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A Proposed Screening Paradigm for Discovery of Covalent Inhibitor Drugs

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Abstract: The *in vitro* and *in vivo* preclinical ADME properties of 10 clinically late stage or marketed covalent inhibitors were evaluated in order to define advancement criteria for discovery of future drugs in this arena.

Our studies revealed the following: After incubating with S9 fractions for 30 minutes, the rat and human *in vitro* stability for these compounds ranged from 1% to 100%. The blood stability ranged from 30% to 100%. There was a broad range of CYP inhibition with prevalence for time-dependent inhibition of at least one enzyme. The Caco-2 permeability (A→B) ranged from negligible (0.6 x 10^{-6} cm/s) to highly permeable (31 x 10^{-6} cm/s) and the efflux ratio also varied widely (0.2-30). Most of the compounds were highly protein bound in both rat and human with binding ≥ 90%. Rat plasma clearance for the 10 compounds ranged from slow (11 mL/min/kg) to very rapid (350 mL/min/kg). The V_{ss} ranged from low (0.67 L/kg) to very high (115 L/kg). MRT's also ranged from short (0.5 hr) to long (7.4 hr). The oral exposures also showed a very broad range with C_{max}'s ranging from 0.01-77 μM and exposure levels ranging from 0.03-106 μM.hr.

In conclusion, the wide range in *in vitro* and *in vivo* ADME data makes these particular ADME assays non-discriminatory in the selection of promising compounds. In our opinion, non-traditional assays such as target mass modification, target confirmation by amino acid sequencing, cellular target occupancy, and target turnover rate data in combination with the pharmacokinetic profiles are the critical considerations for progression of irreversible compounds in early discovery.

Keywords: ADME, covalent, discovery, irreversible, mass-modification, pharmacokinetics, screening.

[#]**Biography:** Mehran Moghaddam, PhD, is the head of Discovery DMPK at Celgene, a global biopharmaceutical company with products in oncology and immune-inflammatory related diseases. He has 18 years of industrial experience with DuPont, Pfizer, and Celgene. His expertise includes animal experimentation, pharmacokinetics, metabolism, metabolite/catabolite identification, and bioanalysis of small and macro-molecules.

INTRODUCTION

Until recently, the pharmaceutical industry has been reluctant to rationally design covalent (irreversible) inhibitors for therapeutic targets due to the perceived likelihood for potential idiosyncratic adverse reactions (IADR's) as a result of off-target covalent binding [1]. Many drugs on the market are indeed irreversible inhibitors of their targets but they were not designed for this mode of action [2]. Their mode of action was, in fact, discovered after proof of their pharmacological activity was already well established. However, recent articles [2, 3] have outlined the renewed interest in designing targeted covalent inhibitors for therapeutic targets that posses the right attributes such as: 1) longer rates of *de novo* synthesis, 2) propensity to confer drug resistance due to mutations in the drug binding site, 3) need for much higher potency of the inhibitor to effectively suppress the target, and 4) need for isoform selectivity. Several covalent inhibitors have been recently approved (Afatinib and Ibrutinib) or are in late stage clinical trials for indications such a hepatitis C, autoimmune diseases and various forms of cancer [4-9].

With the renewed interest in covalent inhibitors comes the responsibility for drug discovery teams to ensure that only those molecules with good drug-like properties are advanced through discovery programs so that they can withstand the rigor of drug development programs. In particular, they need to withstand the nonclinical ADME and toxicology programs and prove their safety at high enough doses to provide sufficient safety margins and high level of confidence for moving into Phase I studies. The traditional small molecule reversible drug discovery workflow includes target identification and validation, lead identification, lead optimization and pre-clinical profiling. Once a target is identified and possibly validated, medicinal chemists evaluate several chemotypes that have the potential to provide potent compounds with good intellectual property protection. Analogs are tested for their ADME properties in assays that generally include *in vitro* metabolic, blood, and plasma stability, membrane permeability and efflux, CYP inhibition, plasma protein binding, intravenous (clearance, volume of distribution, and MRT) and oral pharmacokinetics (absorpiton and bioavailability). Compounds that exhibit good potency, selectivity, and good ADME properties are then advanced into preclinical pharmacology and toxicology studies.

The work presented herein was aimed at determining whether the same workflow and assay criteria can be applied to covalent inhibitors. Our ultimate goal was to design

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appropriate ADME screening paradigms to effectively and efficiently screen out the undesirable covalent inhibitors and only advance those with the highest potential for development.

To that end, we selected 10 recent covalent inhibitors and screened them using our traditional ADME assays. These inhibitors are shown in Fig. **1** and listed in Table **1** and included Abiraterone®, Afatinib®, Boceprevir®, Canertinib®, Carfilzomib®, Dacomitinib®, Ibrutinib®, Neratinib®, Telaprevir®, and Vigabatrin®. Some of these are covalent in the traditional sense since they covalently bind to the target protein and do not dissociate from the target. Others such as Boceprevir® and Telaprevir® are slightly different because even though they bind covalently to the target, they dissociate from it with a very slow off-rate which in turn helps them inhibit the target for prolonged periods of time; in effect very similar to a covalent inhibitor in their mechanism of action [7, 8]. These compounds were attractive choices for retrospective determination of discovery ADME advancement criteria for the next generation of covalent drugs because they have progressed beyond early clinical safety and efficacy criteria and into late stage clinical trials or to the market.

MATERIALS AND METHODS

Chemicals

Abiraterone (Cat # S1123), Afatinib (Cat# S1011), Canertinib (Cat# S1019), Dacomitinib (Cat# S2727), Ibrutinib (Cat# S2680), Iniparib (Cat# S1087), Neratinib (Cat# S2150), and Telaprevir (Cat# S1538) were purchased from Selleckchem (Houston, TX). Boceprevir (Cat # DM-0761) was purchased from Amatek (Berwyn, PA). Carfilzomib (Cat# CT-CARF) was purchased from Chemie Tek (Indianapolis, IN). Vigabatrin (Cat# 9000976) was purchased from Cayman chemical (Ann Arbor, MI).

In Vitro Studies

Liver S9 Metabolic Stability Assay

Incubations were performed at 37°C in a Dubnoff shaking water bath using 2 mL 96-well incubation plates (7-Ethoxycoumarin was used as the control for each incubation). Rat and human S9 protein concentrations were 0.75 mg/mL and 1.2 mg/mL, respectively. The final concentrations of Nicotinamide adenine dinucleotide phosphate (NADPH), Uridine 5'-diphospho-glucuronic acid (UDPGA) and glutathione (GSH) were 1, 0.5 and 2.5 mM, respectively while that of 3' –Phosphoadenosine-5'-phosphosulfate (PAPS) was 0.05 mg/mL. Substrate concentrations were 3μM, incubation time was 30 minutes and all tests were done in triplicate. The incubation was conducted in 200 mM Tris buffer containing 2 mM magnesium chloride, pH 7.4 and the total incubation volume was 0.5 mL. Samples were analyzed *via* liquid chromatography-tandem mass spectrometry (LC-MS/MS) and reported as percentage remaining after 30 minute incubation.

