. The free IC_{50}, if measured, might improve the correlation. Another possibility is that all transporters, which may mediate uptake and efflux of GPa inhibitors (*vide infra*) may not be expressed and function the same in SK-HEP1 cells as in vivo. To fully understand factors in hepatocytes that contribute to the discordance to the in vivo potency is important and deserves additional study.

The effect of the presence of liver homogenate on the binding of a GPa inhibitor to GPa was clearly evident from the present study. As shown in Table 3, the free fraction of a GPa inhibitor in the dialysis buffer ($fu_{GPa\text{-}buffer}$), the condition used in the previous method for IC_{50} determination, does not correlate with the free fraction of a GPA INHIBITOR in the presence of liver homogenate ($fu_{GPa\text{-}liver}$). The latter should be closer to the in vivo physiological conditions. Ercan-Fang et al. also reported that the inhibition potency of a GPa inhibitor to GPa was modulated by endogenous effectors, and the magnitude of shift of in vitro IC_{50} in the presence of all effectors varied for different GPa inhibitors (Ercan-Fang et al., 2005). Therefore, in order to accurately measure the K_d of GPa inhibitor to GPa enzyme that predicts in vivo activity, all cofactors at physiological concentrations should be included in the in vitro assay. The $Kd_{GPa\text{-}liver}$ was obtained under the conditions designed to be close to those in vivo and correlated very well for the in vivo activity determined by free inhibitor concentration in the liver of an ob/ob mouse 3 h post dose at their respective MED for all the GPa inhibitors tested in the present study (see Figure 4).

The K_d determines the binding affinity of a ligand to an enzyme theoretically independent of the enzyme concentration. In the present study, the $Kd_{GPa\text{-}liver}$ was determined using a single GPa and liver homogenate concentration. The liver homogenate concentration (200 mg/mL) used in the competition dialysis assay was under a non-saturation condition (data not shown). The concentration ratio of GPa to liver homogenate was selected for equilibrium dialysis A based on the estimated ratio in a mouse liver (GPa = ~0.1% of wet liver weight). A good correlation (R^2=0.776) shown in Figure 4 between the free inhibitor concentration in the liver of an ob/ob mouse 3 h post dose at the MED and the $Kd_{GPa\text{-}liver}$ supports the validity of the $Kd_{GPa\text{-}liver}$ measurement used in the present study.

It is worthwhile to point out that the measured free inhibitor concentrations in the liver at the MED were all lower than the $Kd_{GPa\text{-}liver}$ (approximately 20% of the $Kd_{GPa\text{-}liver}$) for all tested GPa inhibitors, consistent with estimated low percentage of GPa bound to GPA INHIBITOR (1.4 μM, see results) relative to total GPa concentration (~10 μM, Treadway et al. unpublished data) at the MED. This is expected because only minimum glucose lowering (~20% of E_{max}) was observed at the MED. In order to have the maximum effect on GPa inhibition, the free inhibitor concentration in liver should be ≥10x K_d wherein >90% GPa is bound. This hypothesis was supported by a separate study, in which Compound **4** administered via portal vein infusion to normal male Sprague-Dawley rats blocked 69% and 100% of glucagon-induced glucose excursion in rats at steady-state free plasma concentrations of 1.5 and 2 μM (7.5x and 10x $Kd_{GPa\text{-}liver}$, respectively; T. Checchio and L.J. Yu, unpublished data).

Theoretically, the free liver concentration should be equal to free plasma concentration under equilibrium conditions if the liver distribution is driven by passive diffusion and no significant metabolism occurs. However, a good correlation was observed only for the $Kd_{GPa\text{-}liver}$ with the free liver concentration but not with the free plasma concentration at the MED. This discrepancy may be explained either by non-equilibrium conditions at the sampling times (1 or 3 h post dose) from the in vivo study, or that some of GPa inhibitors may be substrates for liver transporter proteins (Fichtl et al., 1991; Cory Kalvass and Maurer, 2002). These two possibilities may also contribute to the lack of a good agreement between free liver and free plasma concentrations (see Figure 3). Both possibilities could not be rigorously examined in the present study due to the practical limitations of a drug discovery setting and available tools for all liver transporters, although some GPa inhibitors were found to be P-glycoprotein substrates (data not shown). Therefore, this complexity may be avoided by using free concentration in liver to correlate with an in vitro activity.

