TITLE: Application of Target-Mediated Drug Disposition Model to Small Molecule Heat Shock Protein 90 Inhibitors

AUTHORS: Shinji Yamazaki, Zhongzhou Shen, Ying Jiang, Bill J. Smith and Paolo Vicini

ADDRESS: Pharmacokinetics, Dynamics and Metabolism (S.Y., Z.S., Y.J., B.J.S., P.V.), Pfizer Worldwide Research & Development, San Diego, California
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CORRESPONDING AUTHOR:

Shinji Yamazaki, Ph.D.
Pharmacokinetics, Dynamics and Metabolism, La Jolla Laboratories
Pfizer Worldwide Research and Development
10777 Science Center Drive, San Diego, CA 92121, USA
Tel: 858-622-8050 Fax: 858-678-8269
E-mail: shinji.yamazaki@pfizer.com

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ABBREVIATIONS:

$CL$, clearance; $f_u$, unbound fraction; $K_D$, equilibrium dissociation constant; HAI, higher affinity inhibitors; HSP90, heat shock protein 90; LAI, lower affinity inhibitor; LC-MS/MS, liquid-chromatography tandem mass spectrometry; $OFV$, objective function value; PBPK, physiologically based pharmacokinetics; $R_{bp}$, blood-to-plasma concentration ratio; $R_{total}$, maximum receptor capacity; TMDD, target-mediated drug disposition; $V_1$, volume of distribution in central compartment; $V_2$, volume of distribution in peripheral compartment; $V_{ss}$, volume of distribution at steady-state
ABSTRACT

Replacement of hydrogen with fluorine within three pairs of structurally similar small molecule inhibitors of heat shock protein 90 (HSP90) resulted in differences in inhibition constants ($K_i$) in vitro as well as marked differences in rat intravenous pharmacokinetic profiles. The difference in pharmacokinetic profiles between lower and higher affinity inhibitors (LAIs and HAIs, respectively) was characterized by remarkably different estimates for steady-state volumes of distribution ($V_{ss}$: 1.8 to 2.0 versus 10 to 13 L/kg) with comparable clearance estimates (3.2 to 3.5 L/h/kg). When the observed $V_{ss}$ estimates were compared to the values predicted with the tissue-composition-based model, the observed $V_{ss}$ estimates for HAIs were 4 to 8-fold larger than the predicted values whereas the $V_{ss}$ values for LAIs were comparable each other. Accordingly, a negative relationship between in vitro HSP90 $K_i$ versus in vivo $V_{ss}$ estimates was observed among these inhibitors. We therefore hypothesized that pharmacokinetic profiles of these inhibitors could be characterized by target-mediated drug disposition (TMDD) model. In vivo equilibrium dissociation constant ($K_D$) estimates for HAIs due to target binding by TMDD model with rapid binding approximation were 1 to 6 nM (equivalent to 0.3 to 2 nM free drug), which appeared comparable to the in vitro $K_i$ estimates (2 to 3 nM). In vivo $K_D$ values of LAIs were not accurately determined by the TMDD model, likely due to non-specific binding dependent-tissue distribution obscuring TMDD profiles. Overall, these results suggest that the observed large $V_{ss}$ estimates for potent HSP90 inhibitors are likely due to pharmacological target binding.
INTRODUCTION

Typical pharmacokinetic (PK) evaluation for small molecules generally assumes that amounts of drug distributing and binding to pharmacological targets (e.g., receptors and enzymes) are negligible; therefore, the interaction of drugs with pharmacological targets is not considered to affect PK profiles as it is masked by the much larger capacity of non-specific binding to proteins in blood and tissues. However, this assumption is not valid for target-mediated drug-disposition (TMDD), in which a significant proportion of drug, relative to the administered dose, interacts with pharmacological targets. Such an interaction can affect PK profiles, resulting in dose-dependent non-linear PKs. A general modeling framework has been developed to characterize this phenomenon for many biologic drugs, especially monoclonal antibodies (Levy, 1994; Mager and Jusko, 2001). The model employs a system of differential equations including compound- and receptor-dependent parameters such as the concentrations of drug, free target and compound-target complex and the binding and dissociation constants ($k_{on}$ and $k_{off}$, respectively). Furthermore, the TMDD model can be simplified to its quasi-equilibrium or rapid-binding form by replacing $k_{on}$ and $k_{off}$ with an equilibrium dissociation constant ($K_D$) since the in vivo simultaneous estimation of $k_{on}$ and $k_{off}$ often becomes difficult (Wagner, 1971; Mager, 2006; Gibiansky and Gibiansky, 2009; Marathe et al., 2009). A rapid binding approximation of TMDD model was successfully applied to biologic drugs exhibiting non-linear PK profiles (Mager et al., 2003; Mager and Krzyzanski, 2005; Woo et al., 2007); however, TMDD models have to date rarely applied to small molecule drugs.

The pharmacological target under consideration in this study is heat shock protein 90 (HSP90), which is highly conserved and expressed in a variety of different organisms from bacteria to mammals (Csermely et al., 1998). HSP90 is a member of a class of proteins called...
molecular chaperones that regulates conformation, stability and activity of numerous key signaling proteins (Csermely et al., 1998; Whitesell and Lindquist, 2005; Mahalingam et al., 2009). These client proteins are involved in a large variety of biological processes such as cell proliferation, cell transformation and tumor progression. Targeting HSP90 offers an opportunity for the inhibition of multiple pathways in human cancers since a number of mutant oncoproteins requires HSP90 to maintain their stability and function (Mahalingam et al., 2009; Holzbeierlein et al., 2010; Trepel et al., 2010). Extensive efforts in drug discovery with rational drug design have successfully advanced more than ten HSP90 inhibitors into clinical trials with cancer patients (Li et al., 2009; Trepel et al., 2010). Interestingly, it has been reported that small molecule HSP90 inhibitors consistently showed large volumes of distribution in nonclinical species (10 to 500 L/kg) and patients (3 to 20 L/kg) even though their chemical structures vary from natural product analogs such as 17-AAG and 17-DMAG (Goetz et al., 2003; Eiseman et al., 2005; Pacey et al., 2011) to newly synthesized chemical entities such as AT13387, NVP-AUY922, NXD30001 and PF04929113 (Jensen et al., 2008; Woodhead et al., 2010; Zhu et al., 2010; Rajan et al., 2011). Consistently, we observed that the estimates for volumes of distribution of relatively potent small molecule HSP90 inhibitors were larger than those of less potent inhibitors when structurally similar compounds were compared in our drug discovery program. Since HSP90 is one of the most abundant proteins in unstressed eukaryotic cells, accounting for 1–2% of cytosolic protein (Parsell and Lindquist, 1993; Csermely et al., 1998), it may be possible that the target binding of HSP90 inhibitors affects in vivo PK profiles (e.g., TMDD) as is often observed for biologic drugs (Mager et al., 2003; Mager and Krzyzanski, 2005; Woo et al., 2007; Gibiansky and Gibiansky, 2009). As shown in Fig. 1, we selected six in house small molecule HSP90 inhibitors, i.e., three pairs of structurally similar compounds...
showing in vitro lower and higher affinities to HSP90 (henceforth LAIs and HAIs, respectively). The corresponding LAIs and HAIs have exactly the same chemical structures except for the presence of either a hydrogen (A1, B1 and C1 as LAIs) or fluorine (A2, B2 and C2 as HAIs) on the pyrazole ring. The replacement of hydrogen with fluorine enhanced HSP90 inhibition constant ($K_i$) by 3 to 5-fold. In order to investigate our hypothesis, we applied a rapid binding approximation of the TMDD model to characterize in vivo rat PK profiles of these inhibitors. Results were also compared to those from non-compartmental PK and physiologically-based pharmacokinetic (PBPK) modeling analyses. Subsequently, we explored an effect of target affinity on in vivo PK profiles by simulating plasma concentrations of virtual inhibitors having a wide range of binding affinity using the rapid binding approximation model. These results suggest that an interaction of potent small molecule HSP90 inhibitors to their pharmacological target can affect their in vivo PK profiles.
Materials and Methods

Chemicals

All in house HSP90 inhibitors (A1, A2, B1, B2, C1 and C2) were synthesized by Pfizer Worldwide Research and Development (San Diego, CA) (Zehnder et al., 2011). All other commercially available reagents and solvents were of either analytical or high performance liquid chromatography grade.