Table 1. A list of 10 clinically late-stage or marketed covalent inhibitors, the corresponding corporations that develop or market them, their route of administration, therapeutic indications, clinical dosing regimen, and therapeutic target.

Compound	Corporation	Dosing Route / Therapeutic Indication	Clinical Dosing Regimen	Target (De Novo Synthesis Half-life)
Abiraterone®	Johnson & Johnson	Oral / Prostate Cancer	1000 mg/day	CYP17A1 (12-33 hr)
Afatinib®	Boehringer Ingleheim	Oral / NSCLC, Prostate Cancer, Head and Neck Cancer, Glioma	40 mg/day	EGFR (16-24 hr) and Her-2
Boceprevir®	Merck & Co.	Oral / Hepatitis C	800-2400 mg/day	HCV Protease (> 16 hr)
Canertinib®	Pfizer Inc.	Oral / Cancer	≥ 200 mg/day for a week, every other week	EGFR (16-24 hr)
Carfilzomib®	Onyx Pharmaceuticals	Intravenous / Myeloma	~1000 mg infused in 2-10 min, 2 days/week, for 3 weeks, followed by 12 day drug holiday	20S Proteosome (Unknown)
Dacomitinib®	Pfizer Inc	Oral / NSCLC	150 mg/day	EGFR (16-24 hr) and Her 2, Her-4
Ibrutinib®	Pharmacyclics	Oral / CTL, Myeloma, Lymphoma, Autoimmune Diseases	420 mg/day	Btk (16-24 hr)
Neratinib®	Pfizer Inc	Oral / Breast Cancer	160-240 mg/day	EGFR (16-24 hr) and Her-2
Telaprevir®	Vertex Pharmaceuticals	Oral / Hepatitis C	2250 mg/day	HCV Protease (NS3 > 24 hr)
Vigabatrin®	Lundbeck Inc	Oral / Antiepileptic	2000-4000 mg/day	GABA Transaminase (Unknown)

Blood Stability Assay

Whole blood was spiked with test compound at a final concentration of 3μM (in triplicate) and incubated at 37°C in a Dubnoff Shaking water bath. Samples were taken at zero and 60 minutes, extracted *via* protein precipitation and filtration, and analyzed by LC-MS/MS. Blood stability was reported as percentage remaining after 60 minute incubation.

Cocktail CYP Inhibition Assay (Reversible and Time-dependent Inhibition)

A cocktail of midazolam (1.2 μM), phenacetin (90 μM), amodiaquine (2.4 μM), diclofenac (8 μM), dextromethorphan (2.4 μM), bupropion (31 μM), and omeprazole (2.8 μM) was used as probe substrates to assess the inhibitory potencies of the test compounds towards human CYP3A4/5, CYP1A2, CYP2C8, CYP2C9, CYP2D6, CYP2B6, and CYP2C19, respectively. The final reaction mixtures contained 0.16 mg/mL human liver microsomes, 10 pmol/mL rCYP2C19, 5 μM of test compounds and 1 mM NADPH in 0.1 M phosphate buffer (pH 7.4). Experiments were conducted in quadruplicates. The co-incubation assay was conducted to assess the reversible inhibitory potency while the 30 minute pre-incubation in the presence of NADPH was performed to evaluate the time dependent (TDI) inhibitory potential of test compounds.

In the pre-incubation assay, the incubation mixtures containing HLM/rCYP2C19 and NADPH in phosphate buffer (pH 7.4) were pre-warmed for 10 min. The test compounds (or acetonitrile as vehicle control) were added immediately and allowed to incubate for 30 min at 37°C. After 30 min of pre-incubation, the cocktail of probe substrates was added and allowed to incubate for 10 minutes. The reaction was terminated by the addition of acetonitrile containing the IS. In a parallel experiment, the coincubation assay was conducted by pre-warming the incubation mixtures containing HLM/rCYP2C19 and NADPH in phosphate buffer for 10 min. The test compounds followed by the probe substrates were quickly added and allowed to react for 10 minutes at 37°C. After quenching the reaction with the addition of acetonitrile/IS, the samples were analyzed by LC-MS/MS to monitor the formation of OH-midazolam, acetaminophen, desethylamodiaquine, OH-diclofenac, dextrorphan, OH-bupropion, and OH-omeprazole. Percent inhibition of the formation of specific metabolite of each probe substrate was measured using the equation shown below.

$$\% \text{ Inhibition} = (A_{(\text{vehicle control})} - A_{(\text{test compound})})/(A_{(\text{vehicle control})}) \times 100$$

Where $A_{(\text{vehicle control})}$ and $A_{(\text{test compound})}$ are the rates of metabolite formation of probe substrates or the peak area ratios of the metabolites and IS, determined in samples incubated in the vehicle control (acetonitrile) and test compound, respectively.

Caco-2 Assay

A ready-to-use cell culture system that provides a 21-day cell barrier in integrated HTS Transwell®-24 plates purchased from ADMEcell was used for the Caco-2 assay. Polarized cultures of Caco-2 cells were provided on polycarbonate micro-porous filters in HTS Transwell® plates (6.5mm diameter, 0.33cm^2 area and 0.4μm pore diameter). The transport medium used for the permeability studies was Hank's balanced salt solution (HBSS) buffer containing 1.1 mM magnesium chloride, 1.3 mM calcium chloride and 5 mM D-glucose. Prior to the experiment, each monolayer was washed twice with warm buffer. The concentration of test compound in this assay was 10 μM and all measurements were performed in duplicate. Lucifer yellow served as a quality control check for monolayer integrity of all wells and three control compounds were run with each assay (Atenolol, Propranolol, and Vinblastine). Studies were initiated by adding an appropriate volume of buffer containing test compound to either the apical or basolateral side of the monolayer. The monolayers were placed into a standard cell culture incubator (5% CO_2, 37°C) for two hours. Samples were taken from both the apical and basolateral compartments at the end of the two hour incubation and compound concentration was analyzed by LC-MS/MS. Permeability of compounds was determined as the coefficient of apparent permeability (P_{app}, measured in cm/s) calculated according to the following formula:

$$P_{app} = dQ/(dt \cdot A \cdot C_0),$$

where dQ/dt is the amount of compound present in the receiver compartment as a function of time; A is the area of the Transwell (cm^2); and C_0 is the initial concentration of compound applied in the donor compartment.

Plasma Protein Binding Equilibrium Dialysis Assay

Equilibrium dialysis was used to determine the extent of binding of compounds to plasma proteins (Thermo Scientific Pierce RED Device). A semi-permeable membrane separated a protein-containing compartment from a protein-free compartment (phosphate buffered saline, pH 7.4). Compound was spiked into plasma at a final concentration of 1 μM, and the assay was performed in triplicates. Warfarin and Methotrexate were used as controls for this assay. The system was allowed to equilibrate at 37°C for 4 hours with gentle shaking. The test compound concentration in each compartment was quantified by LC-MS/MS. The percentage of free drug (% Free) was calculated according to the following equation:

$$\% \text{ Free} = C_b/C_p \times 100,$$

where C_b is the compound concentration in the buffer chamber and C_p is the compound concentration in the plasma chamber. The final reported value, percentage bound (% Bound) is calculated as: % Bound = 100-% Free.