Lastly, the present study demonstrates an excellent inverse correlation (R^2=0.862, Figure 5) between the $[I]_{b\text{-}GPa}/[I]_{t\text{-}liver}$ ratio and the liver concentration of a GPa inhibitor at the MED. The $[I]_{b\text{-}GPa}/[I]_{t\text{-}liver}$ ratio represents the fraction of a GPa inhibitor bound to GPa over the total GPa inhibitor concentration in the liver of an ob/ob mouse after oral administration of the GPa inhibitor. A high $[I]_{b\text{-}GPa}/[I]_{t\text{-}liver}$ ratio of a GPa inhibitor suggests a high selectivity of the GPa inhibitor to GPa over all other liver proteins and low non-specific binding. Therefore, to achieve the same amount of GPa bound to the inhibitor at the respective MED, the GPa inhibitor with a high $[I]_{b\text{-}GPa}/[I]_{t\text{-}liver}$ ratio

requires a lower liver concentration than one with a low $[I]_{b\text{-}GPa}/[I]_{t\text{-}liver}$ ratio. The very poor $[I]_{b\text{-}GPa}/[I]_{t\text{-}liver}$ ratio for compound **8** may be the reason, at least in part, for its poor in vivo activity.

In summary, the present study has demonstrated a useful in vitro model for determining $Kd_{GPa\text{-}liver}$ that correlates well with the in vivo activities of a series of GPa inhibitors. This model may be particularly useful for studying compounds where binding to the target enzyme in vitro is dependent upon multiple endogenous cofactors present in the target tissue. A selectivity parameter determined by the $[I]_{b\text{-}GPa}/[I]_{t\text{-}liver}$ ratio can also be very useful for understanding in vivo potency and disposition. Improving the selectivity of a GPa inhibitor to GPa over non-specific protein binding in the liver will reduce the exposure required to show efficacy and may help to avoid an undesired effect. The simplicity and usefulness of this model, along with its adaptation to a 96-well format, make it a useful new tool applied to many drugs in pharmacology studies in which the correlation between in vitro and in vivo activities for the target tissue (such as solid tumors, brain and liver) is critical.

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Footnotes:

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Legends for figures.

Figure 1. The diagram of equilibrium dialysis assays.

Figure 2. Lack of correlation between human liver GPa IC_{50} and plasma concentration (1 or 3 h post dose; $R^2 = 0.012$) or liver concentration (3 h post dose; $R^2 = 0.033$) of a GPa inhibitor in vivo from ob/ob mice at the MED.

Figure 3. Lack of correlation between HUMAN LIVER GPa IC_{50} and free plasma concentration (1 or 3 h post dose; $R^2 = 0.497$) or free liver concentration (3 h post dose; $R^2 = 0.184$) of a GPa inhibitor in vivo from ob/ob mice at the MED.

Figure 4. Correlation between $Kd_{GPa\text{-}liver}$ estimated from the equilibrium dialysis studies and the free liver concentration of a GPa inhibitor estimated from ob/ob mice in vivo 3 h post dose at the MED for GPa inhibitors listed in Table 2.

Figure 5. Correlation between the liver concentration of a GPa inhibitor in vivo from the ob/ob mouse 3 h post dose at the MED and the ratio of $[I]_{b\text{-}GPa}$ / $[I]_{t\text{-}liver}$ for the panel of GPa inhibitor listed in Table 1.

