Research Article

Theme: Pharmacokinetic/Pharmacodynamic Modeling and Simulation in Drug Discovery and Translational Research

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Mechanism-Based Pharmacokinetic/Pharmacodynamic Modeling of Rat Prefrontal Cortical Dopamine Response to Dual Acting Norepinephrine Reuptake Inhibitor and 5-HT_{1A} Partial Agonist

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Abstract. Evidence suggests that compounds possessing both norepinephrine reuptake inhibition and 5-HT_{1A} partial agonism (NRI/5-HT_{1A}) activities may have a greater efficacy in treating neuropsychiatric disorders than compounds possessing either activity alone. The objectives of the present study were first to characterize the pharmacokinetic/pharmacodynamic (PK/PD) relationship of the plasma concentrations of atomoxetine (NRI) and buspirone (5-HT_{1A} partial agonist), administered alone and in combination, on the prefrontal cortex dopamine levels in rats, and second to use the model developed to characterize the PK/PD relationship of novel NRI/5-HT_{1A} compounds, PF-04269339 and PF-03529936, in a NRI/5-HT_{1A} drug discovery program. Maximal dopamine elevation was twofold higher after administration of atomoxetine and buspirone in combination, PF-04269339, or PF-03529936 than after administration of atomoxetine or buspirone alone. A mechanism-based extended indirect response model characterized the time profiles of the prefrontal cortex dopamine response to atomoxetine and buspirone, administered alone or in combination. After fixing three mechanism-specific pharmacodynamic parameters ($I_{\max}$ and γ_2 for NRI and γ_1 for 5-HT_{1A}) based on the model for atomoxetine and/or buspirone, the model fitted the exposure–response profiles of PF-04269339 and PF-03529936 well. Good *in vitro*-to-*in vivo* correlation was demonstrated with the compound-specific pharmacodynamic parameters (IC₅₀ for NRI and SC₅₀ and $S_{\max}$ for 5-HT_{1A}) across the compounds. In summary, a piecewise modeling approach was used successfully for the characterization of the PK/PD relationship of novel NRI/5-HT_{1A} compounds on prefrontal cortex dopamine levels in rats. The application and value of the mechanism-based modeling in the dual pharmacology drug discovery program are also discussed.

KEY WORDS: atomoxetine; buspirone; dual pharmacology drug discovery program; indirect response model; NRI/5-HT_{1A}; PK/PD modeling.

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ABBREVIATIONS: ADHD, Attention deficit hyperactivity disorder; ATX, Atomoxetine; BUSP, Buspirone; DA, Dopamine; HPLC, High-performance liquid chromatography; NE, Norepinephrine; NRI, Norepinephrine reuptake inhibitor; PK/PD, Pharmacokinetic/pharmacodynamic; SC, Subcutaneous; 5-HT_{1A}, 5-HT_{1A} subtype of serotonergic receptor.

INTRODUCTION

Dysfunction in the dopamine (DA) and norepinephrine (NE) neuronal systems has been postulated to be involved in various neuropsychiatric conditions (1) and has thus been targeted for drug treatment. Stimulant medications, such as amphetamine and methylphenidate, which increase synaptic concentrations of DA and NE, have been widely used in the treatment of attention deficit hyperactivity disorder (ADHD). However, these stimulant medications are not always well-tolerated. Additionally, DA signaling is a key component of the reward pathway; thus, some stimulants that rapidly and significantly enhance DA signaling in areas like the nucleus accumbens and substantia nigra have been associated with an increased potential for abuse (2).

Atomoxetine (ATX), a norepinephrine reuptake inhibitor (NRI), is the first nonstimulant drug approved for ADHD treatment (3). ATX acts to increase NE signaling throughout

the brain via blockade of the presynaptic norepinephrine reuptake transporter, which is responsible for the synaptic clearance of released neurotransmitter (4). Because there is negligible expression of the dopamine-specific reuptake transporter in the prefrontal cortex, DA released in the synapse is taken up nonselectively by the norepinephrine reuptake transporter. Thus, NRI inhibition also increases DA signaling in the prefrontal cortex, a region of the brain thought to be impaired in ADHD, but has less impact on DA reward pathways. It has been shown that ATX is both safe and effective for long-term use for the treatment of ADHD, although perhaps not as effective or quick acting as stimulants. NRI drugs, such as reboxetine, are also clinically efficacious in the treatment of depression and anxiety (5).

Several lines of evidence suggest adding partial agonist activity at the serotonergic receptor (5-HT_{1A}) to an NRI agent may increase its ability to elevate prefrontal cortex DA signaling and produce efficacy comparable to that of stimulants in the treatment of ADHD. Buspirone (BUSP), the only 5-HT_{1A} partial agonist marketed in the USA, is clinically prescribed for anxiety treatment. However, BUSP also shows efficacy in treating symptoms of ADHD (6), suggesting it acts within the common neuronal pathways of monoamine neurotransmission modulators. Additionally, preclinical evidences suggest BUSP increases extracellular levels of DA in the prefrontal cortex via its activity as a partial agonist for postsynaptic 5-HT_{1A} receptor, which, in turn, act on cortical projection neurons to stimulate subcortical DA systems for DA release (7–9). BUSP also increases prefrontal cortex NE levels, but this effect is believed to be mediated by the α 2-adrenergic receptor antagonist activity of its active metabolite, 1-(2-pyrimidinyl)-piperazine (1-PP) rather than the 5-HT_{1A} partial agonist activity of BUSP (10). 1-PP has low affinity for the 5-HT_{1A} binding site and does not contribute to DA modulation by BUSP. Thus, we hypothesized that novel compounds possessing dual NRI and 5-HT_{1A} partial agonist effect(s) have greater efficacy in elevating prefrontal cortex DA signaling and may provide an important new therapeutic approach in the treatment of ADHD, depression, and/or anxiety. The discoveries of several novel chemical series with favorable NRI and 5-HT_{1A} partial agonist pharmacological profiles in our NRI/5-HT_{1A} program have been reported recently (11,12).