In Vivo Studies

All pharmacokinetic studies were conducted in male CD-IGS rats. The rats were purchased from Charles River laboratories as singly-cannulated animals in the jugular vein, regardless of the route of compound administration. Their body weight was 250-300 g at the time of dosing. Three rats were dosed per compound per route of administration.

Intravenous Dosing

For Vigabatrin the formulation was 5% dextrose in water (D5W). For Abiraterone®, Carfilzomib®, and Telaprevir® the formulation was N,N-dimethylacetamide (DMA), 85%

polyethylene glycol (PEG). For the rest of the compounds, the IV formulation consisted of a 1 mg/mL solution of the compound in 15% DMA, 50% PEG400, and 35% D5W. The formulation was dosed to each animal at 2 mg/kg (2 mL/kg) *via* the jugular cannula. The cannula was rinsed by flushing it using 200 µL of saline containing 20 units/mL of heparin. Blood samples were collected *via* serial sampling at 1, 5, 15, 30 min, 1, 2, 4, 6, 8, and 24 hours post-dose using manual blood sampling. The samples were centrifuged at 12000 rpm for 4 minutes within 30-60 min of collection and the resulting plasma was kept frozen until further analysis. Aliquots of whole blood (25 µL) from the 0.5-hour time points were also frozen for analysis and calculation of blood: plasma ratios.

Oral Dosing

For each compound, the PO formulation consisted of a 1 mg/mL suspension of the compound in 0.5% carboxymethylcellulose (CMC) and 0.25% Tween 80 in water. The formulation was dosed to each animal at 10 mg/kg (10 mL/kg) *via* the oral gavage. Blood samples were collected at 30 min, 1, 2, 4, 6, 8, and 24 hours post-dose using the automated blood sampling (ABS) units. The samples were centrifuged at 12000 rpm for 4 minutes within 30-60 min of collection and the resulting plasma was kept frozen until further analysis.

Sample Analysis and Pharmacokinetic Calculations

A total of 50 microliters (µL) of each plasma sample (diluted or undiluted depending on known or predicted concentrations of compound in the sample) were transferred to 96-well extraction plates. After addition of three (3) volumes (constant amount for each assay) of a methanol/ acetonitrile (1:1) mixture containing internal standard (IS) to the samples, the plate was shaken for 5 minutes on a multi-tube vortexer. After the extraction step, the contents of each well were transferred to a Captiva™ 96-well filtration plate (0.45 µm, Varian, Inc.) and placed on a vacuum apparatus to filter the extract into a receiving plate.

For blood:plasma ratios, the 25 µL blood sample was thawed and mixed with 25 µL of DI water and 25 µL counterpart control plasma. In the meantime, the 25 µL thawed plasma sample was mixed with 25 µL of DI water and 25 µL counterpart control blood. The mixture tubes were then sonicated for 10 minutes. After addition of four (4) volumes of a methanol/acetonitrile (1:1) mixture containing IS to the blood and plasma sample mixtures, the tubes were vortexed and mixed well. After the extraction step, the content of each well was transferred to a Captiva™ 96-well filtration plate (0.45µm, Varian, Inc.) and filtered under vacuum into a receiving plate. For blood-to-plasma ratio determination, a calibration curve was not constructed. Only area ratio of the analyte of interest vs. IS in blood and plasma was used for B/P ratio determination. A 10-point standard curve was prepared for each compound, with concentrations spanning 2-3 orders of magnitude. Samples were analyzed by LC-MS/MS using a MS2 multiple reaction monitoring (MRM) transition of parent ion m/z to the most abundant daughter ion m/z. The dosing formulation aliquots were also analyzed to obtain % recovery. The general mobile phase for all the quantitative work in plasma included acetonitrile or methanol and water both with 0.1% formic acid.

Pharmacokinetic calculations were performed using the non-compartmental analysis in Phoenix WinNonlin 6.1. For the IV studies, plasma clearance (CL), volume of distribution at steady state (V_{ss}), mean residence time (MRT), and blood:plasma ratio (B/P ratio) were reported. For the oral studies, plasma maximal concentrations (C_{max}), time to reach maximal concentration in the plasma (T_{max}), area under the concentration-time curve (AUC), and oral bioavailability (F%) were reported.

RESULTS

The 10 covalent inhibitors (Table **1**) were evaluated in various *in vitro* and *in vivo* discovery ADME assays. The results are listed in Tables **2**-**6**. Table **2** depicts the results from our *in vitro* liver S9 stability assays (30 min incubation)

Table 2. ***In vitro*** **stability of the 10 covalent inhibitors calculated as percent parent remaining following incubation with rat and human liver S9 for 30 minutes and incubation with rat and human whole blood for 60 min.**

Compound	*% Remaining at 30 min*		*% Remaining at 60 min*	
	Rat Liver S9	**Human Liver S9**	**Rat Whole Blood**	**Human Whole Blood**
Abiraterone®	46	3	67	70
Afatinib®	82	85	100	82
Boceprevir®	90	65	32	58
Canertinib®	39	70	72	69
Carfilzomib®	9	1	67	63
Dacomitinib®	87	90	76	100
Ibrutinib®	3	25	79	56
Neratinib®	59	69	NA	85
Telaprevir®	97	80	96	52
Vigabatrin®	100	99	NA	NA

and whole blood stability assays (60 min incubation). The 10 compounds exhibited a broad range of liver S9 stability ranging from 3% - 100% parent remaining in rat liver S9 and 1% - 99% parent remaining in human liver S9 after 30 minutes of incubation. In contrast the whole blood stability was much better for most of the compounds ranging from 32% - 100% parent remaining in rat whole blood and 52% - 100% parent remaining in human whole blood after 60 minutes of incubation.

CYP inhibition potential (reversible and time-dependent) was evaluated for all compounds against a panel of 7 enzymes. These included CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5. All the compounds were weak to moderate inhibitors of these CYP enzymes as demonstrated by <80 % inhibition following co-incubation with 5 μM compound (Table **3**). A majority of the compounds exhibited time-dependent inhibition of at least one enzyme following pre-incubation for 30 minutes. Dacomitinib® was the most significant time-dependent inhibitor and resulted in ~30-fold shift in the % inhibition of CYP2C8 at 5 μM concentration followed by Telaprevir® (~14-fold shift against CYP2C8), Boceprevir® (~9-fold shift against CYP1A2), and Canertinib® (~8-fold shift against CYP3A4/5).