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Table 1. Identity and source of GPa inhibitors studied. [a]Values were calculated by the BioByte ClogP program, which used the Pomona 4.3 algorithm. [b]The original compound ID. [c]Trade name for this compound.

Compound No.	Compound Name	cLogP[a]	Reference
1	5-chloro-N-((S)-1-(3-(N-hydroxyimino)pyrrolidin-1-yl)-1-oxo-3-phenylpropan-2-yl)-1H-indole-2-carboxamide, more polar isomer	4.16	(Hulin et al., 1996b)
2	N-((N-(cyanomethyl)-N-cyclopentylcarbamoyl)methyl)-5-chloro-1H-indole-2-carboxamide	3.12	(Wright et al., 2005)
3 (CP-316819[b])	N-((1R,2S)-1-(N-methoxy-N-methylcarbamoyl)-1-hydroxy-3-phenylpropan-2-yl)-5-chloro-1H-indole-2-carboxamide	3.86	(Hulin et al., 1996a)
4 (CP-368296[b], Ingliforib[c])	5-chloro-N-((2S,3R)-3-hydroxy-4-((3S,4R)-3,4-dihydroxypyrrolidin-1-yl)-4-oxo-1-phenylbutan-2-yl)-1H-indole-2-carboxamide	3.10	(Hulin et al., 1996a)
5	5-chloro-N-((S)-1-(3-hydroxyazetidin-1-yl)-1-oxo-3-phenylpropan-2-yl)-1H-indole-2-carboxamide	3.69	(Hulin et al., 1996b)
6	N-((N-(cyanomethyl)-N-cyclobutylcarbamoyl)methyl)-5-chloro-1H-indole-2-carboxamide	2.56	Pfizer file compound
7 (CP-91149[b])	N-((1R,2S)-1-(dimethylcarbamoyl) -1-hydroxy-3-phenylpropan-2-yl)-5-chloro-1H-indole-2-carboxamide	3.56	(Hoover et al., 1998)
8	5-chloro-N-((2S,3R)-4-(tetrahydro-1,1-dioxido-thiophen-2-yl)-3-hydroxy-4-oxo-1-phenylbutan-2-yl)-1H-indole-2-carboxamide	2.92	(Bussolotti et al., 2004)
9	N-((N-(tetrahydro-2H-pyran-4-yl)-N-(3-hydroxypropyl) carbamoyl)methyl)-5-chloro-1H-indole-2-carboxamide	1.51	Pfizer file compound

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10 (CP-320626[b])	5-chloro-N-((S)-1-(4-hydroxypiperidin-1-yl)-1-oxo-3-(4-fluorophenyl)propan-2-yl)-1H-indole-2-carboxamide	2.54	(Hoover et al., 1998)
11	N-((N-(2-hydroxyethyl)-N-isobutylcarbamoyl)methyl)-5-chloro-1H-indole-2-carboxamide	3.09	(Wright et al., 2005)
12	N-((N-(tetrahydro-2H-pyran-4-yl)-N-(2-hydroxyethyl) carbamoyl)methyl)-5-chloro-1H-indole-2-carboxamide	1.27	(Wright et al., 2005)
13	(E)-3-(4-chlorophenyl)-N-((S)-1-((3S,4R)-3,4-dihydroxypyrrolidin-1-yl)-1-oxo-3-phenylpropan-2-yl)acrylamide	3.20	Pfizer file compound

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Table 2. In vitro and in vivo activities for the panel of GPa inhibitors. [a]The average value from n= 2-5 measurements are used for human liver GPa IC50, in vivo liver concentration at 3 h post dose, and plasma concentration 1 or 3 h post dose from the ob/ob mouse at the MED. The MEDs were confirmed from a repeat study. [b]The value was estimated based on an extrapolation from the exposure at 50 mg/kg, assuming exposure proportional to the dose.