Extracellular DA levels in the prefrontal cortex in rats, measured by intracerebral microdialysis, were used as the primary preclinical efficacy model in the NRI/5-HT_{1A} program. Quantitative characterization of the plasma exposure–response relationship of NRI/5-HT_{1A} compounds on the prefrontal cortex DA levels in the preclinical efficacy model and determining the *in vivo* potency and efficacy specific to the NRI or 5-HT_{1A} pathway, are key steps for dose selection and optimization in drug discovery. However, data from NRI/5-HT_{1A} compounds alone failed to support complex mechanistic models due to overparameterization.

To address the overparameterization issue, the first objective of the present study was to establish an appropriate mechanism-based model for characterization of the exposure–response relationships of NRI/5-HT_{1A} compounds on

prefrontal cortex DA levels in rats based on simultaneous modeling of the effects of ATX, the NRI reference compound, and BUSP, the 5-HT_{1A} reference compound, administered alone and in combination. The second objective was to use the model developed, after fixing appropriate mechanism-specific PD parameters, for novel NRI/5-HT_{1A} compounds, PF-04269339 and PF-03529936, and to assess the correlation between *in vitro* and *in vivo* compound-specific pharmacodynamic parameters across the compounds. Furthermore, a third objective was to explore the application of model-based PK/PD simulation and extrapolation in the NRI/5-HT_{1A} dual pharmacology drug discovery program. A thorough understanding of the preclinical PK/PD relationship and appropriate extrapolation of the preclinical PK/PD relationship to clinical setting can be particularly helpful in predicting the efficacious dose and appropriate dosing regimen for NRI/5-HT_{1A} compounds in early clinical studies. Additionally, PK/PD simulation based on the model developed may provide mechanistic insight to guide lead compound selection and optimization for drug discovery.

METHODS AND MATERIALS

Materials

Atomoxetine, PF-04269339 (Fig. 1), and PF-03529936 (Fig. 1) were synthesized at Pfizer (Ann Arbor, MI, USA). Buspirone-HCl was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and solvents were obtained commercially and were of either analytical or high-performance liquid chromatography (HPLC) grade. A vehicle of 1 % Cremophor/99 % methylcellulose (0.5 % w/v) in sterile saline was used for all compound dosing.

Intracerebral Microdialysis and PK Study in Rats

Prefrontal cortex extracellular DA concentration was monitored via intracerebral microdialysis. Freely moving male Sprague–Dawley rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA). On arrival at the animal facility, rats were housed two per cage, maintained under a 12/12-h light–dark cycle, and given free access to food and water for 1 week before surgery. On day 8, rats were anesthetized with 2 % isoflurane mixed with air and oxygen, and a guide cannula (MD-2251, Bioanalytical Systems, West Lafayette, IN, USA) was implanted stereotactically in the left prefrontal cortex [(anterior to bregma), 3.2 mm; L (lateral from

Fig. 1. Chemical structures of PF-04269339 and PF-03529936

the midsagittal suture), 0.7 mm; and V (ventral from the dura surface), 1.5 mm] and fixed with two anchor screws and dental cement. After surgery and recovery from anesthesia, the rats were housed singly, each in a cage located in an isolation box with free access to food and water and maintained on a 12/12-h light–dark cycle. On day 9, a 4-mm microdialysis probe (MD-2204, Bioanalytical Systems) was inserted into the brain through the guide. The inlet tubing of the probe was connected to a syringe pump and the outlet tubing was attached to a refrigerated fraction collector through a two-channel liquid swivel. Artificial Cerebrospinal fluid (T-5481, CMA Microdialysis, Solna, Sweden) containing NaCl (147 mM), KCl (2.7 mM), CaCl₂ (1.2 mM), and MgCl₂ (0.85 mM) was perfused overnight at a rate of 1.0 μ L/min. On day 10, the perfusion rate was increased to 2 μ L/min, and dialysate was collected at 30-min intervals (60 μ L per sample), starting from 1.5 h pre-dose to 5 h post-dose. In the microdialysis studies for ATX and BUSP, male Sprague–Dawley rats, three per dose group, were dosed via subcutaneous (SC) injection, at 1.5 h after the start of the day 10 study, with ATX alone (0.1, 0.3, or 1 mg/kg), BUSP alone (0.1, 0.3, 1, 3, or 10 mg/kg), ATX (0.1, 0.3, or 1 mg/kg) and BUSP (3 mg/kg) in combination, or ATX (1 mg/kg) and BUSP (0.3, 1, 3, or 10 mg/kg) in combination. Dose levels in the microdialysis study for PF-04269339 were 1, 3, and 10 mg/kg, whereas the microdialysis study for PF-03529936 used 0.1, 1, and 10 mg/kg. Four baseline samples were collected every 30 min from each rat, followed by vehicle or drug administration by SC injection at 1.5 h after the study start. Samples were then collected every 30 min over the next 4 h. All samples were collected into preservative solution (10 mM citrate acid+1 mM EDTA, 10 μ L/60 μ L sample) to prevent the degradation of neurotransmitters.

Blood samples for ATX and BUSP were collected in a parallel study for PK parameter estimates. In the PK study for ATX and BUSP, male Sprague–Dawley rats, three per dose group, were dosed via SC injection with ATX alone (0.3 or 3 mg/kg), BUSP alone (3 mg/kg), or ATX (0.3 or 3 mg/kg) and BUSP (3 mg/kg) in combination. Plasma samples (with EDTA) were collected from each rat pre-dose and at 0.083, 0.167, 0.33, 0.5, 1, 2, 4, and 7 h post-dose. Dose levels in the PK study for PF-04269339 included 1 and 10 mg/kg, whereas the PK study for PF-03529936 used 10 mg/kg. All rats were fed throughout the study.