Ibrutinib® (P_{app} = 31 X 10^{-6} cm/s) was the most permeable of all the compounds, as determined in the Caco-2 permeability assay, and also had one of the lowest efflux ratios (B→A / A→B = 0.4) (Table **4**). Carfilzomib® on the other hand exhibited the lowest permeability (Papp = 0.6 X 10^{-6} cm/s). The rank order for A→B permeability was Ibrutinib® > Dacomitinib® > Canertinib® > Vigabatrin® >

Table 3. ***In vitro*** **CYP inhibition potential of the 10 irreversible inhibitors reported as % inhibition following incubation with 5 μM compound. The % inhibition was compared following co-incubation and pre-incubation with the compound evaluate for time-dependent inhibition of the enzyme (Fold-shift).**

Compound	*REVERSIBLE INHIBITORY POTENTIAL*						
	Mean % Inhibition following co-incubation with 5 μM compound						
	CYP1A2	**CYP2C8**	**CYP2D6**	**CYP2C19**	**CYP2B6**	**CYP2C9**	**CYP3A4/5**
Abiraterone®	52.0	43.5	62.8	20.8	19.7	18.1	36.8
Afatinib®	6.0	6.0	25.6	2.4	28.5	29.1	1.1
Boceprevir®	6.2	0.0	5.6	21.9	18.3	15.7	40.8
Canertinib®	4.8	0.0	11.8	40.0	20.8	19.3	1.0
Carfilzomib®	4.0	41.6	18.9	36.1	25.4	15.2	68.6
Dacomitinib®	2.7	1.3	78.5	47.6	30.7	12.6	24.3
Ibrutinib®	0.0	58.1	16.4	34.7	61.2	48.6	38.7
Neratinib®	5.7	31.3	11.2	28.9	26.1	33.3	19.1
Telaprevir®	6.5	1.5	10.1	37.8	20.0	13.2	69.6
Vigabatrin®	3.5	6.7	4.7	12.9	15.8	3.9	4.0
Compound	*TIME DEPENDENT INHIBITION POTENTIAL*						
	Mean % Inhibition following pre-incubation with 5 μM compound (Fold-Shift)						
	CYP1A2	**CYP2C8**	**CYP2D6**	**CYP2C19**	**CYP2B6**	**CYP2C9**	**CYP3A4/5**
Abiraterone®	57.9 (0.9)	67.8 (0.6)	67.0 (0.9)	7.0 (0.3)	17.4 (0.9)	17.2 (0.9)	52.0 (1.4)
Afatinib®	0.0 (0.0)	10.7 (1.7)	1.7 (0.1)	5.4 (2.3)	16.3 (0.6)	18.5 (0.6)	0.0 (0.0)
Boceprevir®	57.9 (9.3)	0.0 (0.0)	0.0 (0.0)	20.4 (0.9)	6.2 (0.3)	1.4 (0.1)	81.4 (2.0)
Canertinib®	3.7 (0.8)	0.0 (0.0)	2.8 (0.2)	15.4 (0.4)	28.2 (1.4)	12.4 (0.6)	7.7 (7.7)
Carfilzomib®	2.9 (0.7)	5.0 (0.1)	0.3 (0.0)	21.1 (0.6)	27.3 (1.1)	11.1 (0.7)	83.8 (1.2)
Dacomitinib®	0.0 (0.0)	40.4 (31.1)	75.1 (1.0)	12.2 (0.3)	29.8 (1.0)	13.8 (1.1)	19.7 (0.8)
Ibrutinib®	0.0 (0.0)	41.9 (0.7)	2.2 (0.1)	4.1 (0.1)	40.4 (0.7)	30.5 (0.6)	52.1 (1.3)
Neratinib®	1.5 (0.3)	8.0 (0.3)	0.1 (0.0)	17.6 (0.6)	24.2 (0.9)	34.6 (1.0)	29.8 (1.6)
Telaprevir®	0.0 (0.0)	21.2 (14.1)	0.0 (0.0)	15.1 (0.4)	14.7 (0.7)	12.3 (0.9)	86.6 (1.2)
Vigabatrin®	1.5 (0.4)	19.5 (2.9)	0.04 (0.0)	3.7 (0.3)	8.0 (0.5)	0.4 (0.1)	11.7 (2.9)

Afatinib® > Telaprevir® > Boceprevir® > Neratinib® > Carfilzomib®. The efflux ratio also showed a very broad range (0.2-30). The Caco-2 permeability and efflux ratio for Abiraterone® was not measurable due to very low percent recovery of the compound. It was quite noteworthy that the high permeability compounds had smaller efflux ratios and the low permeability compounds had higher efflux ratios. Rat and human plasma protein binding was high for all compounds (≥90% bound) except Canertinib® which exhibited moderate (86%) plasma protein binding in human (Table **4**).

In vivo rodent studies constitute an important stage gate for progressing compounds with good drug-like properties through the discovery pipeline. So, we evaluated the 10 covalent inhibitors in rat pharmacokinetic studies. In contrast to small molecule kinase inhibitors approved by the FDA over the past decade (O'Brien and Moghaddam, 2013), the majority of which show rat plasma clearances of less than or equal to hepatic blood flow, the rat plasma clearance of these 10 covalent inhibitors showed a very broad range. Neratinib® and Vigabatrin® had the lowest plasma clearance of 10 and 11 mL/min/kg, respectively. The other end of the spectrum featured Boceprevir® with a plasma clearance of 353 mL/min/kg (Table **5**). A very broad range was also observed with the volume of distribution at steady state (V_{ss}) for these compounds. Neratinib®, Telaprevir®, and Vigabatrin® had the lowest V_{ss} which was close to total body water in the rat (1.1 L/kg, 0.9 L/kg, and 0.7 L/kg, respectively). Carfilzomib® on

Table 4. Apical to basolateral permeability (A→B) and efflux potential (B→A/A→B) of the 10 covalent inhibitors across Caco-2 cells. Binding of the 10 compounds to plasma proteins in rat and human reported as percent bound following equilibration of the incubation mixture for 4 hours.

Compound	*Caco-2 Assay*		*Plasma Protein Binding*	
	P_{app} A→B (X 10^{-6} cm/s)	Efflux Ratio (B→A / A→B)	Rat (% Bound)	Human (% Bound)
Abiraterone®	NA	NA	100	100
Afatinib®	1.8	30	100	98
Boceprevir®	1.0	20	98	98
Canertinib®	5.8	2.0	NA	86
Carfilzomib®	0.6	18	100	99
Dacomitinib®	12	2.0	99	98
Ibrutinib®	31	0.4	100	99
Neratinib®	0.9	6.0	NA	100
Telaprevir®	1.5	12	99	90
Vigabatrin®	2.4	0.2	NA	90

Table 5. Plasma pharmacokinetic parameters for the 10 covalent inhibitors following a bolus intravenous dose of 2 mg/kg to male CD-IGS rats.

Compound	CL (mL/min/kg)	Vss (L/kg)	MRT (hr)	B/P Ratio
Abiraterone®	144	22.6	2.5	1.2
Afatinib®	182	35.7	3.2	1.5
Boceprevir®	353	11.0	0.52	1.3
Canertinib®	53.7	7.27	2.3	NA
Carfilzomib®	297	115	6.4	0.80
Dacomitinib®	69.2	24.7	6.0	2.9
Ibrutinib®	41.6	18.8	7.4	0.62
Neratinib®	9.72	1.13	2.0	0.59
Telaprevir®	21.2	0.882	0.69	0.95
Vigabatrin®	11.1	0.666	1.0	0.75

Table 6. Plasma pharmacokinetic parameters for the 10 covalent inhibitors following oral administration of 10 mg/kg dose as a suspension/solution to male CD-IGS rats.