Compound No.	Human liver GPa IC_{50} (μM)[a]	MED (mg/kg)	Liver concentration (μM) at MED[a]	Plasma concentration (μM) at MED[a]
1	0.023	5	1.4	0.066
2	0.030	5	3.6	0.060
3	0.040	5	1.7	0.30
4	0.055	5	1.8	0.50
5	0.062	2.5	0.8	0.20
6	0.11	25	14.5	2.7
7	0.12	25	4.7[b]	2.0[b]
8	0.128	>25	>64	12.8

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9	0.14	10	7.9	0.80
10	0.155	15	27.0	2.0
11	0.18	10	2.3	0.37
12	0.253	2.5	1.0	0.14
13	0.46	2.5	7.4	5.4

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Table 3. Summary of binding parameters determined from all equilibrium dialysis assays and distribution ratios of total or bound GPa inhibitor in the GPa chamber and in the liver homogenate chamber. The mean ± SD from n=3-5 measurements are used for all measured parameters. The calculated parameters are based on the mean value of measured parameters.

Compound No.	$fu_{GPa\text{-}liver}$	$fu_{GPa\text{-}buffer}$ (± SD)	uFu_{liver} (± SD)	$Kd_{GPa\text{-}liver}$ (μM)	$[I]_{t\text{-}GPa}$ / $[I]_{t\text{-}liver}$ (± SD)	$[I]_{b\text{-}GPa}$ / $[I]_{t\text{-}liver}$
1	0.041	0.050 (±0.002)	0.0032 (±0.0002)	0.07	0.39 (±0.01)	0.37
2	0.038	0.130 (±0.007)	0.0050 (±0.0002)	0.07	0.64 (±0.08)	0.62
3	0.026	0.058 (±0.003)	0.0040 (±0.0003)	0.05	0.76 (±0.03)	0.74
4	0.11	0.102 (±0.006)	0.0160 (±0.0007)	0.20	0.64 (±0.02)	0.57
5	0.043	0.076 (±0.006)	0.0058 (±0.0001)	0.08	0.65 (±0.01)	0.62
6	0.23	0.097 (±0.005)	0.0120 (±0.0015)	0.42	0.23 (±0.02)	0.18
7	0.064	0.192 (±0.004)	0.0040 (±0.0003)	0.13	0.30 (±0.01)	0.28
8	0.13	0.21 (±0.017)	0.0033 (±0.0003)	0.3	0.12 (±0.008)	0.10
9	0.35	0.210 (±0.020)	0.0380 (±0.0017)	0.75	0.55 (±0.01)	0.36
10	0.28	0.097 (±0.003)	0.0075 (±0.0005)	0.69	0.076 (±0.005)	0.055
11	0.13	0.22 (±0.006)	0.025 (±0.001)	0.26	0.82 (±0.07)	0.71
12	0.19	0.255 (±0.003)	0.044 (±0.001)	0.39	0.94 (±0.02)	0.76

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13	0.36	0.234 (±0.005)	0.023 (±0.0004)	0.92	0.30 (±0.01)	0.19

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Figure 1

Fig. 1a. Equilibrium dialysis A.

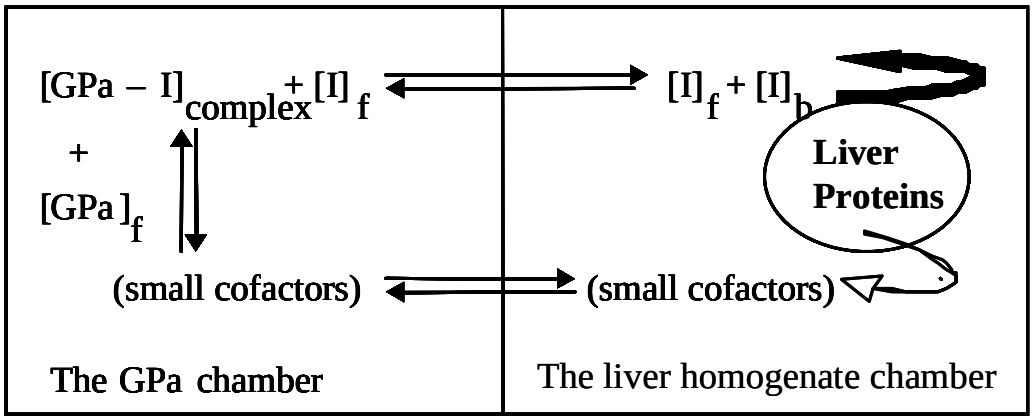

Fig 1b. Equilibrium dialysis B.