All of the procedures in this publication were conducted in full compliance with local, national, ethical, and regulatory principles and local licensing regulations, per the spirit of Association for Assessment and Accreditation of Laboratory Animal Care International's expectations for animal care and use/ethics committees (http://www.aaalac.org/education/module_1.cfm) and in full compliance with Pfizer Animal Care and Use Committee guidelines.

DA and Drug Exposure Analysis

Dialysate samples were assayed for DA using HPLC coupled with electrochemical detection. In all experiments, standard solutions of DA were used to determine HPLC retention time and to quantify peak areas. Chromatographic separation was carried out using a Waters HPLC, model 2795 (Waters Corp., Milford, MA, USA), connected to a TSK

column (150 $\times$ 2 mm $\times$ 5 μ m; Tosoh Bioscience, Montgomeryville, PA, USA) with a Javelin guard column (20 $\times$ 3 mm; Thermo Electron Corp., Woburn, MA, USA) maintained at 32 °C. The flow rate of the HPLC was set at 0.46 mL/min. DA was detected using a mobile phase containing 75 mM sodium acetate (pH 4.4), 0.1 mM EDTA, 0.8 mM HSA, and 6.5 % methanol. A sample of 25 μ L brain dialysate was injected for monoamine detection using an electrochemical detector (Antec, Leyden, Netherlands) with a glassy carbon electrode (0.56 V). Each chromatographic peak was identified and mapped using the Millennium software (Waters Corp.). Data were reported as the concentrations of DA directly measured in the dialysate samples. It should be noted that, to determine actual concentrations in the extracellular fluid, a more elaborate *in vivo* microdialysis procedure for measurement of *in vivo* probe recovery of DA in individual rats is necessary (13). No attempt to correct for *in vitro* probe recovery was made in the present study given that *in vitro* recovery cannot be directly extrapolated to *in vivo* samples; it was felt that it is better to report the data as directly measured in dialysate samples.

Plasma concentrations of ATX, BUSP, PF-04269339, and PF-03529936 in rats were determined using liquid chromatography, tandem mass spectrometry (LC/MS/MS). Standard curves were prepared in blank rat plasma in the range of 1.0–5,000 ng/mL for ATX and 0.610–2,500 ng/mL for BUSP, PF-04269339, and PF-03529936. Traditional protein precipitation extraction was used, where 150 μ L of risperidone in acetonitrile as an internal standard was added to 50 μ L of plasma sample or standard. Samples were vortex-mixed for 1 min, centrifuged (3,000 rpm, 10 min), and 100 μ L of the supernatant was transferred to a clean 96-well plate. Then, 10 μ L of sample was injected. Separation was achieved using a Hypersil Gold C18 2.1 $\times$ 50 mm $\times$ 5 μ m column at a flow rate of 400 μ L/min using a 3-min gradient method, starting at 90 % acetonitrile and 10 % 0.1 % formic acid in H₂O+10 mM NH₄OAc. LC/MS/MS analysis was performed using an autosampler (HTS PAL Leap), a pump (Shimadzu LC-10 ADVP), and a mass spectrometer (AB Sciex 4000 triple-quadrupole) with positive electrospray ionization in multiple reaction-monitoring mode.

In Vitro Plasma Protein Binding

Plasma-free fractions of ATX, BUSP, PF-04269339, and PF-03529936 in rat and human plasma were determined using an equilibrium dialysis technique. Protein binding was measured using a 96-well Teflon dialysis chamber (HTDialysis LLC, Gales Ferry, CT, USA) with a semipermeable membrane (Spectra/Por4; Spectrum, Laguna Hills, CA, USA) of 12–14 kDa molecular mass cut-off. Pilot experiments revealed that: (1) all compounds were stable in plasma; (2) protein binding reached equilibrium at 37 °C for 6 h; and (3) minimal difference in protein binding was observed at concentrations between 10 nM and 1 μ M for the four compounds. For final protein binding measurements, an aliquot of plasma (0.15 mL) of 1 μ M of each test compound was placed in half of the wells in triplicate. The second half of the wells contained an equal volume of potassium buffer (100 mM, pH 7.4). The plate was covered with a top seal film

to avoid evaporation and was incubated at 37 °C for 6 h. After incubation, the plasma (0.02 mL) and buffer (0.08 mL) were transferred to separate tubes containing either 0.08 mL of blank buffer or 0.02 mL of blank plasma, respectively. Samples were extracted with 0.3 mL of an acetonitrile-methanol mixture (1:1, v/v) containing the internal standard and were analyzed by liquid chromatography-tandem mass spectrometry, as described above. The free fraction (f_u) was calculated using the equation $f_u = C_{\text{buffer}}/C_{\text{plasma}}$, where C_{buffer} and C_{plasma} denote the concentrations of the test compound in buffer and plasma, respectively, after the incubation.

PK Analysis

Given that the PK and microdialysis samples were not from the same individual rats and were collected in separate studies, no population PK modeling was attempted. Instead, a naïve-pooled compartmental PK analysis was used for ATX, BUSP, and the two PF compounds using plasma exposure data obtained in the satellite pharmacokinetic studies. One- and two-compartment models were tested for all compounds studied. For SC administration of ATX and BUSP, zero- and first-order absorption models were tested. The model was parameterized using absorption rate constant (k_a , h⁻¹), SC clearance (CL/F, L/h/kg), and SC volume of distribution (Vd/F, L/kg). For SC administration of the two PF compounds, because the maximal exposure was observed at the first sampling time, at 0.5 h, the plasma exposure-time profiles were modeled using IV infusion models rather than absorption models. Residual variability in the PK modeling was characterized by a proportional error model. The difference in the objective function value produced by NONMEM was used to discriminate between nested models using the likelihood ratio test.