Compound	C_{max} (μM)	T_{max} (hr)	$AUC_{0\text{-}inf}$ (μM*hr)	F%
Abiraterone®	0.0296	2.0	0.106	3.1
Afatinib®	0.0856	2.7	0.586	31
Boceprevir®	0.134	0.33	0.267	29
Canertinib®	0.255	2.3	0.878	14
Carfilzomib®	NC	NC	NC	NC
Dacomitinib®	0.282	4.7	4.30	69
Ibrutinib®	0.627	0.75	1.49	16
Neratinib®	0.634	4.0	5.13	16
Telaprevir®	0.00547	0.83	0.0273	0.23
Vigabatrin®	76.6	0.42	106	91

the other hand exhibited the highest V_{ss} (115 L/kg) that was ~ 164 fold total body water. Several others including Abiraterone®, Afatinib®, Ibrutinib®, and Dacomitinib® also exhibited high V_{ss} in the 19-36 L/kg range. These high V_{ss} could not be readily explained with the compounds blood:plasma ratios which were modest and ranged from 0.6-2.9 (Table **5**). The mean residence times (MRT) in plasma were short (<1 hr) for the hepatitis C drugs Boceprevir® and Telaprevir® and moderate to high (1-7.5 hr) for the remaining 8 compounds.

The plasma exposures (C_{max} and AUC) following oral administration at 10 mg/kg were minimal for 9 out of the 10 compounds (Table **6**). Vigabatrin had excellent plasma exposure with a mean C_{max} of 76 μM and mean AUC of 106 μM·hr, resulting in oral bioavailability of 91%. The oral exposure for Carfilzomib® was not calculable since the plasma concentrations at all time points were below the limit of quantitation (BLQ < 2 nM). The T_{max} values were in the 0.3-4.7 hr range, but most of the compounds had rapid absorption.

DISCUSSION

We subjected 10 late stage covalent inhibitors, which have progressed beyond early clinical safety and efficacy criteria, to our *in vitro* and *in vivo* discovery ADME screening assays to understand if they followed a particular trend with respect to discovery ADME selection criteria. The 10 inhibitors represent a broad variety of chemical templates and are designed to inhibit targets such as cytochrome P450 17A1 (steroid 17-alpha monooxygenase; CYP17A1), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (Her2), 20S proteosome, Bruton's tyrosine kinase (Btk), and the NS3 protease of hepatitis C virus (HCV protease) [10-19]. These targets have long rates of *de novo* synthesis, making them good candidates for inhibition using targeted covalent inhibitors. Because these compounds have passed rigorous testing in preclinical assays resulting in their advancement into the clinic and the market, we believe they served as good models for discovery of successful covalent inhibitors. It was, therefore, of interest to us to investigate their preclinical virtues in order to set up stage gate criteria for discovery of future covalent inhibitor drugs. In doing so, we wanted to determine whether the same workflow and assay criterion that has been successfully applied to the reversible inhibitors [20] is applicable for screening covalent inhibitors, too. The analysis of our results, as presented in the ensuing discussion, argues that targeted covalent inhibitors do not always conform to the criteria set forth for reversible inhibitors.

The 3 key screening assays used as stage gate criteria in early discovery to select compounds for assessment of *in vivo* pharmacokinetic characteristics include metabolic stability, blood stability, and Caco-2 permeability/efflux ratios.

In vitro metabolic stability determined by rat and human liver microsomes, S9 fractions, or hepatocytes serve as the first ADME gate for evaluating new chemical entities. Typically, compounds that have low metabolic stability are not good candidates for advancement since this parameter is often indicative of rapid clearance from the systemic circulation due to first pass effect. In our organization, a S9 stability value of less than 70% parent remaining after 1 hour of incubation at 3 μM substrate concentration has been determined to be unfavorable for reversible inhibitors [20]. For covalent inhibitors, we shortened the incubation time to 30 minutes because the 60 minute incubation rendered all of them unstable. Even with the shortened incubation time of 30 minutes, Ibrutinib® and Carfilzomib® had rat liver S9 stability of <10% and failed this screening criteria. Similarly, Abiraterone®, Ibrutinib® and Carfilzomib® had human liver S9 stability of ≤ 25% and would not have progressed further using the screening criteria for reversible inhibitors. Since these are highly successful clinical inhibitors, our data suggested that liver S9 stability was not a viable stage gate criterion for screening covalent inhibitors.

On the other hand, most of the compounds had good rat and human whole blood stability. This bodes well for the compounds especially because poor whole blood stability could signal metabolic instability in whole blood and/or indiscriminate bonding to blood/plasma proteins, the latter being of significant concern with regards to toxicological consequences and idiosyncratic adverse drug reactions [1]. Therefore, good whole blood stability (≥ 50% stable following 60 min incubation, based on data presented in Table **2**, can potentially be used as a stage gate for advancing discovery stage covalent inhibitors.

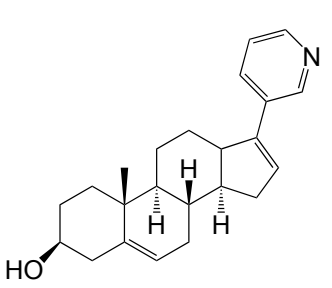
Caco-2 permeability and efflux ratios were not effective stage gates either because according to our internal guidelines [20] at least 6 out of the 10 compounds had poor Caco-2 permeability (A→B P_{app} < 8X 10^{-6} cm/s) and at least 4 compounds had high efflux ratio (B→A/A→B > 8) (Table **4**). So, it appears that of these three screening assays, the whole blood stability assay might be the only one that could be potentially useful as a stage gate for advancing covalent inhibitors.

Two other *in vitro* assays typically included as screens in drug discovery are the Cytochrome P450 inhibition and plasma protein binding. Cytochrome P450 inhibition is one of the key screening activities in drug discovery to ensure that significant inhibitors of P450 enzymes are not advanced into successive studies. Inhibition of these drug metabolizing enzymes gives rise to significant drug-drug interaction potential in the clinic and is a major liability in a poly-pharmacy environment [21]. All the compounds were weak to moderate inhibitors of these CYP enzymes as demonstrated by <80 % inhibition following co-incubation with 5 μM compound (Table **3**). However, a majority of the compounds exhibited time-dependent inhibition of at least one enzyme following pre-incubation for 30 minutes. Dacomitinib® was the most significant time-dependent inhibitor and resulted in ~30-fold shift in the % inhibition of CYP2C8 at 5 μM concentration followed by Telaprevir® (~14-fold shift against CYP2C8), Boceprevir® (~9-fold shift against CYP1A2), and Canertinib® (~8-fold shift against CYP3A4/5). As with any other drug discovery program, weak or non-inhibitors of cytochrome P450 enzymes along with a lack of time-dependent inhibition are always desired. While elimination of CYP inhibition should always be the goal, our data point to the fact that clinically successful molecules may have paved the way for more tolerance in assessing covalent inhibitors particularly when addressing unmet clinical needs or life-threatening diseases. We recommend that in the case of covalent inhibitors with propensity for CYP inhibition and/or time-dependent inhibition, a rigorous attempt be made to demonstrate a lack of non-specific or promiscuous bonding to circulating proteins to address the obvious safety concerns.