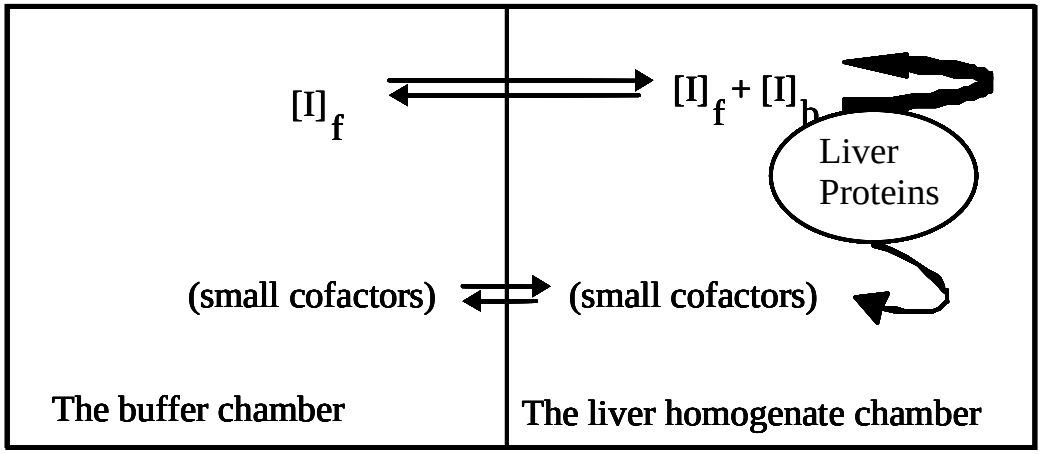

Fig 1c. Equilibrium dialysis C.