In Vivo PK Study

All of the procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and with Pfizer Animal Care and Use Committee guidelines. Male Sprague-Dawley rats (Charles River Laboratories, Hollister, CA), weighing 0.25 to 0.28 kg, were used for the PK studies. Each animal was housed in stainless cages or metabolic cages (urine collection) under controlled conditions (20 - 26°C, 30-70% relative humidity and 12-hour light/dark cycle) with free access to food and water throughout the study. All HSP90 inhibitors (A1, A2, B1, B2, C1 and C2) were dissolved in PEG400/saline (4:6, v/v) as the intravenous formulation. All dose levels of the inhibitors were expressed as free base equivalents. Animals (n=2 per group) were given each inhibitor intravenously at the dose of 2.5 mg/kg through a catheter implanted into the jugular vein. Additionally, in order to investigate dose-dependent PK profiles, A1 and A2 were intravenously administered to the animals (n=2 per group) at the doses of 0.75 and 7.5 mg/kg in addition to 2.5 mg/kg. Consideration was given to the reduction in animal use to align the number of animals per group with the study objectives. Blood samples were collected from all animals at pre-determined time points up to 24 hours post-dose with K₂EDTA or K₃EDTA as the anticoagulant. Plasma samples were harvested by centrifugation at 1500 × g for 10 minutes and
were stored at approximately -20°C until analysis. Urine samples were also collected over 24 hours post-dose with a minimal cage rinse and the samples were stored at approximately -20°C until analysis.

Bioanalytical Assay for HSP90 Inhibitors

Concentrations of each inhibitor in plasma and urine were determined by a liquid chromatography tandem mass spectrometry (LC-MS/MS) method following protein precipitation of plasma samples. The LC-MS/MS system consisted of Shimadzu LC-10AD vp pumps (Shimadzu, Columbia, MD), a CTC PAL autosampler (Leap Technologies, Carrboro, NC), and a Sciex API 4000 mass spectrometer (AB Sciex, Foster City, CA) equipped with a turbo ion spray source. Chromatographic separation of the analytes was achieved using Zorbax, XDB-phenyl, 5 μm, 2.1 × 50 mm column (Agilent technologies, Palo Alto, CA). The mobile phase consisting of 0.1% formic acid in water (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) was delivered at a flow rate of 0.6 ml/min under a 3.0-min gradient elution program. The initial solvent composition of 2% B was held for 0.5 min, increased linearly to 90% B over 1.5 min, and then held at 90% B for an additional 0.2 min. The column was then equilibrated at the initial conditions (2% B) for 0.8 min. The mass spectrometry was operated in the positive ionization mode using multiple reaction monitoring. The ion source parameters were optimized for each inhibitor: turbo ion spray voltage, 5.0 kV; turbo ion spray temperature, 500°C; declustering potential, 81-96 V; collision energy, 33-35 V; entrance potential, 10 V; collision cell exit potential, 14 V; dwell times, 100 ms; and mass resolution (quadrupoles Q1 and Q3), 0.7 mass units (peak width at half-height). Each analyte was monitored at specific precursor ion→product ion transitions of m/z 516→417 for A1, 534→435 for A2, 512→417 for B1, 530→435 for B2, 500→391 for C1, 518→435 for C2 and 386→122 for buspirone as a generic
internal standard. Data were processed using AB Sciex Analyst software (v. 1.4.2). The calibration curve range was 0.25 to 500 ng/mL. The back-calculated calibration standard concentrations were within 15% of their theoretical concentrations with coefficients of variation of less than 15%. The precision and accuracy of the quality control samples were within 15%.

**HSP90 Biochemical Assay**

Compounds were evaluated for potency against recombinant human HSP90 using a scintillation proximity competition binding assay as previously reported (Kung et al., 2010). Briefly, the incubation mixture consisting of 100 mM Hepes buffer (pH 7.5) containing 5 nM HSP90, 150 mM KCl, 50 nM ³H-ligand, various concentrations of HSP90 inhibitors. A tritiated proprietary small molecule ligand was used as the displacement ligand with either full length or N-terminal HSP90. The reaction was carried out in a 96-well plate with the addition of the labeled ligand at the start of incubation. HSP90 inhibitors in 100% DMSO were diluted to 11 different concentrations prior to addition to the HSP90/³H-ligand solution. The %inhibition values (Y) were plotted against log HSP90 inhibitor concentration (X) and then the $K_i$ value was determined as follows:

$$Y = A + \left(\frac{(B - A)}{1 + 10^{(\log IC_{50} - X)\gamma}}\right)$$  \hspace{1cm} (1)

$$K_i = IC_{50} / \left[1 + (L/K_d)\right]$$  \hspace{1cm} (2)

where $A$ is the lower plateau, $B$ is the upper plateau, $\gamma$ is the Hill coefficient, $L$ is the total concentration of ³H-ligand (200 nM) and $K_d$ is the dissociation constant for the ligand (240 nM).

**In Vitro Plasma Protein Binding**

Plasma protein binding of HSP90 inhibitors was determined in male Sprague-Dawley rats at 0.3 to 3 μM (0.15 to 1.5 μg/mL) using the equilibrium dialysis technique as described previously (Yamazaki et al., 2008). Briefly, the study was conducted in a 96-well Teflon®
dialysis chamber (HTDialysis LLC, Gales Ferry, CT) using a semi-permeable membrane (Spectra/Por4®, Spectrum, Laguna Hills, CA) with a 12,000-14,000 Da molecular mass cut-off. After the incubation at 37°C for 6 hours, aliquots of plasma and buffer samples were extracted with aliquots of acetonitrile: methanol mixture (1:1, v/v) containing the internal standard and analyzed by LC-MS/MS as described above. The unbound fraction in plasma ($f_{u,\text{plasma}}$) was calculated by the following equation:

$$f_{u,\text{plasma}} = \frac{C_{\text{buffer}}}{C_{\text{plasma}}}$$

where $C_{\text{buffer}}$ and $C_{\text{plasma}}$ denote the concentrations of HSP90 inhibitors in buffer and plasma, respectively, after the incubation.

**Blood-to-Plasma Concentration Ratio**

The blood-to-plasma concentration ratios of HSP90 inhibitors were determined by incubating the compounds with whole blood from male Sprague-Dawley rats as described previously (Yamazaki et al., 2008). Briefly, HSP90 inhibitors (final 1 μM), dissolved in acetonitrile:water (1:1, v/v), was added to whole blood, and was incubated at 37°C for 1 hour. Aliquots of the spiked whole blood and the harvested plasma were transferred into a tube with acetonitrile:methanol (1:1, v/v) containing the internal standard (1 μM). An appropriate volume of all samples was analyzed by LC-MS/MS. All of the incubations were performed in triplicate. The blood-to-plasma concentration ratio ($R_{bp}$) and the unbound fraction in whole blood ($f_{u,\text{blood}}$) were calculated by the following equations:

$$R_{bp} = \frac{C_{\text{blood}}}{C_{\text{plasma}}}$$

$$f_{u,\text{blood}} = f_{u,\text{plasma}} / R_{bp}$$

where $C_{\text{blood}}$ and $C_{\text{plasma}}$ denote the drug concentrations in whole blood and plasma, respectively, after the incubation.
**In Vitro Non-Specific Binding**

In vitro non-specific bindings of HSP90 inhibitors, A1 and A2, were determined in liver microsomes using the equilibrium dialysis technique as described above. Dialysis mixtures contained liver microsomes (0.5 mg/mL), 125 mM MgCl₂ and A1 or A2 (1 µM) in 100 mM potassium phosphate, pH 7.4, in a final volume of 0.2 mL. All of the incubations were performed in triplicate. The unbound fraction in microsomes \( f_{u,\text{mic}} \) was calculated by the following equation:

\[
 f_{u,\text{mic}} = \frac{C_{\text{buffer}}}{C_{\text{mic}}}
\]

where \( C_{\text{buffer}} \) and \( C_{\text{mic}} \) denote the drug concentrations in buffer and microsomes, respectively, after the incubation.