PK parameters obtained were fixed in the subsequent PK/PD modeling to simulate plasma concentrations at specific time points in the microdialysis studies to drive time-dependent PD modeling. The plasma concentrations of BUSP after SC administration of 0.1, 0.3, 1, and 10 mg in the microdialysis study were extrapolated using the PK parameters estimated based on the 3 mg data in the PK study, given the dose proportional PK of BUSP observed in our pilot single time point dose response study and reported for BUSP in literature (10). Similarly, the plasma concentrations of PF-03529936 after SC administration of 0.1 and 1 mg in the microdialysis study were extrapolated using the parameters estimated based on the 10 mg data, given that dose proportional PK observed in the pilot dose response study.

PK/PD Modeling and Simulation

After fixing the PK parameters, prefrontal cortex DA responses in individual rats were modeled using a population modeling approach. An extended indirect response model (Fig. 2) with dual pharmacodynamic interactions was proposed, based on our understanding of the underlying mechanisms of action for NRI and 5-HT_{1A} activities on DA levels. In this model, the DA level at baseline and its turnover rate are governed by the balance of formation and dissipation rates. NRI

compounds such as ATX inhibit the rate of dissipation of DA through specific inhibition of presynaptic NE reuptake transporters. 5-HT_{1A} partial agonists such as BUSP increase the formation rate of DA through activation of postsynaptic 5-HT_{1A} receptors. The direct effects of NRI compounds on the rate of dissipation and 5-HT_{1A} compounds on the rate of formation of DA were described by sigmoidal E_{max} models. With the extended indirect response model, the time profiles of DA responses following administration of a NRI and a 5-HT_{1A} compound alone or in combination, or a NRI/5-HT_{1A} dual pharmacology compound can be described by the following differential equation:

$$\frac{dDA}{dt} = K_{\text{in}} \times \left(1 + \frac{S_{\text{max}} \times C_p^{\gamma_1}}{SC_{50}^{\gamma_1} + C_p^{\gamma_1}} \right) - K_{\text{out}} \times \left(1 - \frac{I_{\text{max}} \times C_p^{\gamma_2}}{IC_{50}^{\gamma_2} + C_p^{\gamma_2}} \right) \times DA \quad (1)$$

where, DA is the DA levels in the extracellular fluid of the prefrontal cortex, which are expressed as the DA concentrations in the intracerebral dialysate, C_p refers to the concentrations of the test compounds at the prefrontal cortex NRI and/or 5-HT_{1A} binding site, which are assumed to be equal to the plasma-free concentration of the tested compound, K_{in} (nanomole per hour) represents the apparent zero-order rate constant for the production of DA in the prefrontal cortex, K_{out} (per hour) defines the first-order rate constant for dissipation of DA in the prefrontal cortex, SC_{50} refers to the concentration of 5-HT_{1A} partial agonist causing half-maximal effects (S_{max}) for the stimulation of DA formation, and IC_{50} refers to the concentration of the NRI compound causing half-maximal effects (I_{max}) for the inhibition of DA degradation. γ_1 and γ_2 are the steepness factors for the modulation of K_{in} and K_{out} through the 5-HT_{1A} and NRI activities, respectively.

The pre-dose ($t \leq 0$) DA level is described by the baseline DA, that is equal to $K_{\text{in}}/K_{\text{out}}$. The population baseline DA level (E_0) was estimated from the modeling and K_{in} was calculated as a secondary parameter from the baseline E_0 and K_{out} estimates. Given the considerable variability in baseline DA levels in individual rats, inter-individual variability was estimated for E_0 using an exponential variance model. Residual variability was characterized using an additive error model.

Model validation was based on several criteria, such as the objective function value, parameter estimates, biological plausibility, and visual assessment of the goodness-of-fit plots. Additionally, to assess the predictive performance of the PK/PD models, visual predictive check was conducted based on the simulation of 1,000 datasets using the parameter estimates and variability estimates from the dataset. The 5th, 50th, and 95th percentiles were calculated from the simulated profiles for the predictive checks and were superimposed on the raw data to allow assessment of model predictability.

After successful model evaluation, the model was used for simulating the steady-state PK/PD relationships for the various compounds in the rat model and extrapolating the preclinical relationship to clinical setting. Based on Eq. (1), the DA increase relative to its baseline level at steady-state

Fig. 2. Schematic representation of the extended indirect response model used to describe the prefrontal cortex dopamine responses for atomoxetine, buspirone, or novel NRI/5-HT_{1A} compounds

(net DA change=0) can be described using the following expression

$$\frac{[DA]_{ss}}{[DA]_{baseline}} = \frac{1 + \frac{S_{max} \times C_{p,ss}^{\gamma_1}}{SC_{50}^{\gamma_1} + C_{p,ss}^{\gamma_1}}}{1 - \frac{I_{max} \times C_{p,ss}^{\gamma_2}}{IC_{50}^{\gamma_2} + C_{p,ss}^{\gamma_2}}} \quad (2)$$

Additionally, the preclinical PK/PD model was used as the basis for reverse translation of the PK/PD relationship of ATX in ADHD patients to the rat efficacy model. When extrapolating PK/PD relationships from the rat efficacy model to patients or *vice versa*, additional terms are included to account for differences in plasma protein binding and receptor binding affinity for the reference compounds or novel NRI/5-HT_{1A} compounds in human *versus* preclinical species. For example, the cross-species scaling of efficacious exposure between rat and human for NRI agent including ATX can be conducted based on the following equation:

$$IC_{50, human} = IC_{50, rat} \times \frac{K_{i, NET, human}}{K_{i, NET, rat}} \times \frac{f_{u, rat}}{f_{u, human}} \quad (3)$$

where, $K_{i, NET}$ is the *in vitro* NET binding affinity in rat or human and f_u is the plasma-free fraction measured in rat or human. Our in-house data showed that the *in vitro* rat NET binding affinity for ATX was eightfold higher than that to human NET (binding K_i values of 7.03 and 0.88 nM to human and rat NET, respectively). The plasma-free fraction of ATX in human was 8.45-fold higher than that in rat (plasma-free fractions of 1.3 and 11 % in human and rat, respectively).