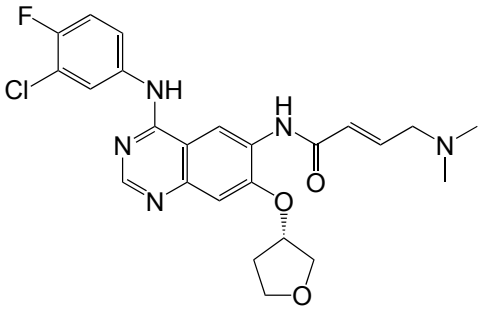
Rat and human plasma protein binding did not prove to be an effective stage gate criterion either since the majority of the compounds were highly protein bound in rat and/or human plasma (Table **4**).

Next, we evaluated the pharmacokinetic parameters of covalent inhibitors to see if any useful trends could be deduced to help in selecting discovery compounds for further pharmacological and toxicological studies. We have previously reported that in our organization, compounds with plasma clearance ≤ 43 mL/min/kg in rats have the best chance of yielding oral exposures worthy of pharmacological evaluations [20]. By that standard, several of the 10 covalent inhibitors are extremely rapidly cleared compounds. These include Abiraterone®, Afatinib®, Boceprevir®, Carfilzomib®. Dacomitinib®, and Canertinib®. Except Carfilzomib®, the other compounds appear to be relatively metabolically stable in the rat liver S9 assay. However, it is interesting to note that most of these rapidly cleared compounds also exhibit very large volumes of distribution suggesting that the rapid clearance may be a combination of metabolic clearance and rapid partitioning into tissue depots and perhaps bonding to their cellular target. Such depots are most likely the tissue compartments since the blood partitioning of these compounds appears to be minimal. Also, the non-specific tissue bonding hypothesis appears to have little or no toxicological consequences in these particular cases since these compounds have continued into late stage development.

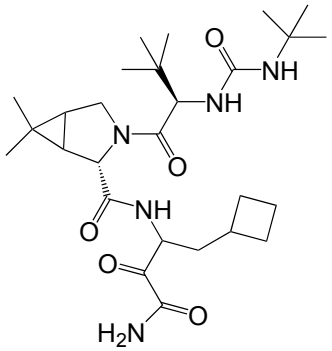
The absolute plasma exposure of all compounds (except Vigabatrin®) following oral administration of a 10 mg/kg dose was low to moderate with mean C_{max} ≤ 0.63 μM and mean $AUC_{0\text{-}inf}$ in the 0.03-5.13 μM·hr range. Vigabatrin® stood out with excellent plasma exposure resulting in a mean C_{max} of 77 μM, $AUC_{0\text{-}inf}$ of 106 μM·hr, and oral bioavailability of 91%. Even though the oral bioavailability for some of the compounds was acceptable (Boceprevir® = 29%, Afatinib® = 31%, and Dacomitinib® = 69%), the absolute plasma exposure was poor-to-moderate for these compounds in our view [20]. As > 50% of these compounds would be screened out based on these results, the plasma clearance and oral exposure screen do not provide effective stage gates in isolation. However, the exposure data in the context of a threshold value (for example, IC_{50} or EC_{90} based on target *de novo* synthesis half-lives and cellular pharmacology data could be very effective in screening compounds designed for covalent inhibition. The rationale behind this is that a long rate of *de novo* synthesis or resynthesis half-life is one of the preferred attributes for a therapeutic target selected for covalent inhibition. Since covalent inhibitors completely nullify the activity of the target by covalently binding to the catalytically critical target site, *de novo* synthesis is the only mechanism by which the target can regain its activity. Therefore, a longer rate of *de novo* synthesis of the target bodes well for longer duration of action of a covalent inhibitor with a poor to modest pharmacokinetic profile (rapid clearance and modest absolute exposure and oral bioavailability). In contrast, in the case of a reversible inhibitor the long duration of action is attained *via* sustained inhibition of the target due to long and adequate exposure of the target to the reversible inhibitor. This requires inhibitors with low clearance and "good" pharmacokinetic profiles. Covalent inhibition can happen within a short period of time (typically 30 min-1 hour, internal communications) and therefore sustained plasma exposure may not be required for favorable efficacy. As a result, compounds with relatively poor pharmacokinetics, or rather "adequate PK", may be advanced through the discovery value chain. "Adequate PK" for a covalent inhibitor can be defined as provision of high enough circulating concentrations, in context of an inhibition



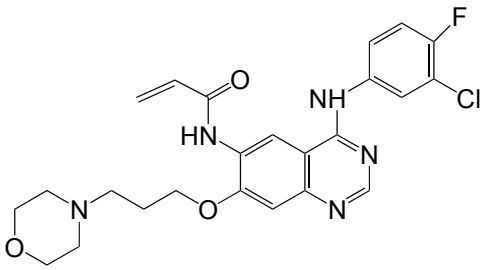
Abiraterone



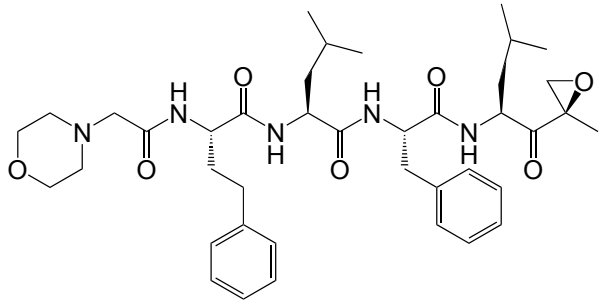
Afatinib



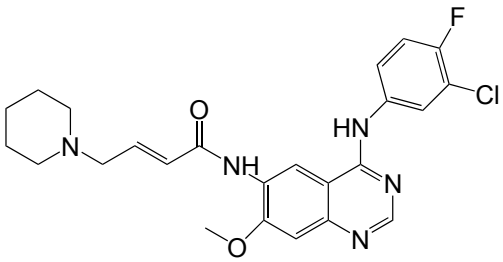
Boceprevir



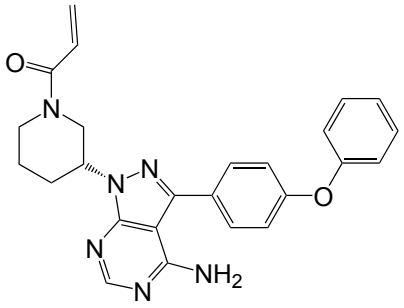
Canertinib



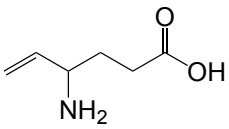
Carfilzomib



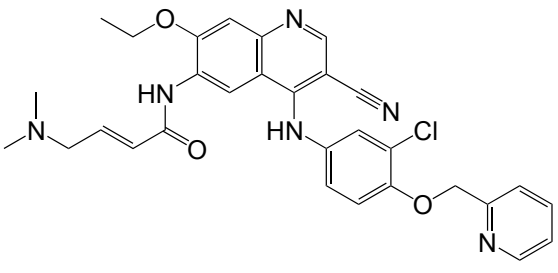
Dacomitinib



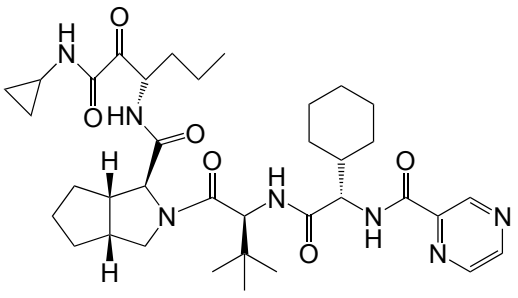
Ibrutinib



Vigabatrin



Neratinib



Telaprevir

Fig. (1). Chemical structures of the 10 covalent inhibitors.