**In Vitro Metabolic Clearance**

Pooled liver microsomes of male Sprague-Dawley rats (n = 50) were purchased from BD Gentest (Woburn, MA). The incubation mixture consisted of liver microsomes (0.5 mg/mL), 125 mM MgCl₂ and A1 or A2 (0.078 to 10 µM) in 100 mM potassium phosphate, pH 7.4. The reaction was initiated by the addition of NADPH (final 1 mM) and the mixture (0.2 mL) was incubated at 37°C for 0, 5, 10, 15, 30, 45 and 60 minutes. The reaction was terminated by the addition of cold acetonitrile (0.3 mL) containing the internal standard (1 µM) followed by vortexing for 15 minute on a SP Multi-tube Vortexer (Baxter, McGaw Park, IL). The sample was centrifuged (Allegra 6KR with GH-3.8A rotor) at 2000 \( \times \) g for 10 minutes and the supernatant (0.1 mL) was mixed with an equal volume of water in an HPLC plate for LC-MS/MS analysis. All of the incubations were performed in triplicate. Michaelis-Menten parameters (\( K_m \) and \( V_{max} \)) for A1 and A2 were estimated by nonlinear regression analysis.
Non-compartmental PK Analysis

For the in vivo PK studies, single-dose plasma concentration-time profiles of each inhibitor were analyzed by model-independent methods. Plasma concentration at time zero after an intravenous bolus administration was calculated in the same manner as the physiologically based pharmacokinetic (PBPK) modeling (i.e., dose amount divided by blood volume of 54 mL/kg followed by the correction for $R_{bp}$) as described below, to assess the difference between the observed versus PBPK model-simulated PK parameters. The area under the plasma concentration-time curve from time zero to the last time point ($t_{last}$) with a quantifiable concentration ($C_{last}$), $AUC_{0\text{-}last}$, was calculated using the linear trapezoidal rule. The area under the plasma concentration-time curve was then extrapolated to infinity ($AUC_{0\text{-}\infty}$):

$$AUC_{0\text{-}\infty} = AUC_{0\text{-}last} + \frac{C_{last}}{\lambda}$$ (6)

where $\lambda$ was the elimination rate constant determined by linear regression of the last two to four quantifiable data points in the log plasma concentration-time curves.

The apparent terminal half-life ($t_{1/2}$) was calculated as follows:

$$t_{1/2} = \frac{ln(2)}{\lambda}$$ (7)

Plasma clearance ($CL_p$), blood clearance ($CL_b$) and $V_{ss}$ were calculated by the following equations:

$$CL_p = \frac{Dose}{AUC_{0\text{-}\infty}}$$ (8)

$$CL_b = CL_p / R_{bp}$$ (9)

$$V_{ss} = CL_p \cdot AUMC_{0\text{-}\infty} / AUC_{0\text{-}\infty}$$ (10)

where $AUMC_{0\text{-}\infty}$ was the area under the first moment of the plasma concentration-time curve.
from time zero to infinity:

\[ AUMC_{0-\infty} = AUMC_{0-\text{last}} + \frac{C_{\text{last}}}{\lambda^2} + \frac{C_{\text{last}} \cdot t_{\text{last}}}{\lambda} \]  \hspace{1cm} (11)

**PBPK Modeling**

The GastroPlus 5.3 PBPK model and its built-in mass balance differential equations were used for all simulations (Simulations Plus Inc., Lancaster, CA). In brief, the PBPK model was composed of 14 tissue compartments, including lung, spleen, liver, gut, adipose tissue, muscle, heart, brain, kidney, skin, testes, bone and rest of the body, which were linked by the venous and arterial blood circulation. It was assumed that drug distributes instantaneously and homogenously within each tissue compartment, and uptake of drug within each tissue compartment was limited by the blood flow (i.e., perfusion rate-limited distribution).

Physiological parameters for tissue volume and blood flows used for the present study were previously reported (Yamazaki et al., 2011b). The observed in vivo \( CL_h \) values were used as the hepatic clearance inputs for the PBPK model. Renal clearances of these inhibitors were set to null based on in vivo PK results (<5% of the dose administered as parent drug in urine). Tissue-to-plasma partition coefficients \( k_p \) for each tissue compartment were predicted from physico- and bio-chemical parameters (Rodgers et al., 2005).

**TMDD Model**

The details of the general TMDD model have been introduced previously (Levy, 1994; Mager and Jusko, 2001). As shown in Fig. 2, drug in the central compartment \( (C) \), i.e., plasma, binds to free receptors \( (R) \) at the second-order rate \( (k_{on}) \) to form a drug-receptor complex \( (RC) \), which dissociates at the first-order rate \( (k_{off}) \) or be internalized by the first-order rate process of endocytosis \( (k_{int}) \). Drug in the central compartment can also be directly eliminated at a first-order rate \( (k_{el}) \) or be distributed a non-specific binding dependent tissue compartment \( (A_T) \) at a
first-order process ($k_{pt}$ and $k_{tp}$). Free receptor can be synthesized at a zero-order rate ($k_{syn}$) and degraded at a first-order rate ($k_{deg}$). The drug input rate ($In(t)$) to the central compartment accounts for the intravenous bolus administration. Drug, free receptor and drug-receptor complex are expressed in nanomolar concentrations, and the model equations are as follows:

$$
\frac{dC}{dt} = \ln(t) - k_{on} \cdot R \cdot C + k_{off} \cdot RC - k_{el} \cdot C
$$

$$
\frac{dA_T}{dt} = k_{pt} \cdot C \cdot V_1 - k_{tp} \cdot A_T
$$

$$
\frac{dR}{dt} = k_{syn} - k_{on} \cdot R \cdot C + k_{off} \cdot RC - k_{deg} \cdot R
$$

$$
\frac{dRC}{dt} = k_{on} \cdot R \cdot C - (k_{off} + k_{int}) \cdot RC
$$

where $V_1$ denotes the apparent volume of distribution in the central compartment.

In a rapid binding approximation model (Mager and Krzyzanski, 2005; Gibiansky and Gibiansky, 2009; Marathe et al., 2009), the equilibrium between the binding and dissociation is assumed to be achieved instantly since the binding of drug to free receptor and dissociation of the drug-receptor complex are often of several orders of magnitude faster than the remaining system process:

$$
R \cdot C / RC = k_{off} / k_{on} \approx K_D
$$

where $K_D$ denotes the equilibrium dissociation constant.

In addition, the baseline of free receptor ($R(0)$) and the maximum receptor capacity ($R_{total} = R + RC$) are considered to remain constant assuming the receptor turnover rate and the internalization rate of the drug-receptor complex are slow with respect to the experimental time scale (i.e., $k_{syn} = k_{deg} = 0$ and $k_{int} = 0$, respectively). These assumptions allow to posit $R_{total}(0) = R(0) = k_{syn} / k_{deg}$. Collectively, the rapid binding approximation model with slow receptor turnover and negligible internalization can be described by the following single equation (Wagner, 1971; Mager and Krzyzanski, 2005):
The rapid binding approximation model with a non-specific binding dependent tissue compartment ($A_T$) is henceforth referred to as two-compartment rapid binding approximation model. Since the presence of a non-specific binding dependent tissue compartment is optional depending upon the characteristics of PK profiles, the two-compartment rapid binding approximation model can be simplified to the following equation without $A_T$ (henceforth referred to as one-compartment rapid binding approximation model):

$$\frac{dC}{dt} = \frac{\ln(t) - (k_{el} + k_{pt}) \cdot C + k_{ip} \cdot A_T / V_C}{1 + R_{total} \cdot K_D / (K_D + C)^2}$$

(14)

For HSP90 inhibitors, their bindings to pharmacological target are expected to be as rapid as many other biologics (Mager et al., 2003; Mager and Krzyzanski, 2005; Woo et al., 2007) and $R_{total}$ can be assumed to remain constant because of high HSP90 abundance accounting for 1–2% of the total proteins in normal cells (Parsell and Lindquist, 1993; Csermely et al., 1998). The internalization rate of HSP90 inhibitor-receptor complex can be assumed to be negligible because these inhibitors are considered to bind reversibly to the ATP-binding pocket of HSP90 in the cytosolic fraction without any elimination process through the formation of inhibitor-receptor complex. Collectively, the assumptions underlying a rapid binding approximation model appear to be valid for HSP90 inhibitors.