All PK/PD analyses were performed using the NONMEM software (ver. VII; Icon Development Solutions, Ellicott City, MD, USA) and S-Plus (ver. VII; Insightful Corporation, Seattle, WA, USA). Simulations of the human DA response for ATX at its therapeutic dose were conducted using the Berkeley Madonna software (University of California, Berkeley, CA, USA).

RESULTS

Pharmacokinetics of ATX and BUSP

The *in vitro* properties, including human NET binding K_i , 5-HT_{1A} binding K_i , and 5-HT_{1A} intrinsic efficacy, of the four tested compounds are summarized in Table I. The rank order of the *in vitro* NET binding K_i was ATX < PF-04269339 < PF-03529936. The rank order of the *in vitro* 5-HT_{1A} binding K_i was PF-03529936 < PF-04269339 < BUSP. Lastly, the rank order of the *in vitro* intrinsic activity to 5-HT_{1A} receptor was PF-03529936 ≈ BUSP < PF-04269339.

The observed and model-fitted plasma concentration–time profiles of ATX and BUSP are presented in Fig. 3. The pharmacokinetic parameter estimates for ATX and BUSP are presented in Table II. A one-compartment PK model with first-order absorption and first-order elimination best described the naïve-pooled exposure data of ATX and BUSP administered alone or in combination. All parameters were estimated with good precision except for some variation in the K_a estimate, which may reflect a large inter-individual variability in the rate of absorption following the subcutaneous administrations. No appreciable difference in the plasma concentration–time profiles, in terms of C_{max} and C_{trough} at 7 h post-dose, was observed for ATX or BUSP, when they were administered alone or in combination. Similarly, little difference (<20 %) was observed in the pharmacokinetic parameter estimates of ATX and BUSP between administration alone and in combination.

PK/PD Relationship of ATX and BUSP Alone or in Combination

A dose-dependent increase in the prefrontal cortex DA levels in rats was observed after administration of ATX and BUSP alone or in combination. Additionally, there was an apparent delay in the DA response, relative to the plasma concentrations of ATX and/or BUSP. Specifically, the maximum plasma concentrations of ATX or BUSP were observed at 0.5 h post-dose, whereas the maximum DA increase following ATX and/or BUSP treatment was observed at least 1 h post-dose.

Table I. Comparison between the Affinity and Intrinsic Efficacy of NRI/5-HT_{1A} Agents with Atomoxetine and Buspirone at 5-HT_{1A} Receptors and Norepinephrine Reuptake Transporters

Receptor	K_i (nM)				Intrinsic efficacy (% 5-HT)		
	Atomoxetine	Buspirone	PF-04269339	PF-03529936	Buspirone	PF-04269339	PF-03529936
5-HT _{1A}	–	20	5	0.33	58	74	55
NET	7	–	18	79	–	–	–

Data are reproduced from internally generated datasets at Pfizer Global Research and Development, Ann Arbor, MI, USA. Receptor binding studies used membranes from cells transfected with cloned receptors. Intrinsic efficacy was determined by comparing the maximal response of each agonist in a FLIPR assay (9) compared with the maximal response induced by 5-HT_{1A}

The observed and model-fitted population and individual prefrontal cortex DA response *versus* time profiles for ATX and/or BUSP are presented in Fig. 4. The pharmacodynamic parameter estimates are listed in Table III. The extended indirect response model sufficiently characterized the time

profiles of the prefrontal cortex DA levels for ATX and BUSP after administration alone or in combination. E_0 , the population baseline DA level, was 0.303 nM, with an inter-individual variation of 49.6 %. The high inter-individual variability in E_0 may be partially due to potential variability

Fig. 3. Observed and model-fitted plasma concentrations of atomoxetine (*top panel*) and buspirone (*bottom panel*) in rats after subcutaneous administration of atomoxetine and buspirone alone or in combination. Observation (OBS): circles indicate the observed concentrations in each rat ($n=2$ or 3/group). Prediction (PRED): solid lines are model-fitted plasma concentrations

Table II. Pharmacokinetic Parameter Estimates of Atomoxetine, Buspirone, PF-04269339, and PF-03259936 in Rats

Compounds for analysis	Group	K_a (h^{-1})	Cl/F (L/kg/h)	V_1/F (L/kg)	Q (L/kg/h)	V_2 (L/kg)
Atomoxetine	Atomoxetine 0.3 mg/kg	24.2 (23.7)	6.49 (0.318)	12.9 (1.04)	–	–
	Atomoxetine 0.3 mg/kg+buspirone 3 mg/kg	30.3 (26.6)	5.66 (0.310)	10.0 (0.819)	–	–
	Atomoxetine 3 mg/kg	19.4 (4.06)	4.33 (0.299)	12.8 (0.866)	–	–
	Atomoxetine 3 mg/kg+buspirone 3 mg/kg	9.14 (2.52)	4.08 (0.221)	10.5 (1.20)	–	–
buspirone	Buspirone 3 mg/kg	7.43 (1.37)	2.40 (0.126)	5.20 (0.287)	–	–
	Atomoxetine 0.3 mg/kg+buspirone 3 mg/kg	9.78 (3.84)	1.90 (0.173)	4.85 (0.608)	–	–
	Atomoxetine 3 mg/kg+buspirone 3 mg/kg	4.89 (1.88)	2.39 (0.249)	4.20 (0.756)	–	–
PF-04269339	PF-04269339 1 and 10 mg/kg	–	7.63 (0.411)	25.4 (1.45)	–	–
PF-03529936	PF-03529936 10 mg/kg	–	3.84 (0.358)	2.30 (0.977)	7.96 (2.83)	5.74 (0.499)