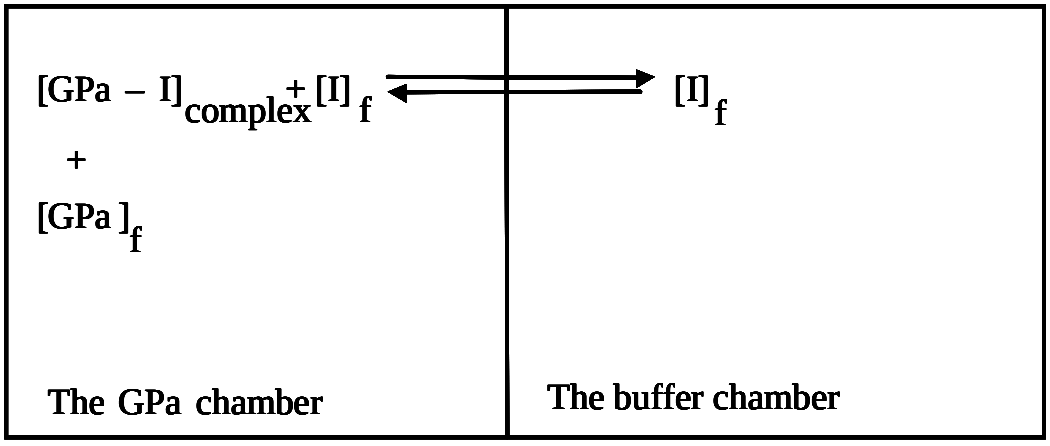

Figure 2