The parameters of the HSP90 inhibitors were estimated for all compounds simultaneously by a one- or two-compartment rapid binding approximation model using NONMEM 7 with the subroutine ADVAN8 (University of California at San Francisco, San Francisco, CA) in the population model. That is, all inhibitors shared the same PK parameters for either the one- or two-compartment model (e.g., $V_1$, $k_{el}$, $k_{pt}$, $k_{ip}$ and $R_{total}$), while the $K_D$ values...
were estimated for each inhibitor. Additionally, in order to investigate an effect of the
relationship between $K_D$ versus $R_{total}$ on PK profiles, plasma concentrations of virtual inhibitors
having $K_D$ ranging from 0.1 to 1000 nM were simulated by the one- and two-compartment rapid
binding approximation models with assumed $R_{total}$ of 100, 1000 and 10000 nM. In the PK
simulation as representative examples, $CL$ and $V_1$ in the one-compartment model were set equal
to 3 L/h/kg and 1 L/kg, respectively, whereas $CL$, $V_1$, $Q$ (inter-compartmental $CL$) and $V_2$ in the
two-compartment model were set to 3 L/h/kg, 1 L/kg, 3 L/h/kg and 3 L/kg, respectively. The
value of $R_{bp}$ was set as unity to calculate plasma concentrations. The simulation was also
performed by NONMEM 7 with the subroutine ADVAN8. The initial condition at time zero for
the central compartment was dose amount (mg/kg), which was converted to concentration using
the $V_1$ estimate. Residual variability was characterized by a proportional error model. Model
selection was based on a number of criteria such as the NONMEM objective function value
(OFV), estimates, standard errors and scientific plausibility as well as exploratory analysis of the
goodness-of-fit plots. The difference in the OFV between two nested models was compared with
a $\chi^2$ distribution in which a difference of 6.63 was considered significant at the 1% level
(Wahlby et al., 2001).
RESULTS

In Vitro and In Vivo PK

There was no marked difference in the $f_{u,\text{plasma}}$ values (0.30 to 0.33) between all of the HSP90 inhibitors used in the present study, and these $f_{u,\text{plasma}}$ values were concentration-independent at the concentrations tested. Their $R_{bp}$ values were also comparable among these HSP90 inhibitors (1.5 to 1.8). Therefore, the mean values of 0.32 for $f_{u,\text{plasma}}$ and 1.6 for $R_{bp}$ were consistently used for these inhibitors in the present study. For in vitro metabolic clearance in rat microsomes, the estimates for $K_m$ and $V_{max}$ were comparable between A1 and A2: $K_m$ of 9.9 and 10 μM, respectively, and $V_{max}$ of 0.28 and 0.35 nmol/min/mg protein, respectively. The $K_m$ values for both A1 and A2 were corresponding to total plasma concentrations of approximately 30 μM following the correction for $f_{u,\text{plasma}}$.

Estimates for non-compartmental single-dose PK parameters of the HSP90 inhibitors in rats are summarized in Table 1. Plasma concentrations of LAIs, A1, B1, and C1, declined bi-exponentially with apparent terminal $t_{1/2}$ of 0.5 to 1.4 hours. The estimates for $CL_p$ and $V_{ss}$ were roughly comparable among LAIs (e.g., 3.0 to 3.5 L/h/kg and 1.8 to 3.1 L/kg, respectively). The calculated $CL_b$ values from $CL_p$ with $R_{bp}$ were approximately 2 L/h/kg. Assuming the liver was the main organ for elimination, the $CL_b$ values were corresponding to hepatic extraction ratio of approximately 0.5 relative to rat hepatic blood flow of 4.2 L/h/kg (Yamazaki et al., 2011b), suggesting that these HSP90 inhibitors were moderate $CL$ compounds in rats. In contrast, plasma concentrations of HAIs, A2, B2, and C2, declined multi-exponentially with longer apparent terminal $t_{1/2}$ of 3.0 to 8.7 hours. In addition, the $MRT$ estimates for HAIs (3.0 to 4.0 hours) at the dose of 2.5 mg/kg were approximately 6-fold longer than those of the corresponding LAIs (0.5 to 0.6 hours). The estimates for $V_{ss}$ of HAIs (10 to 13 L/kg) were also
approximately 6-fold larger than the corresponding LAIs (1.8 to 2.0 L/kg), whereas the estimates for $CL_p$ were comparable between these inhibitors (3.2 to 3.5 L/h/kg). Thus, a negative relationship characterized by switch-like behavior between $K_i$ and $V_{ss}$ was observed as shown in Fig. 3. In the dose-proportional studies of A1 and A2 at the doses of 0.75 to 7.5 mg/kg, the estimates of $CL_p$ and $V_{ss}$ for A1 were comparable (3.0 to 3.3 L/h/kg and 2.0 to 3.1 L/kg, respectively) at these doses. In contrast, although the $CL_p$ estimates for A2 were consistent (2.6 to 3.2 L/h/kg), the $V_{ss}$ estimate at the highest dose of 7.5 mg/kg was 2 to 3-fold lower than those at the doses of 0.75 and 2.5 mg/kg, suggesting a dose-dependent saturation of tissue distribution at the dose of 7.5 mg/kg.

**PBPK Modeling**

The PBPK model-simulated and observed plasma concentration-time profiles of HSP90 inhibitors are graphically presented in Fig. 4. Overall, the PBPK model-predicted plasma concentration-time profiles of LAIs reasonably matched the observed profiles. The predicted $V_{ss}$ values (3.1 to 3.7 L/kg) by the tissue composition-based model were comparable to the observed values (1.8 to 3.1 L/kg) (Table 2). The ratios of observed to predicted $t_{1/2}$ were within 1.4-fold. In contrast, the PBPK model significantly under-predicted plasma concentration-time profiles of HAIs, especially in the apparent terminal phase. The predicted $t_{1/2}$ values were 4 to 9-fold shorter than the observed $t_{1/2}$ (Table 2). These under-predictions for $t_{1/2}$ were largely due to 3 to 8-fold under-prediction for $V_{ss}$ (1.7 to 3.0 L/kg versus 5.2 to 13 L/kg). Collectively, the PBPK model reasonably matched the observed plasma concentrations for LAIs, but significantly under-predicted the $t_{1/2}$ of HAIs due to the under-predicted $V_{ss}$ estimates. This suggests that the PBPK model cannot account for the PK disposition mechanism of HAIs.
Rapid Binding Approximation Model

The one- and two-compartment rapid binding approximation model-fitted plasma concentration-time profiles of HSP90 inhibitors along with the observed data are graphically presented in Fig. 5 and 6, respectively. The estimates for PK parameters are summarized in Table 3. Both the one- and two-compartment rapid binding approximation models reasonably fit the observed plasma concentrations of all inhibitors, with \( OFV \) of 1863 and 1694, respectively. Thus, based on the \( OFV \) values, the goodness-of-fit for the two-compartment model was better than that for the one-compartment model. The estimates for \( K_D \) by the one-compartment model were 447 to 832 nM for LAIs and 3.0 to 6.3 nM for HAIs (Table 3). Thus, the \( K_D \) estimates for HAIs were greater than 70-fold lower than those for the corresponding LAIs. The \( K_D \) values estimated by the two-compartment model were 1.0 to 5.8 nM for HAIs (Table 3) whereas those for LAIs could not be estimated because the model assumed negligible HSP90 binding for LAIs as discussed later. It might be worth noting that the \( K_D \) estimates for HAIs showed larger coefficients of variation (3 to 5-fold) compared to those for LAIs in the one-compartment model while the coefficients of variation for HAIs were approximately 2-fold larger in the two-compartment model compared to the one-compartment model. The estimates for \( R_{total} \) were relatively consistent between the one- and two-compartment analyses (i.e., 2380 and 2710 nM, respectively). Thus, the estimated \( R_{total} \) values were at least 300-fold higher than the \( K_D \) estimates for HAIs.

Furthermore, the sensitivity analysis for the in vivo \( K_D \) values for LAIs was performed in order to investigate an effect of in vivo \( K_D \) values on the two-compartment rapid binding approximation model. As the representative examples, the sensitivity analysis results assuming that the \( K_D \) values of LAIs were equal to or 10-fold higher than the in vitro \( K_i \) estimates
following the correction for $f_{u, plasma}$ are summarized in Table 3. The rapid binding approximation models with the assumed different in vivo $K_D$ values of LAIs reasonably fit the observed plasma concentrations of all inhibitors with the $K_D$ estimates ranging from 2 to 11 nM for HAIs. Thus, the $K_D$ estimates for HAIs were comparable between these two analyses, suggesting the precise $K_D$ values of LAIs were not key parameters to accurately estimate the $K_D$ values for HAIs.