Parameter estimates are listed with their standard error in parentheses. Parameters are defined in the text

in the *in vivo* probe recovery of DA in the different microdialysis studies. The baseline DA level was governed by a dissipation rate constant K_{out} of 3.61 h^{-1} and a production rate constant K_{in} of 1.48 nM h^{-1} , which was calculated based on the E_0 and K_{out} estimates. The IC_{50} estimate of ATX was 4.67 ng/mL , equivalent to 1.93 nM in free concentration. The SC_{50} estimate of BUSP was 91.2 ng/mL , equivalent to 130 nM in free concentration. These pharmacodynamic parameters were estimated with good precision. No strong correlation ($R^2 > 0.9$) was observed between parameter estimates.

Additional model validation results, including goodness-of-fit and visual predictive check plots, are provided as [supplementary materials](#). The goodness-of-fit plots for ATX

and/or BUSP did not show any systematic deviation between the observed and the individual model-predicted data or trend in individual weighted residuals *versus* time (Electronic Supplementary Material (ESM) Fig. 1). An overlay of the observed data and the visual predictive check predicted data confirmed that the model is able to capture the majority of the prefrontal DA observations, which fell within the 90 % prediction intervals (ESM Fig. 2).

Pharmacokinetics and PK/PD Relationships for PF-04269339 and PF-03529936

One- and two-compartment IV infusion models adequately described the plasma concentration–time profiles of

Fig. 4. Observed and model-fitted dopamine concentrations in the dialysate after administration of vehicle, atomoxetine, buspirone, or atomoxetine and buspirone in combination in rats. Response (RESP): circles represent the observed dopamine concentrations in individual rats. Prediction (PRED): solid lines represent the model-fitted population dopamine concentrations. Individual prediction (IPRE): dashed lines represent the model-fitted dopamine concentrations in individual rats. The dashed vertical line at 1.5 h refers to the time of drug administration at 1.5 h after the start of microdialysis study

Table III. Pharmacodynamic Parameter Estimates of Atomoxetine, Buspirone, PF-04269339, and PF-03529936 for the Prefrontal Cortex Extracellular Dopamine Response in rats

Parameter	Atomoxetine	Buspirone	PF-04269339	PF-03529936
NRI activity, IC ₅₀ (ng/mL)	4.67 (1.31)	–	3.40 (2.81)	89.2 (33.6)
$I_{\max}$	0.552 (0.0509)	–	0.552 (fixed)	0.552 (fixed)
γ_2	1 (fixed)	–	1 (fixed)	1 (fixed)
5HT- _{1A} activity, SC ₅₀ (ng/mL)	–	191 (25.1)	153 (49.3)	69.1 (9.83)
$S_{\max}$	–	0.913 (0.155)	1.35 (0.445)	1.24 (0.344)
γ_1	–	2.50 (0.594)	2.50 (fixed)	2.50 (fixed)
E_0 (nM)	0.303 (0.0131)	–	0.313 (0.0295)	0.0831 (0.00941)
K_{out} (h ⁻¹)	3.61 (0.320)	–	5.27 (0.669)	3.32 (0.790)
Inter-individual variation in E_0 (% of CV)	49.6	–	35.8	43.8
Residual error, SD, nM	0.0894	–	0.0800	0.0331

Parameter estimates are listed with their standard error in parentheses. Parameters are defined in the text

PF-04269339 and PF-03529936, respectively (ESM Figs. 3 and 4). Pharmacokinetic parameter estimates for both compounds are presented in Table II.

The observed and model-fitted population and individual prefrontal cortex DA response–time profiles of PF-04269339 and PF-03529936 are shown in Figs. 5 and 6, respectively. Pharmacodynamic parameters for both compounds are listed in Table III. After fixing the three mechanism-specific pharmacodynamic parameters ($I_{\max}$ = 0.552 and γ_2 = 1 for NRI activity and γ_1 = 2.50 for 5-HT_{1A} activity), the extended indirect response model sufficiently fitted the DA response–time profiles for both compounds. The goodness-of-fit plots did not show any systematic

deviation between observations and population or individual predictions or any trends in individual weighted residuals *versus* time for both PF compounds (ESM Figs. 5 and 7). The visual predictive check demonstrated that the current model was able to capture the majority of the observed individual data for both compounds (ESM Figs. 6 and 8). Comparison of the overlap of the simulated distribution in visual predictive check with the observations for PF-03529936 showed considerable similarity. However, it should be noted that the model tended to slightly underestimate the DA response at 3 mg and overestimate the response at 10 mg for PF-04269339. The model-predicted variability for PF-04269339 also appeared

Fig. 5. Observed and model-fitted dopamine concentrations in the dialysates after administration of vehicle or PF-04269339 of 1, 3, and 10 mg/kg in rats. Response (RESP): circles represent the observed dopamine concentrations in individual rats. Prediction (PRED): solid lines represent the model-fitted population dopamine concentrations. Individual prediction (IPRE): dashed lines represent the model-fitted dopamine concentrations in individual rats. The dashed vertical line at 1.5 h refers to the time of drug administration at 1.5 h after the start of microdialysis study

Fig. 6. Observed and model-fitted dopamine concentrations in the dialysates after administration of vehicle or PF-03529936 of 0.1, 1, and 10 mg/kg in rats. Response (RESP): circles represent the observed dopamine concentrations in individual rats. Prediction (PRED): solid lines represent the model-fitted population dopamine concentrations. Individual prediction (IPRE): dashed lines represent the model-fitted dopamine concentrations in individual rats. The dashed vertical line at 1.5 h refers to the time of drug administration at 1.5 h after the start of microdialysis study

to be greater than observed particularly at 10 mg (ESM Fig. 6).