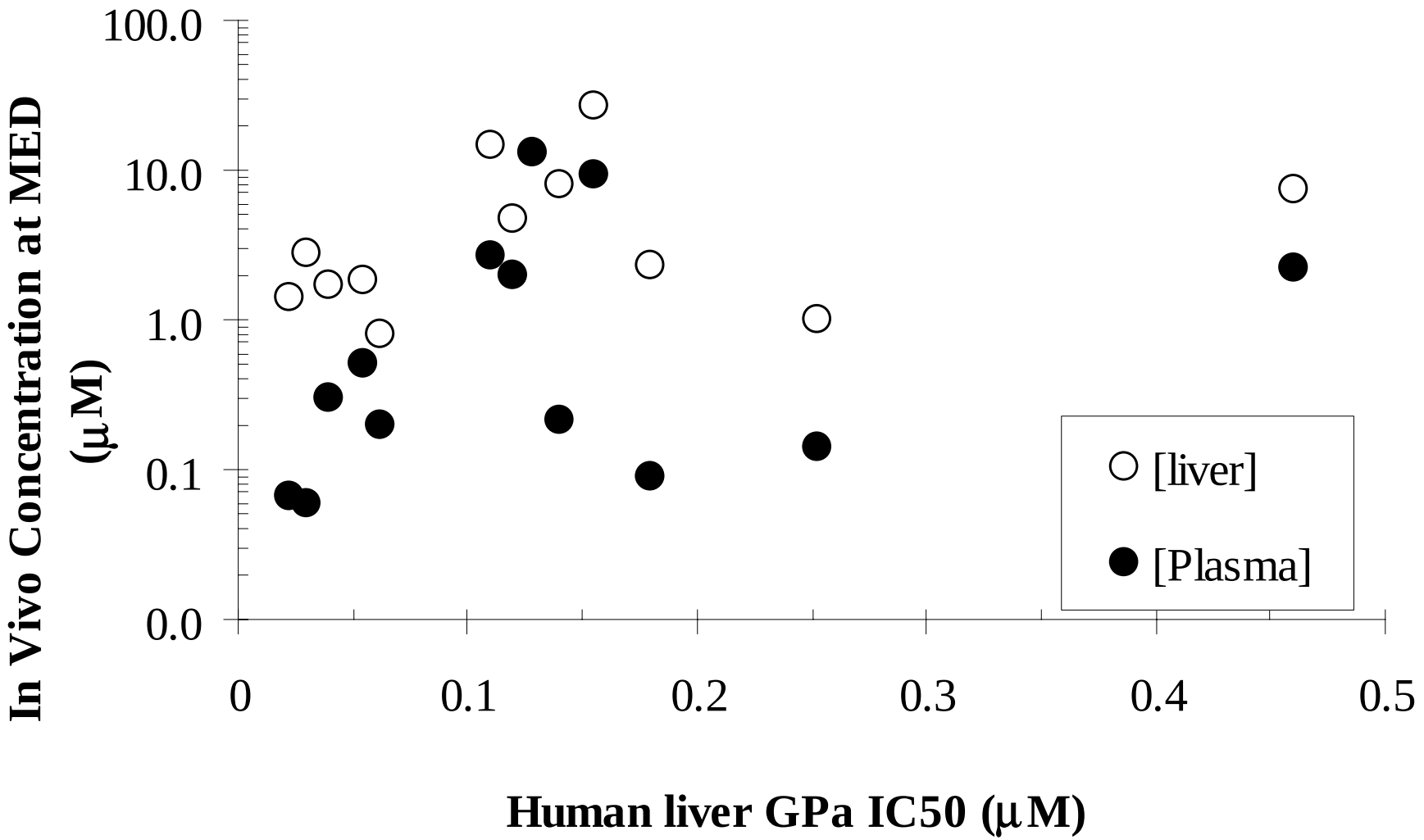

Figure 3

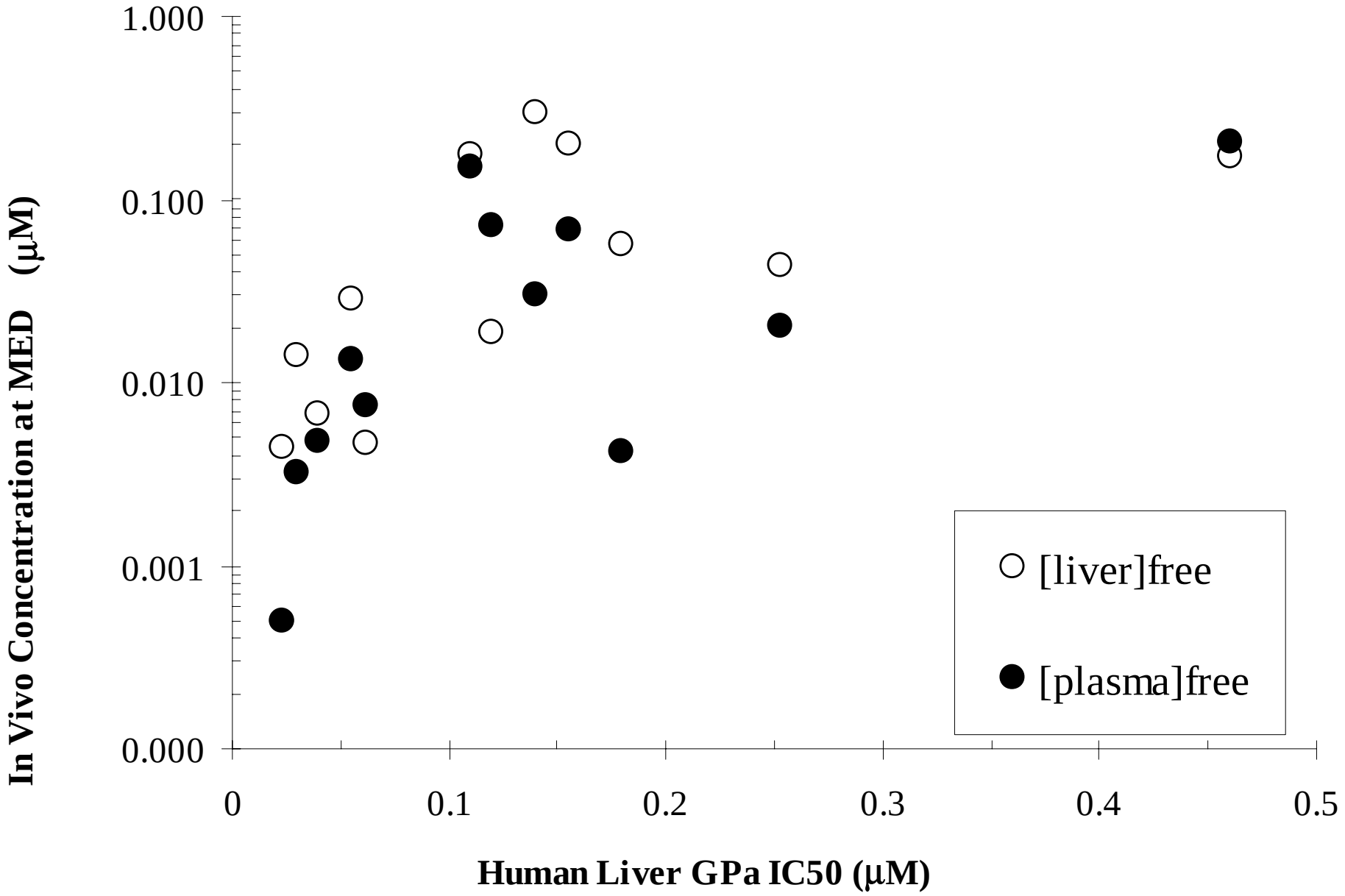