**PK Simulation of HSP90 Inhibitors by Rapid Binding Approximation Model**

In order to investigate an effect of the relationship between $K_D$ and $R_{total}$ on plasma concentration-time profiles, plasma concentrations of virtual inhibitors having $K_D$ of 0.1 to 10000 nM were simulated by one- and two-compartment rapid binding approximation models with $R_{total}$ of 100 to 10000 nM (Figs. 7 and 8, respectively). The simulated plasma concentration-time profiles by the one-compartment model showed near mono-exponential declines when the ratio of $R_{total}$ to $K_D$ was unity or less, indicating target binding could not alter plasma concentration-time profiles in such cases. In contrast, when the ratio of $R_{total}$ to $K_D$ was greater than unity, the simulated plasma concentration-time profiles showed apparent bi-exponential declines due to target binding. Additionally, a one-compartment model simulation with a wide range of $CL$ and $V_1$ values was performed to understand the relationship between TMDD-dependent $t_{1/2}$ and systemic $t_{1/2}$ derived from the input parameters of $CL$ and $V_1$ (data now shown). In order to differentiate TMDD-dependent $t_{1/2}$ from systemic $t_{1/2}$, TMDD-dependent $t_{1/2}$ should obviously be longer than systemic $t_{1/2}$ (based, e.g., on higher $CL$ and/or smaller $V_1$). In other words, systemic $t_{1/2}$ tended to mask TMDD-dependent $t_{1/2}$ when lower $CL$ and/or larger $V_1$ yielded longer systemic $t_{1/2}$ than TMDD-dependent $t_{1/2}$. This phenomenon was more pronounced in the two-compartment rapid binding approximation model simulations. For example, the two-compartment rapid binding approximation model-simulated PK profiles with
the aforementioned $CL$ and $V$ parameters were apparently near bi-exponential even though the $R_{total}$ to $K_D$ ratios were greater than unity. As a result, TMDD-dependent $t_{1/2}$ would be difficult to differentiate from the systemic $t_{1/2}$ in the apparent terminal phase. Therefore, it would be required (if possible) to have intensive sampling time points with high-sensitivity quantitative analysis (e.g., 12 to 24 hours post-dose) to “experimentally” determine TMDD-dependent $t_{1/2}$ in the low nM to pM range.

In addition, non-compartmental PK analysis was performed based on these one- and two-compartment rapid binding approximation model-simulated PK profiles, to assess an effect of target binding on non-compartmental PK parameters and place our data analysis in perspective. As shown in Table 4, the estimates for $CL_p$ varied from 1.1 to 3.0 L/h/kg in the one-compartment model-simulation. The $CL_p$ estimates were comparable to the simulation input value (3 L/h/kg) when plasma concentrations rapidly declined to approximately below 10 nM, even though the apparent $t_{1/2}$ estimates calculated from the plasma concentrations from 20 to 24 hours post-dose varied from 0.2 to 25 hours with the MRT estimates ranging from 0.3 to 3 hours. This was because the concentrations below 10 nM did not significantly contribute to total AUC estimates. On the other hand, the $V_{ss}$ estimates varied from 1.0 to 21 L/kg, which appeared to be related to the $R_{total}$ to $K_D$ ratios. The $V_{ss}$ estimates were comparable to the simulation input value (1 L/kg) when the $R_{total}$ to $K_D$ ratios were unity or less. In contrast, the $V_{ss}$ estimates were larger (or much larger) than the input value when the $R_{total}$ to $K_D$ ratios were greater than unity. A similar trend was observed for the estimates for $CL_p$ and $V_{ss}$ for the two-compartment model-simulation (Table 5). Summarizing the simulation results, the estimates for $CL_p$ and $V_{ss}$ tended to be different from the input values when $R_{total}$ was larger. This trend should depend upon plasma concentration levels relative to $R_{total}$ values. Moreover, the extrapolated $AUC$ values from the
last time point (i.e., 24 hours) to infinity were greater than 10% of $AUC_{0-\infty}$ in some cases; therefore, the non-compartmental PK parameters might not be accurately determined from the simulated plasma concentrations up to 24 hours post-dose. Collectively, the simulation results suggest that TMDD can significantly alter plasma concentration-time profiles when TMDD-dependent $t_{1/2}$ was longer than systemic $t_{1/2}$ (which was derived from $CL$ and $V$ estimates) and the $R_{total}$ to $K_D$ ratio was greater than unity (Fig. 9). In such cases, it would be difficult to accurately determine PK parameter (e.g., $CL_p$ and $V_{ss}$) by non-compartmental PK analysis.
DISCUSSION

Replacement of hydrogen with fluorine within three pairs of structurally similar small molecule HSP90 inhibitors resulted in marked differences in the rat PK profiles related to their HSP90 binding affinities. The difference in PK profiles between LAIs and HAIs featured remarkably different $V_{ss}$ estimates (1.8 to 2.0 and 10 to 13 L/kg, respectively). This difference was unlikely due to plasma protein binding, which was kept consistent among these inhibitors.

In the dose-proportional studies with each representative inhibitor (A1 and A2), the $CL_p$ estimates for A1 and A2 (3.0 to 3.3 and 2.6 to 3.1 L/h/kg, respectively) were comparable at the doses of 0.75 to 7.5 mg/kg. The consistent $CL_p$ values were apparently in line with the comparable in vitro metabolic kinetic parameters for A1 and A2: $K_m = 9.9$ to 10 μM and $V_{max} = 0.28$ to 0.35 nmol/min/mg protein. The $K_m$ values were corresponding to total plasma concentrations of approximately 30 μM following the correction for $f_{u,plasma}$. Their renal clearances were minimal in rats (<5% of systemic $CL$). These findings, therefore, suggest linear in vivo $CL$ for A1 and A2 at these doses, where total plasma concentrations (protein-bound plus unbound) at the initial time point of 2 minutes were below 8 μM. In contrast, the $V_{ss}$ estimates for A2 considerably decreased from 13 to 5.2 L/kg with the increase in doses, whereas those for A1 were relatively consistent (2.0 to 3.1 L/kg). Dose-dependent $V_{ss}$ estimates for A2 does not appear to be related to plasma protein binding because of the concentration-independent $f_{u,plasma}$.

Assuming non-specific binding dependent $V_{ss}$ of 2 L/kg (mean $V_{ss}$ value of LAIs), approximate $V_{ss}$ of 10 L/kg for A2 was considered to result from its binding to HSP90 at the doses of 0.5 and 2.5 mg/kg while approximate $V_{ss}$ of 7 L/kg could be saturated at the dose of 7.5 mg/kg. As shown in Fig. 4 and Table 2, PBPK model-simulated PK profiles reasonably matched the observed profiles of LAIs, whereas the model significantly under-predicted PK profiles of HAIs.
The predicted $V_{ss}$ values for LAIs with the tissue composition-based model were 3.1 to 3.7 L/kg, which were comparable to the observed $V_{ss}$ of 1.8 to 3.1 L/kg. In contrast, the predicted $V_{ss}$ values for HAIs were 1.7 to 3.0 L/kg which were 4 to 8-fold lower than the observed values (10 to 13 L/kg) at the dose of 2.5 mg/kg. The passive permeability of both LAIs and HAIs was moderate (5 to $10 \times 10^{-6}$ cm/sec) in the recently reported permeability assay system using low-efflux Madin-Darby canine kidney cells (Di et al., 2011). Calculated LogP values for LAIs and HAIs were 2.3 to 2.4 and 2.6 to 2.7, respectively, whereas measured LogD values were 2.5 to 2.6 and 2.2 to 2.5, respectively. Sensitivity analysis for $V_{ss}$ revealed that LogD of approximately 4 would be required to yield comparable $V_{ss}$ values observed for HAIs (data not shown).

Collectively, the PK difference between LAIs and HAIs could not be explained by their physico- and bio-chemical properties such as plasma protein binding, permeability and lipophilicity. As shown in Fig. 3, these HSP90 inhibitors showed a negative relationship between $V_{ss}$ versus in vitro $K_i$ values, suggesting that the PK difference could be related to binding affinities to their pharmacological target. Accordingly, these findings positioned these small molecule HSP90 inhibitors as interesting compounds to investigate the application of TMDD model that has been proposed to characterize dose-dependent non-linear PKs of some biologic drugs due to high affinity to their targets.