For PF-04269339, the IC₅₀ estimate of its NRI activity was 3.40 ng/mL, equivalent to 2.38 nM free. The SC₅₀ estimate of its 5-HT_{1A} activity 153 ng/mL, equivalent to 108 nM free. For PF-03529936, its NRI IC₅₀ and 5-HT_{1A} SC₅₀ estimates were 89.2 ng/mL (63.7 nM free) and 69.1 ng/mL (49.4 nM free), respectively. A qualitative correlation between the *in vivo* and *in vitro* drug-related properties was observed for the four compounds. Specifically, the rank order of the *in vivo* NRI IC₅₀ estimates was ATX < PF-04269339 < PF-03529936, which is consistent with the rank order of their *in vitro* NET binding *K_i*. The rank order of the *in vivo* 5-HT_{1A} IC₅₀ estimates was PF-03529936 < PF-04269339 < BUSP, which is consistent with the rank order of their *in vitro* 5-HT_{1A} binding *K_i*. Lastly, the rank order of the *in vivo* S_{max} of the 5-HT_{1A} activity was consistent with the *in vitro* 5-HT_{1A} intrinsic activity in that PF-03529936 ≈ BUSP < PF-04269339.

PK/PD Simulations

A simulation of the steady-state prefrontal cortex DA response in rats following ATX and BUSP administration singly or in combination using the pharmacodynamic parameter estimates is presented in Fig. 7. It showed that the administration of ATX alone could increase the prefrontal cortex DA level in rats by 2.2-fold, maximally. BUSP alone could cause a maximum 1.91-fold increase. The combination of ATX and BUSP could lead to a 4.2-fold maximal increase

of the DA level, greater than either agent alone. This simulation underlined the potential therapeutic advantage of the proposed NRI/5-HT_{1A} therapeutic agents, compared with NRI or 5-HT_{1A} agents alone.

The simulation of steady-state exposure–response relationship of prefrontal cortex DA responses in rats was also used as the basis for cross-species PK/PD scaling to support human efficacious dose projection for NRI/5-HT_{1A} compounds. First, based on the human PK parameters for ATX reported in the literature (14), the C_{max} of

Fig. 7. Simulation of steady-state dopamine response versus plasma concentration relationship in rats following single or co-administration of atomoxetine and buspirone

ATX was approximately 710 ng/mL in patients following 30 mg twice a day, the efficacious dose of ATX for ADHD treatment in adolescents and children. After subsequent correction of eightfold higher NET *in vitro* binding affinity in rat than in human and a 8.45-fold higher plasma free fraction in human than in rat, the C_{eff} of 710 ng/mL in human can be reverse translated to a C_{eff} of 11 ng/mL in rat. Based on the steady-state exposure–response relationship, the C_{eff} of 11 ng/mL for ATX in rats was associated with a 1.6-fold increase in prefrontal cortex DA levels (ESM Fig. 9). Based on this clinical-to-preclinical reverse translation, a steady-state twofold increase of prefrontal cortex DA levels was set as the minimal efficacious response criteria for NRI/5-HT_{1A} compounds in the rat efficacy model. Based on this criteria, the steady-state concentrations of PF-04269339 and PF-03529936 associated with a twofold increase of the prefrontal DA levels in rats would be 30 and 56 ng/mL, respectively (ESM Fig. 9). Furthermore, the simulation suggested that at the targeted minimal efficacy level in rats (*i.e.*, a twofold increase of the prefrontal cortex DA levels), the *in vivo* effect of PF-04269339 is mediated primarily via its 5-HT_{1A} activity while the effect of PF-03529936 is mediated by almost equal contribution of its NRI- and 5-HT_{1A} activities.

DISCUSSION

The present study provides a novel example of the development and application of an integrated mechanism-based PK/PD model in a dual-pharmacology drug discovery program. The prefrontal cortex DA levels in rats following the administrations of ATX, the NRI reference compound, and BUSP, the 5-HT_{1A} reference compound, alone or in combination, and following the administrations of two novel NRI/5-HT_{1A} compounds, were modeled using a piecewise approach.

Prior to the use of ATX and BUSP as the reference compounds in the microdialysis study, we wanted to rule out any potential pharmacokinetic interaction between these two drugs. The possibility was generally considered to be low, based on our understanding of the absorption and elimination mechanisms of ATX and BUSP. Both drugs have high biological membrane permeability and rapid and near-complete absorption (15). Whereas the elimination of ATX from the body is mediated primarily by CYP2D6, BUSP is eliminated primarily by CYP3A4-mediated metabolism (16–18). Additionally, neither agent modulates CYP enzyme activity (16–18). As observed in the present study, the plasma exposure–time profiles and pharmacokinetic parameter estimates for ATX and BUSP were comparable following either single or co-administration, supporting the absence of a pharmacokinetic interaction between ATX and BUSP in the subsequent microdialysis studies.

The prefrontal cortex DA responses were delayed relative to the plasma concentrations of ATX and/or BUSP. Rather than fitting various PK/PD models empirically to characterize the temporal delay, an extended indirect response–interaction model was proposed, based on our understanding of the mechanisms of action of ATX and

BUSP. The basic indirect response model was first established in 1993 by Dyneka *et al.* (19). It characterizes pharmacodynamic effect on biological turnover process that is governed by the dynamic equilibrium between K_{in} and K_{out} . With this model, the delay between drug exposure and response is caused by the time needed for the direct effects of drugs on K_{in} or K_{out} be fully reflected in the measured physiological response. In 2004, Earp *et al.* (20) expanded the basic indirect response model into a diverse array of extended indirect response models. The model used in the present study belongs to a class of the extended indirect response models characterizing interaction between agents acting noncompetitively on K_{in} and K_{out} by opposing mechanisms (20).