Figure 4

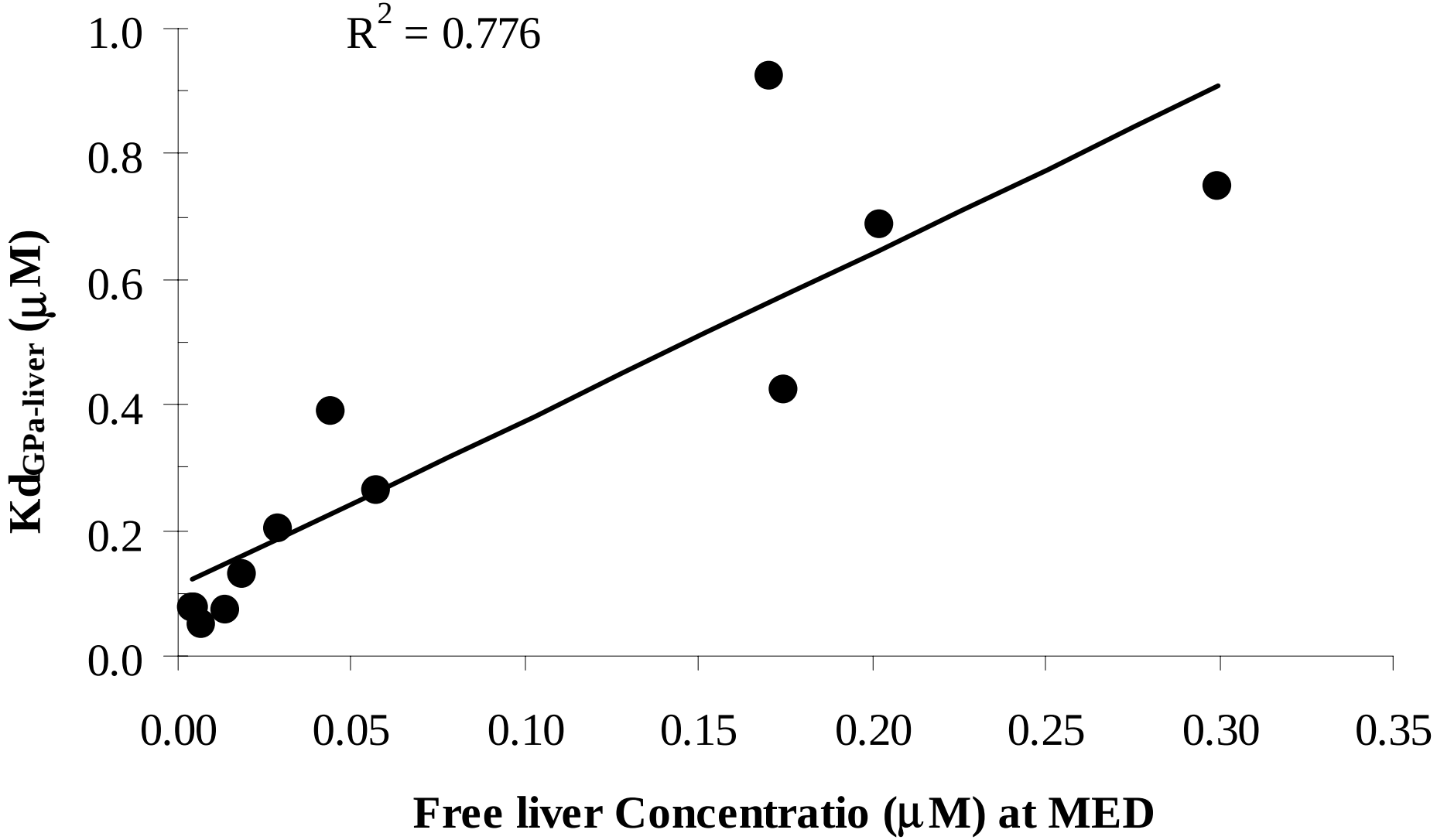

Figure 5