A general TMDD model has been developed to describe dose-dependent non-linear PKs, where high affinity binding of drugs to their pharmacological targets significantly alters their PK profiles (Levy, 1994; Mager and Jusko, 2001). In many cases, such drugs are biologics showing concentration-dependent elimination due to receptor-mediated endocytosis or internalization. While application of TMDD model to small molecules has been rarely reported, the non-linear PK profiles of imirestat (aldose reductase inhibitor) and bosentan (endothelin receptor
antagonist) were successfully characterized by TMDD model (Mager and Jusko, 2001); however, their non-linear PK mechanisms have not been fully understood. HSP90 is one of the most abundant intercellular proteins and undergoes conformational changes during the course of its ATP cycle (Parsell and Lindquist, 1993; Csermely et al., 1998). Most of HSP90 inhibitors including the present in house compounds were reversibly associated with the ATP binding pocket located in the N-terminal domain (Wayne et al.,; Zehnder et al., 2011). Because of the high whole-body HSP90 abundance and the binding mechanism of HSP90 inhibitors, we hypothesized that potent small molecule HSP90 inhibitors could exhibit non-linear PKs characterized by the TMDD model. Moreover, among the proposed TMDD models, the rapid binding approximation model assumes the rapid-binding of a drug to its pharmacological target, the slow receptor turnover rate and the negligible drug-receptor complex internalization rate. These assumptions led to Wagner’s non-linear tissue-binding model described by a single equation (eq. 14) (Wagner, 1971), and appeared valid for HSP90 inhibitor as mentioned in Materials and Methods. Accordingly, we applied the one- and two-compartment rapid binding approximation models to characterize in vivo rat PK profiles of our HSP90 inhibitors in order to investigate our hypothesis. The one-compartment rapid binding approximation model adequately fit the PK profiles of all inhibitors (Fig. 5). The estimated $K_D$ values for HAIs were 3 to 6 nM (equivalent to 1 to 2 nM free), which appeared comparable to the in vitro $K_i$ estimates of 2 to 3 nM (Table 3). The estimated $K_D$ values for LAIs were 447 to 832 nM (143 to 266 nM free), which were much higher than the in vitro $K_i$ values of 7 to 10 nM. This model assumed negligible non-specific binding dependent tissue distribution. That is, a whole-body tissue distribution of the inhibitors was assumed to be exclusively due to their binding to HSP90. This assumption appeared invalid because the $V_{ss}$ values predicted by the tissue-composition-based
model with their physico- and bio-chemical properties were 2 to 4 L/kg. However, it would be difficult, if not impossible, for LAIs to differentiate their target-binding from non-specific binding dependent tissue distribution on the apparent bi-exponential declines without accurate in vivo $K_D$ inputs. Conversely, the two-compartment rapid binding approximation model assumed that a whole-body tissue distribution of LAIs was completely derived from non-specific binding dependent tissue distribution. That is, the difference in the PK profiles between LAIs and HAIs was assumed to be due to the target-binding affinity for HAIs. The estimated in vivo $K_D$ values for HAIs were 1 to 6 nM (equivalent to 0.3 to 2 nM free), which appeared comparable to the in vitro $K_i$ values of 2 to 3 nM (Table 3). When in vivo $K_D$ values for LAIs were assumed to be either equal to or 10-fold higher than the in vitro $K_i$ values, the $K_D$ estimates for HAIs were 3 to 11 nM for both cases with a little difference in the other PK parameters (Table 3). This sensitivity analysis suggests that target-binding of LAIs may be masked by non-specific binding dependent tissue distribution. These considerations were further explored by the simulation approach (Figs. 7 and 8). Apparent bi-exponential declines of plasma concentrations were clearly simulated by the one-compartment rapid binding approximation model when an abundance of pharmacological targets was sufficient enough for inhibitor’s affinity, e.g., $R_{total} > K_D$ (Fig. 7). In contrast, the target binding-dependent distribution tended to be masked by non-specific binding dependent tissue distribution in the two-compartment rapid binding approximation model-simulated PK profiles even though $R_{total}$ was higher than $K_D$ (Fig. 8). Obviously, in order to characterize TMDD profiles, systemic $t_{1/2}$ derived from the input parameters of $CL$ and $V$ should be shorter than TMDD-dependent $t_{1/2}$. In such cases, TMDD profiles would be likely or possibly observed when $R_{total}$ was higher than $K_D$ (Fig. 9). This relationship between $R_{total}$ and $K_D$ (i.e., the effect of $R_{total}$ to $K_D$ ratios on $V_{ss}$) in the rapid binding
approximation model was also suggested mathematically by Mager and Jusko (2001). Additionally, non-compartmental PK analysis for these types of TMDD profiles might lead to over- or under-estimation of $CL$ and/or $V_{ss}$ as summarized in Tables 4 and 5. An appropriate modeling approach would be required to determine accurate PK parameters. For our HSP90 inhibitors, it still remained unclear why these inhibitors showed a threshold-type of negative relationship between $V_{ss}$ and $K_i$ since the difference in the in vitro $K_i$ values was within 5-fold. The difference in $K_i$ values might be just at the threshold between target binding versus non-specific binding dependent tissue distribution.

In conclusion, the plasma concentration-time profiles of in house HSP90 inhibitors in rats were characterized in relation to their pharmacological target affinity by the rapid binding approximation model. The modeling results suggest that large $V_{ss}$ of HAIs are likely due to their target binding affinity. The estimates for in vivo $K_D$ values of HAIs were comparable to their in vitro $K_i$ values. To our knowledge, this is the first report of structure-activity relationship of small molecule compounds showing the effect of target binding affinity on their in vivo PK profiles. As shown in the present study, a modeling and simulation framework is a powerful tool to provide insights in which factors determine observed PK profiles. It has been reported that HSP90-dependent biomarker responses such as AKT degradation and HSP70 induction to inhibitors were sustained beyond what was expected from their PK profiles (Caldas-Lopes et al., 2009; Mehta et al., 2011; Yamazaki et al., 2011a). These sustained effects on HSP90-dependent client proteins could be related to extensive tumor distribution of HSP90 inhibitors due to their pharmacological target affinity. Therefore, it would be interesting to investigate a relationship between pharmacological responses and tumor distribution of HSP90 inhibitors.
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Authorship Contribution

Participated in research design: Shen and Yamazaki

Conduct experiments: Jiang and Shen

Performed data analysis: Shen, Vicini and Yamazaki

Wrote or contribute to the writing of the manuscript: Shen, Smith, Vicini and Yamazaki
References


Footnotes

Send reprint requests to: Shinji Yamazaki, Ph.D., PK, Dynamics and Metabolism, Pfizer Worldwide Research and Development, 10777 Science Center Drive, San Diego, CA 92121.

E-mail: shinji.yamazaki@pfizer.com

Current affiliation (Zhongzhou Shen): Dart NeuroScience, San Diego, California.
Legends for Figures

Fig. 1. Chemical structures of HSP90 inhibitors used in the present study.

Fig. 2. Schemes of a full target-mediated drug disposition model (A) and its rapid-binding approximation model (B). In(t), drug infusion rate; C, free drug (nM); V1, central compartment volume of free drug (L/kg); R, free receptor (nM), RC, drug-receptor complex (nM); ke, the first-order elimination rate constant (h⁻¹); k syn, the zero-order receptor synthesis rate constant (nM·h⁻¹); k deg, the first-order receptor degradation rate constant (h⁻¹); kon, the second-order drug-receptor complex formation rate constant (nM⁻¹·h⁻¹); k off, the first-order drug-receptor complex dissociation rate constant (h⁻¹); k int, the first-order internalization rate constant (h⁻¹).

Fig. 3. Relationship between in vitro Ki and in vivo rat steady-state volume of distribution (Vss) for HSP90 inhibitors. Six HSP90 inhibitors are represented by each symbol from the left to the right, ie, B2, A2, C2, B1, C1 and A1.

Fig. 4. PBPK model-simulated and observed plasma concentration-time profiles of HSP90 inhibitors in rats after a single intravenous administration. The x-axis represents the time after dosing in hours and the y-axis represents the observed (OBS) and PBPK model-simulated (PRED) plasma concentrations of HSP90 inhibitors in nanomolars on a logarithmic scale.
**Fig. 5. One-compartment rapid binding approximation model-fitted and observed plasma concentration-time profiles of HSP90 inhibitors in rats after a single intravenous administration.** The x-axis represents the time after dosing in hours and the y-axis represents the observed (OBS) and rapid binding approximation model-fitted (PRED) plasma concentrations of HSP90 inhibitors in nanomolars on a logarithmic scale.

**Fig. 6. Two-compartment rapid binding approximation model-fitted and observed plasma concentration-time profiles of HSP90 inhibitors in rats after a single intravenous administration.** The x-axis represents the time after dosing in hours and the y-axis represents the observed (OBS) and rapid binding approximation model-fitted (PRED) plasma concentrations of HSP90 inhibitors in nanomolars on a logarithmic scale.