The extended indirect response model well characterized the time course of the DA response to ATX and BUSP, administered alone or in combination. In addition to the proposed noncompetitive interaction, we also considered the possibility of additional pharmacodynamic interaction between ATX and BUSP when they were administered together, which could be either additional synergism between the NRI and 5-HT_{1A} pathways or tolerance in their dopaminergic effects. To assess this possibility, we added additional scaling factors, one at a time, to pharmacodynamic parameters (*i.e.*, the S_{max} in increasing K_{in} and the I_{max} in decreasing K_{out}) in the ATX and BUSP combination group, and rerun the model. The model fitting and objective function did not change appreciably when the scaling factors were added. Therefore, parameters on additional pharmacodynamic interaction between ATX and BUSP were not included in the final PK/PD model in the present study.

The model development based on simultaneous PK/PD modeling of ATX and BUSP, administered alone and in combination, was a key step in the PK/PD analysis in the NRI/5-HT_{1A} program. When the extended indirect response model was first used to characterize the exposure–response relationship of NRI/5-HT_{1A} compounds alone, we ran into parameter identification issue. When data fail to support fully mechanistic models, alternative modeling strategies are often necessary to avoid overparameterization. Examples of these strategies include using a more empirical model, fixing various rate constants, or developing a collapsed mechanistic model, (21). In the present study, the PK/PD modeling of the reference compounds ATX and BUSP allowed us to estimate mechanism-specific PD parameter values and fix them to reduce the number of parameters when applying the model to dual-mechanism compounds. Three PD parameters were assumed to be mechanism-specific, including (1) the I_{max} reflecting the maximal efficacy, (2) γ_2 , reflecting the steepness factor of prefrontal cortex DA enhancement via the NRI mechanism, (3) γ_1 reflecting the steepness factor for the 5-HT_{1A} pathway. In a similar PK/PD modeling of the effects of ATX on prefrontal cortex NE (data not shown), the I_{max} estimate was 62.7 % and γ_2 estimate was 1. The I_{max} and γ_2 for NE elevation were similar to the I_{max} and γ_2 for DA elevation in rats, supporting the use of these two parameters as the mechanism-specific pharmacodynamic parameters for the NRI pathway. The γ_1 is greater than 1, suggesting a steep exposure–response relationship for DA enhancement via the 5-HT_{1A} agonist. Nevertheless, any mechanistic interpretation of the γ_1 estimate needs to be taken with caution. The model is still semimechanistic since it contains an empirical

sigmoidal $E_{\max}$ equation. The operational model of an agonist (22) may be able to provide a more accurate characterization of 5-HT_{1A} agonist. However, it will be overparameterized with the current dataset.

After fixing these mechanism-specific parameters, the indirect response drug interaction model adequately described the time-response profiles of the prefrontal cortex DA levels in rats for PF-04269339 and PF-03529936. Nevertheless, the model tended to slightly under estimate the DA response for PF-04269339 at 3 mg and over estimate the response at 10 mg. This mismatch may be due to some dose nonlinearity in the PD of PF-04269339 that the current model has not accounted for. Additional investigations including perhaps adding a dose lower than 3 mg may be required to refine the model for PF-04269339 in rats. No significant mismatch was observed for PF-03529936. Furthermore, the observed good qualitative correlation between the *in vitro* and *in vivo* PD parameters across the compounds supports the use of the semimechanistic PK/PD model in characterizing the pharmacological properties of NRI/5-HT_{1A} compounds. No assessment of the quantitative *in vitro*-to-*in vivo* correlations for the test compounds was attempted because of the lack of an *in vitro* functional model for rat NRI or 5-HT_{1A}.

In the present study, the mechanism-based model demonstrated greater descriptive power, compared with empirical model. For example, the $T_{\max}$ of the plasma concentration for PF-03529936 was approximately 1 and 3 h after dosing in the 0.1 and 10 mg/kg dose groups, respectively. A shift of $T_{\max}$ to a later time as dose increases is a unique characteristic profile of a drug possessing an indirect response mechanism (19). The delayed response and dose-dependent shift of $T_{\max}$ were well captured with the indirect response model. Furthermore, the mechanism-based modeling has provided mechanistic insights to guide lead optimization and series selection in the drug discovery program. For example, the simulation of NRI- or 5-HT_{1A} specific activity *in vivo* suggests that the minimal efficacious effect of PF-04269339, *i.e.*, corresponding to a twofold increase of the prefrontal cortex DA levels in rats, is mediated predominantly via its 5-HT_{1A} activity whereas similar effect is mediated by almost equal contribution of NRI- and 5-HT_{1A} activity for PF-03529936 (ESM Fig. 9). Thus, new chemical series with *in vitro* pharmacodynamic properties similar to that of PF-03529936 may have more favorable dual pharmacology properties *in vivo* as compared with those similar to PF-04269339. This mechanistic insight provided a quantitative rationale for the team to direct our exploration of chemical series by tuning down the *in vitro* binding affinity towards NET and significantly tuning up the *in vitro* affinity towards 5-HT_{1A}.

In summary, the present study showed that the extended indirect response model can be used to describe the PK/PD relationship of novel NRI/5HT_{1A} compounds on prefrontal cortex dopamine levels. A piecewise modeling approach was necessary to address the overparameterization issue. Application of the model provided important quantitative insights to guide human dose projections and lead compound selection and optimization in the NRI/5-HT_{1A} discovery program. Furthermore, compared with empirical modeling, mechanism-based PK/PD modeling allows for: (1) more

accurate data description, (2) differentiation between compound-specific and mechanism-specific pharmacodynamic properties, (3) greater predictive power for scaling to humans, and (4) *in vitro*-to-*in vivo* correlation and more mechanistic insight to guide lead optimization and biomarker validation.

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