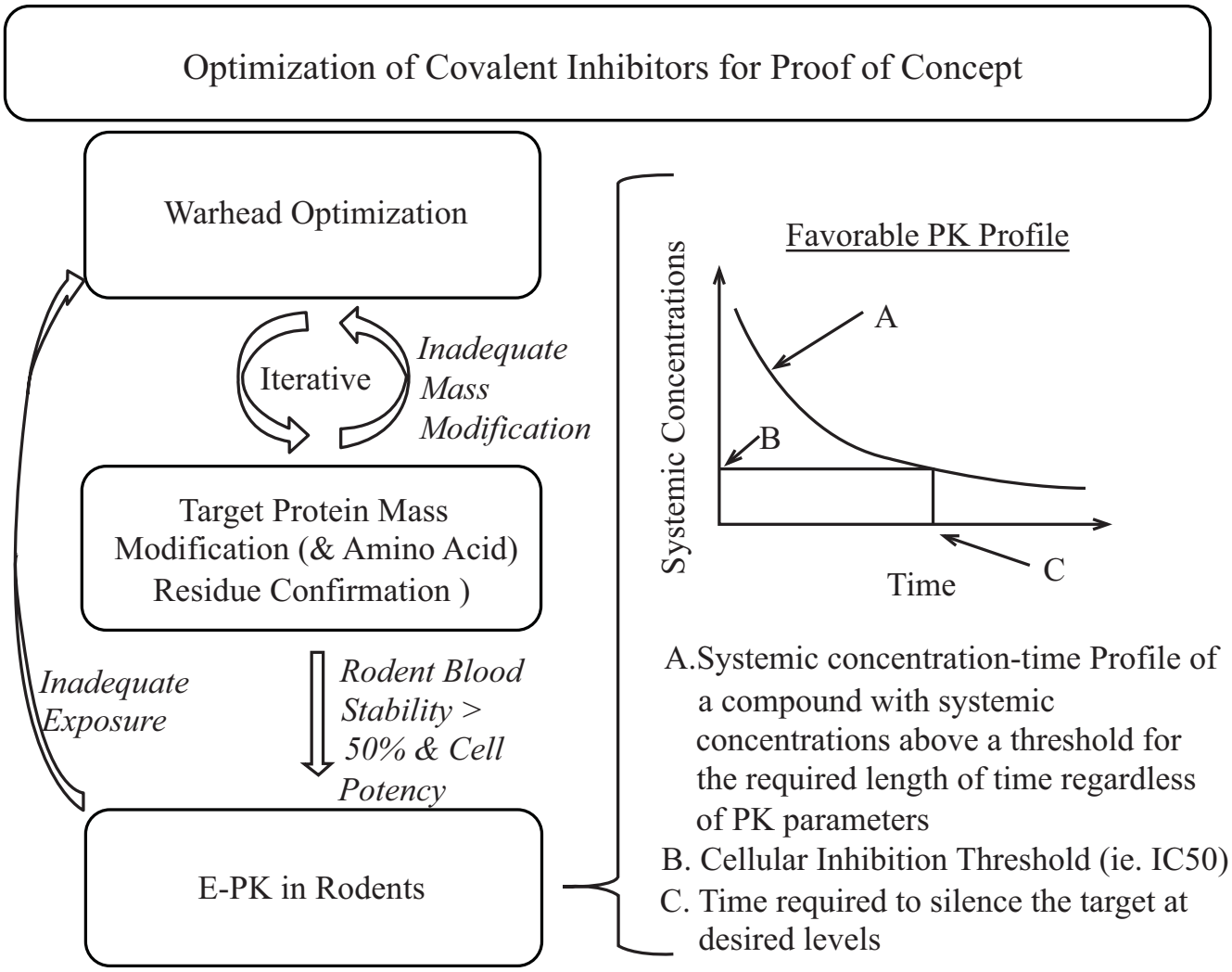


Fig. (2). In this stage, the goal is to analog potent reversible inhibitors with "warheads" to obtain compounds with acceptable target protein mass modification. Once these covalent inhibitors are identified, those with rodent blood stability > 50% and favorable cellular potency are recommended to enter "exploratory PK" (E-PK). E-PK refers to truncated PK studies, iv, ip, or po, in mouse or rats with multiple samplings over a short period of time adequate for silencing the target protein at an adequate level (ie. 2 hours). The compounds regarded as eligible for E-PK are potent and exhibit acceptable levels of target protein mass modification. The purpose of an E-PK study is to quickly identify the "Good enough" compounds that can enter pharmacology to demonstrate proof of concept. At this stage long term considerations, such as metabolic stability, higher species PK, and toxicology and are not of great concerns. However, if after a reasonable effort, adequate *in vivo* exposures were not achieved, a limited set of diagnostic DMPK studies can be deployed to assess the cause of poor exposure to allow optimization.

threshold value, for a long enough time to covalently bond and silence the target protein, therefore resulting in a pharmacologically meaningful effect. In order to understand what "adequate" PK for irreversibly inhibiting different targets may be, one needs to answer two questions: 1) Approximately what level of target silencing will yield the desired response, and 2) How long does it take to reach that level of silencing. This argues that compounds may be selected to enter full pharmacological evaluations based on their "adequate PK" and acceptable cellular pharmacology. Indeed, the uncoupling of pharmacokinetics and pharmacodynamics has been touted as a significant advantage for the development of targeted covalent inhibitors [2].