**Fig. 7. One-compartment rapid binding approximation model-simulated plasma concentration-time profiles of virtual inhibitors with different $K_D$ values in rats after a single intravenous administration.** The x-axis represents the time after dosing in hours and the y-axis represents the observed (OBS) and rapid binding approximation model-simulated (PRED) plasma concentrations of virtual inhibitors in nanomolars on a logarithmic scale. $RT$ and $K_D$ represent maximum receptor capacity (nM) and equilibrium dissociation constant (nM), respectively.
Fig. 8. Two-compartment rapid binding approximation model-simulated plasma concentration-time profiles of virtual inhibitors with different $K_D$ values in rats after a single intravenous administration. The x-axis represents the time after dosing in hours and the y-axis represents the observed (OBS) and t rapid binding approximation model-simulated (PRED) plasma concentrations of virtual inhibitors in nanomolars on a logarithmic scale. $RT$ and $K_D$ represent maximum receptor capacity (nM) and equilibrium dissociation constant (nM), respectively.

Fig. 9. Proposed possible occurrence for target-mediated drug disposition of small molecule.

To characterize target-mediated drug disposition (TMDD), TMDD-dependent half-life ($t_{1/2}$) should be longer than systemic $t_{1/2}$ derived from systemic clearance ($CL$) and volume of distribution at steady-state ($V_{ss}$). The plots were basically generated from one-compartment rapid binding approximation model-simulated plasma concentration-time profiles of virtual inhibitors with different $K_D$ values described in Fig.7 and Table 4. $R_{total}$, $K_D$ and $t_{1/2,app}$ represent maximum receptor capacity, equilibrium dissociation constant and apparent terminal half-life, respectively.
## TABLE 1.
Non-compartmental pharmacokinetic parameter estimates of HSP90 inhibitors in male Sprague-Dawley rats after a single intravenous administration.

<table>
<thead>
<tr>
<th>HSP90 Inhibitor</th>
<th>Dose (mg/kg)</th>
<th>$CL_p$ (L/h/kg)</th>
<th>$V_{ss}$ (L/kg)</th>
<th>MRT (h)</th>
<th>$t_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0.75</td>
<td>3.3</td>
<td>3.1</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>3.3</td>
<td>2.0</td>
<td>0.6</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>3.0</td>
<td>2.0</td>
<td>0.7</td>
<td>1.4</td>
</tr>
<tr>
<td>B1</td>
<td>2.5</td>
<td>3.5</td>
<td>1.8</td>
<td>1.3</td>
<td>0.5</td>
</tr>
<tr>
<td>C1</td>
<td>2.5</td>
<td>3.4</td>
<td>1.8</td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>A2</td>
<td>0.75</td>
<td>3.1</td>
<td>11</td>
<td>3.6</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>3.2</td>
<td>13</td>
<td>4.0</td>
<td>8.7</td>
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<tr>
<td></td>
<td>7.5</td>
<td>2.6</td>
<td>5.2</td>
<td>2.1</td>
<td>6.0</td>
</tr>
<tr>
<td>B2</td>
<td>2.5</td>
<td>3.4</td>
<td>10</td>
<td>8.0</td>
<td>3.0</td>
</tr>
<tr>
<td>C2</td>
<td>2.5</td>
<td>3.4</td>
<td>11</td>
<td>8.3</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Pharmacokinetic parameter estimates are expressed as mean ($n = 2$/group).

A1, B1 and C1 are low-affinity HSP90 inhibitors whereas A2, B2 and C2 were their corresponding high-affinity inhibitors.
## TABLE 2
Physiologically-based pharmacokinetic model-simulated pharmacokinetic parameters of HSP90 inhibitors in male Sprague-Dawley rats after a single intravenous administration.

<table>
<thead>
<tr>
<th>HSP90 Inhibitor</th>
<th>Dose mg/kg</th>
<th>Predicted AUC\textsubscript{0-\infty} nM·h (ratio\textsuperscript{b})</th>
<th>Predicted V\textsubscript{ss} L/kg (ratio\textsuperscript{b})</th>
<th>Predicted t\textsubscript{1/2} h (ratio\textsuperscript{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>A\textsubscript{1}</td>
<td>0.75</td>
<td>613 (1.4)</td>
<td>3.6 (1.2)</td>
<td>1.2 (0.92)</td>
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<tr>
<td></td>
<td>2.5</td>
<td>1777 (1.2)</td>
<td>3.6 (1.7)</td>
<td>1.1 (0.84)</td>
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<tr>
<td></td>
<td>7.5</td>
<td>6502 (1.3)</td>
<td>3.6 (1.7)</td>
<td>1.2 (0.90)</td>
</tr>
<tr>
<td>B\textsubscript{1}</td>
<td>2.5</td>
<td>1718 (1.2)</td>
<td>3.1 (1.7)</td>
<td>0.91 (0.72)</td>
</tr>
<tr>
<td>C\textsubscript{1}</td>
<td>2.5</td>
<td>1794 (1.2)</td>
<td>3.7 (2.0)</td>
<td>1.1 (0.90)</td>
</tr>
<tr>
<td>A\textsubscript{2}</td>
<td>0.75</td>
<td>623 (1.4)</td>
<td>1.7 (0.15)</td>
<td>1.2 (0.28)</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1784 (1.2)</td>
<td>1.7 (0.13)</td>
<td>1.3 (0.16)</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>7150 (1.3)</td>
<td>1.7 (0.32)</td>
<td>1.1 (0.18)</td>
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<tr>
<td>B\textsubscript{2}</td>
<td>2.5</td>
<td>1735 (1.2)</td>
<td>1.9 (0.19)</td>
<td>0.85 (0.11)</td>
</tr>
<tr>
<td>C\textsubscript{2}</td>
<td>2.5</td>
<td>1759 (1.2)</td>
<td>3.0 (0.27)</td>
<td>0.89 (0.11)</td>
</tr>
</tbody>
</table>

A\textsubscript{1}, B\textsubscript{1} and C\textsubscript{1} are low-affinity HSP90 inhibitors whereas A\textsubscript{2}, B\textsubscript{2} and C\textsubscript{2} were their corresponding high-affinity inhibitors.

\textsuperscript{a} The ratios of model-simulated to observed parameters are expressed in parentheses.
**TABLE 3**

Pharmacokinetic parameter estimates of HSP90 inhibitors by rapid binding approximation models in male Sprague-Dawley rats after a single intravenous administration.

<table>
<thead>
<tr>
<th>Model</th>
<th>$V_i$</th>
<th>$k_{el}$</th>
<th>$k_{pt}$</th>
<th>$k_{ip}$</th>
<th>$R_{total}$</th>
<th>HSP90 Inhibitor</th>
<th>$K_D$, nM</th>
<th>$OFV^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$L/kg$</td>
<td>$h^{-1}$</td>
<td>$h^{-1}$</td>
<td>$h^{-1}$</td>
<td>$nM$</td>
<td>A1</td>
<td>A2</td>
<td>B1</td>
</tr>
<tr>
<td>One-cmpt$^b$</td>
<td>2.5</td>
<td>3.2</td>
<td>–</td>
<td>–</td>
<td>2380</td>
<td>447</td>
<td>6.3</td>
<td>710</td>
</tr>
<tr>
<td></td>
<td>(0.29)</td>
<td>(0.27)</td>
<td></td>
<td></td>
<td>(11)</td>
<td>(33)</td>
<td>(1.3)</td>
<td>(35)</td>
</tr>
<tr>
<td>Two-cmpt$^c$</td>
<td>1.3</td>
<td>4.4</td>
<td>5.6</td>
<td>1.9</td>
<td>2710</td>
<td>–</td>
<td>5.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>(0.23)</td>
<td>(0.77)</td>
<td>(1.8)</td>
<td>(0.53)</td>
<td>(705)</td>
<td>(2.4)</td>
<td>(0.48)</td>
<td>(0.68)</td>
</tr>
<tr>
<td>Two-cmpt$^d$</td>
<td>1.3</td>
<td>4.7</td>
<td>6.0</td>
<td>2.1</td>
<td>1540</td>
<td>21</td>
<td>11</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>(0.13)</td>
<td>(0.45)</td>
<td>(0.56)</td>
<td>(0.43)</td>
<td>(368)</td>
<td>(3.4)</td>
<td>(0.95)</td>
<td>(1.4)</td>
</tr>
<tr>
<td>Two-cmpt$^e$</td>
<td>1.4</td>
<td>5.0</td>
<td>6.3</td>
<td>2.1</td>
<td>1370</td>
<td>210</td>
<td>11</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>(0.13)</td>
<td>(0.47)</td>
<td>(0.40)</td>
<td>(0.37)</td>
<td>(274)</td>
<td>(2.4)</td>
<td>(1.3)</td>
<td>(1.8)</td>
</tr>
</tbody>
</table>

Precision of the estimates is expressed as standard error in parentheses.

A1, B1 and C1 are low-affinity HSP90 inhibitors whereas A2, B2 and C2 were their corresponding high-affinity inhibitors.

$-$: not applicable

$^a$ Objective function values estimated with NONMEM.

$^b$ Rapid binding approximation model with a central compartment.

$^c$ Rapid binding approximation model with central and peripheral compartments assuming no target-mediated drug disposition for A1, B1 and C1.

$^d$ Rapid binding approximation model with central and peripheral compartments assuming in vivo $K_D = K_i,\text{vivo, total}$ for A1, B1 and C1.

$^e$ Rapid binding approximation model with central and peripheral compartments assuming in vivo $K_D = 10 \times K_i,\text{vivo, total}$ for A1, B1 and C1.
### TABLE 4
Non-compartmental pharmacokinetic parameter estimates for one-compartment rapid binding approximation model-simulated plasma concentrations of virtual compounds in rats after a single intravenous administration

<table>
<thead>
<tr>
<th>$R_{total}$</th>
<th>$K_D$</th>
<th>$CL_p$</th>
<th>$V_{ss}$</th>
<th>$t_{1/2}^a$</th>
<th>MRT</th>
<th>$AUC_{0-\infty}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td>nM</td>
<td>L/h/kg</td>
<td>L/kg</td>
<td>h</td>
<td>h</td>
<td>nM·h (%)</td>
</tr>
<tr>
<td>10000</td>
<td>0.1</td>
<td>2.6</td>
<td>11</td>
<td>27</td>
<td>4.3</td>
<td>1889 (5.5)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.1</td>
<td>20</td>
<td>26</td>
<td>9.6</td>
<td>2332 (13)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.5</td>
<td>21</td>
<td>22</td>
<td>14</td>
<td>3404 (20)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.1</td>
<td>9.7</td>
<td>13</td>
<td>9.1</td>
<td>4716 (12)</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1.1</td>
<td>2.2</td>
<td>2.5</td>
<td>2.0</td>
<td>4448 (&lt;0.1)</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>1.8</td>
<td>1.1</td>
<td>0.5</td>
<td>0.6</td>
<td>2779 (&lt;0.1)</td>
</tr>
<tr>
<td>1000</td>
<td>0.1</td>
<td>2.9</td>
<td>4.4</td>
<td>25</td>
<td>1.5</td>
<td>1732 (1.7)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.7</td>
<td>7.7</td>
<td>22</td>
<td>2.8</td>
<td>1839 (3.6)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.5</td>
<td>6.4</td>
<td>13</td>
<td>2.5</td>
<td>1977 (3.0)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.5</td>
<td>2.1</td>
<td>2.5</td>
<td>0.8</td>
<td>1995 (&lt;0.1)</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>2.6</td>
<td>1.2</td>
<td>0.5</td>
<td>0.5</td>
<td>1946 (&lt;0.1)</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>2.8</td>
<td>1.0</td>
<td>0.3</td>
<td>0.4</td>
<td>1778 (&lt;0.1)</td>
</tr>
<tr>
<td>100</td>
<td>0.1</td>
<td>3.0</td>
<td>1.8</td>
<td>21</td>
<td>0.6</td>
<td>1684 (0.4)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.9</td>
<td>1.8</td>
<td>12</td>
<td>0.6</td>
<td>1698 (0.4)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.9</td>
<td>1.2</td>
<td>2.5</td>
<td>0.4</td>
<td>1700 (&lt;0.1)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.9</td>
<td>1.1</td>
<td>0.5</td>
<td>0.4</td>
<td>1700 (&lt;0.1)</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>3.0</td>
<td>1.0</td>
<td>0.3</td>
<td>0.3</td>
<td>1695 (&lt;0.1)</td>
</tr>
<tr>
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<td>10000</td>
<td>3.0</td>
<td>1.0</td>
<td>0.2</td>
<td>0.3</td>
<td>1678 (&lt;0.1)</td>
</tr>
</tbody>
</table>

Inputs of $CL$ and $V_1$ were set to 3 L/h/kg and 1 L/kg, respectively.

$^a$ Apparent terminal half-life ($t_{1/2}$) was calculated from the simulated plasma concentrations during 20 to 24 hours post-dose.

$^b$ Percent of extrapolated $AUC$ beyond 24 hours in the calculation of $AUC_{0-\infty}$ is expressed in parentheses.
**TABLE 5**

Non-compartmental pharmacokinetic parameter estimates for two-compartment rapid binding approximation model-simulated plasma concentrations of virtual compounds in rats after a single intravenous administration

<table>
<thead>
<tr>
<th>$R_{\text{total}}$</th>
<th>$K_D$</th>
<th>$CL_p$</th>
<th>$V_{ss}$</th>
<th>$t_{1/2}^a$</th>
<th>MRT</th>
<th>$AUC_{0-\infty}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td>nM</td>
<td>L/h/kg</td>
<td>L/kg</td>
<td>h</td>
<td>h</td>
<td>nM·h (%)</td>
</tr>
<tr>
<td>10000</td>
<td>0.1</td>
<td>2.7</td>
<td>11</td>
<td>17</td>
<td>4.1</td>
<td>1845 (4.6)</td>
</tr>
<tr>
<td>1</td>
<td>2.2</td>
<td>20</td>
<td>19</td>
<td>8.8</td>
<td>2239 (12)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>22</td>
<td>19</td>
<td>14</td>
<td>3274 (20)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.1</td>
<td>12</td>
<td>13</td>
<td>11</td>
<td>4656 (14)</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>3.7</td>
<td>3.4</td>
<td>3.3</td>
<td>10000</td>
<td>4451 (0.4)</td>
<td></td>
</tr>
<tr>
<td>10000</td>
<td>2.9</td>
<td>1.7</td>
<td>1.6</td>
<td>2782</td>
<td>(&lt;0.1)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2.9</td>
<td>6.0</td>
<td>13</td>
<td>2.1</td>
<td>1718 (1.3)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.8</td>
<td>9.1</td>
<td>14</td>
<td>3.3</td>
<td>1812 (3.3)</td>
<td></td>
</tr>
<tr>
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<td>2.5</td>
<td>9.8</td>
<td>11</td>
<td>3.9</td>
<td>1962 (3.8)</td>
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</tr>
<tr>
<td>100</td>
<td>2.5</td>
<td>5.5</td>
<td>3.4</td>
<td>2.2</td>
<td>1998 (0.2)</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>2.6</td>
<td>3.9</td>
<td>1.7</td>
<td>1.5</td>
<td>1949 (&lt;0.1)</td>
<td></td>
</tr>
<tr>
<td>10000</td>
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<td>3.8</td>
<td>1.5</td>
<td>1782</td>
<td>(&lt;0.1)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>3.0</td>
<td>4.5</td>
<td>9.4</td>
<td>1.5</td>
<td>1682 (0.3)</td>
<td></td>
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<tr>
<td>1</td>
<td>2.9</td>
<td>4.8</td>
<td>8.2</td>
<td>1.6</td>
<td>1698 (0.5)</td>
<td></td>
</tr>
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<td>10</td>
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<td>4.5</td>
<td>3.3</td>
<td>1.5</td>
<td>1704 (&lt;0.1)</td>
<td></td>
</tr>
<tr>
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<td>2.9</td>
<td>4.1</td>
<td>1.7</td>
<td>1.4</td>
<td>1703 (&lt;0.1)</td>
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</tr>
<tr>
<td>1000</td>
<td>2.9</td>
<td>4.0</td>
<td>1.5</td>
<td>1.4</td>
<td>1699 (&lt;0.1)</td>
<td></td>
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<tr>
<td>10000</td>
<td>3.0</td>
<td>4.0</td>
<td>1.5</td>
<td>1682</td>
<td>(&lt;0.1)</td>
<td></td>
</tr>
</tbody>
</table>

Inputs of $CL$, $V_1$, $Q$ and $V_2$ were set to 3 L/h/kg, 1 L/kg, 3 L/h/kg and 3 L/kg, respectively.

$^a$ Apparent terminal half-life ($t_{1/2}$) was calculated from the simulated plasma concentrations during 20 to 24 hours post-dose.

$^b$ Percent of extrapolated $AUC$ beyond 24 hours in the calculation of $AUC_{0-\infty}$ is expressed in parentheses.
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
When TMDD-dependent $t_{1/2}$ is longer than $t_{1/2}$ derived from CL and $V_{ss}$

Likely ($R_{total} > K_D$)

Unlikely ($R_{total} \leq K_D$)

Figure 9