These findings clearly demonstrate that traditional ADME screening paradigms cannot be effective in screening for covalent inhibitors as demonstrated by the ADME data generated on 10 successful covalent inhibitors. The segregation of low and high potential compounds based on ADME data would have resulted in multiple false negatives - a high proportion of good compounds would have been discarded. Covalent inhibitors need to be looked at in a different light and non-traditional selection paradigms need to be implemented in order to effectively screen for compounds with high potential. These non-traditional compound selection considerations include:

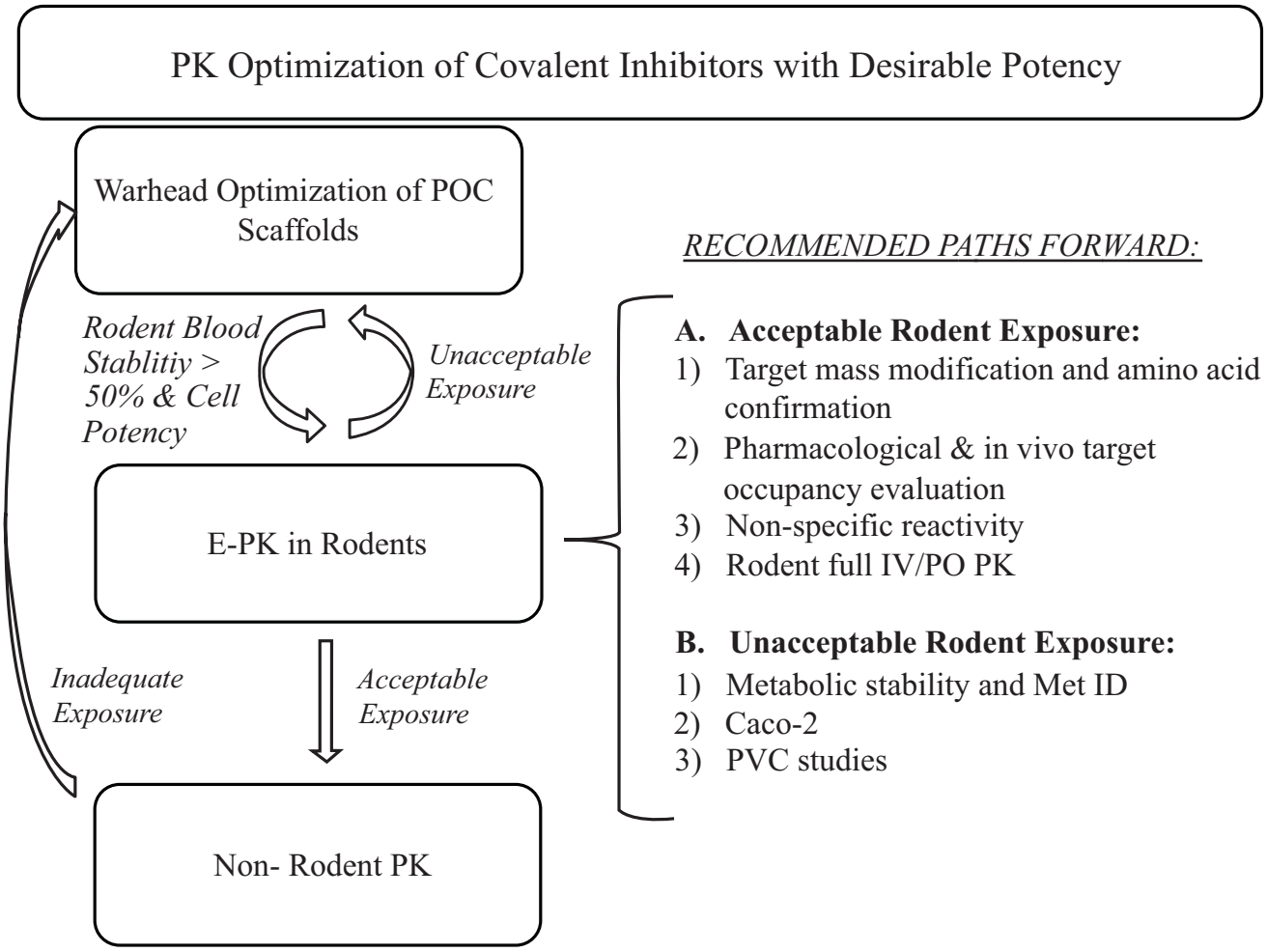


Fig. (3). At this stage, the goal is to obtain compounds worthy of development. Only compounds with desirable potency and blood stability should be advanced to E-PK. A. Target mass modification (including amino acid) and *in vivo* occupancy should be confirmed for compounds with acceptable rodent exposure. It is further recommend to evaluate such compounds for non-specific reactivity toward blood (rat, dog, monkey, and human), glutathione (with and without metabolic activation), and CYP's. In order to construct a shortlist of high potential compounds, it is recommended to evaluate IV and/or PO PK parameters in full PK studies to advance compounds with the best probability of success in non-rodent PK/toxicology and clinical development. B. In order to rescue chemotypes with poor PK, one may deploy diagnostic ADME assays. These can include stability assays to determine metabolically labile sites, Caco-2 permeability/efflux and portal vein cannulated (PVC) rodent studies to optimize oral bioavailability.

1. ***In vitro mass modification assay:*** An assay to determine the extent of modification of the target by the covalent inhibitor. A 100% mass modification implies complete covalent bonding, and therefore total inhibition. The extent of mass modification can be determined using mass spectrometry and the desired level of mass modification/inhibition can be determined for each target using *in vitro* cell washout assays.
2. ***Target covalent binding site confirmation by amino acid sequencing:*** An assay to determine the exact location and nature of covalent modification. This clarifies whether the correct amino acid residue has been modified by the compound of interest. This can be a measure of the specificity of the covalent inhibitor.
3. ***Exploratory pharmacokinetics:*** The C_{max} and exposure levels should be evaluated in context of the target turnover rate or *de novo* synthesis half-life, location of the target (circulating cells vs. deep tissue), and cellular potency of the compound. If the therapeutic target has a long half-life and is readily accessible, then "adequate" pharmacokinetic profiles necessary to achieve sufficient concentrations above the cellular inhibition thresholds (ie. IC_{50}) for a long enough time (typically 30min-1 hour) to silence the target may be acceptable. In such cases, an exploratory 2 hour PK study should suffice in identification of promising compounds. However, if the target protein has a short resynthesis rate and/or is not readily accessible, then prolonged exposure may be needed to silence the target (for once a day dosing). This will

require compounds with slow clearance, long half-life, and viable bioavailability.

We have combined all this information to recommend a workflow for screening covalent inhibitors. This is shown in Figs. **2** and **3**.

CONCLUSION

Our analysis indicates that ADME criteria applied to the selection of reversible inhibitors cannot be used in the traditional sense to discover and develop covalent inhibitors. Non-traditional assays such as mass modification, amino acid sequencing, kinetics of enzyme inhibition, and target occupancy, and target turnover in combination with "adequate" PK profiles are the critical considerations for progression of such compounds. However, it is important to indicate that discovery teams should not completely ignore parameters such as clearance and solubility as these will facilitate the conduct of higher species PK and toxicology work and always improve development potential of drugs.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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Declared none.

LIST OF ABBREVIATIONS

ADME	=	Absorption, distribution, metabolism, excretion
AUC	=	Area under the concentration-time curve
CYP	=	Cytochrome P450
Caco-2	=	Carcinoma of the colon
CL	=	Clearance
E-PK	=	Exploratory 2 hour truncated pharmacokinetic study
F	=	Bioavailability
MRT	=	Mean residence time
NA	=	Not available
NC	=	Not calculable
Vss	=	Volume of distribution at steady state

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