Electronic Supplementary Information

Physicochemistry of cereblon modulating drugs determines pharmacokinetics and disposition

Nikki R. Kong, Hu Liu, Jianwei Che and Lyn H. Jones*

Center for Protein Degradation, Dana-Farber Cancer Institute, 360 Longwood Avenue, Boston, 02215, USA

Contents

Hazards

All compounds (thalidomide, EM-12, pomalidomide, lenalidomide and iberdomide) are teratogenicity risks. Follow additional guidance in safety data sheets.

Molecular orbital and desolvation energy calculations

Jaguar (Schrödinger release 2021-1) was used to assess electronic structures.[1] Density functional theory (DFT) based *ab initio* calculations with B3LYP-D3 functional and the 6-31G**++ basis set were used. Fully analytical instead of pseudopotential was chosen with maximum grid density. Implicit water was modeled by the Poisson Boltzmann Finite element (PBF) method. The chirality for all molecules was defined as S.

Chrom LogD

Chromatographic LogD measurements used a protocol similar to that previously reported[2] – further details are provided below.

Sample preparation

All compounds were analyzed in duplicate as a DMSO solution with a concentration of 1 mg/mL.

Standard solutions

Solutions containing the following neutral compounds with well-established LogD values in the literature were prepared in DMSO, except for hexachlorobenzene which was 90% DMSO 10% $CHCl_3$. Standard LogDs: methylphenyl sulfone (0.5), acetophenone (1.58), thymol (3.21), biphenyl (3.76), hexachlorobenzene (5.69), 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethene (6.18).

LC-UV analysis

This was carried out using an Agilent 1200 HPLC system with a variable wavelength UV Detector. The mobile phase consisted of: (A) 95% [10 mM NaH_2PO_4 adjusted to pH 7.40 with 2 M NaOH] + 5% MeCN and (B) 25% [10 mM NaH_2PO_4 adjusted to pH 7.40 with 2 M NaOH] and 75% MeCN. The flow rate was 1.8 mL/min. The column was a Phenomenex Luna C18 (2), 4.6 mm x 50 mm, 3μm) maintained at a temperature of 37˚C. Injection volume was 2 μL for samples (test) and 3 μL for standards. Detection wavelength was 210 nm.

The standard solutions were analyzed both before and after test sample analysis. The retention time of each component was measured, and the mean value calculated. The published values of LogD for the standards above were plotted versus RT ($r^2 = 0.9961$).

Calculation of compound LogD

Test compound solutions were analyzed by LC-UV. The retention time was measured and the LogD of the test compound was then calculated using the calibration line generated above.

Kinetic Solubility

A stock solution of the compound (10 mM in DMSO) was added to aqueous PBS buffer to achieve a final concentration of 200 µM. The mixture was shaken at room temperature for 4 hours, then allowed to stand for 30 minutes at room temperature before filtration. The filtrate was diluted with DMSO before LC-MS/MS analysis. The MS detection was performed using a SCIEX API 4000 Q trap instrument. Each compound was analyzed by reversed phase HPLC using a Kinetex 2.6µ C18 100Å column (3.0 mm x 30 mm, Phenomenex). The solubility of the test compound was determined based on the calculated concentration.

Plasma stability

Plasma and test compound (100 µM in DMSO) were added to the individual wells of a 96-well microtiter plate to provide a final compound concentration of 1 µM. The plate was incubated at 37 ˚C with gentle agitation. During the incubation, aliquots were withdrawn at 0, 15, 30, 60, 120 and 240 minute time points and quenching solution (25/50 ng/mL terfenadine/tolbutamide in ACN/MeOH (1:1, v/v)) was added. After mixing, the quenched aliquots were centrifuged, and the supernatant withdrawn for analysis by LC-MS/MS. The MS detection was performed using a SCIEX API 4000 Q trap instrument. Each compound was analyzed by reverse phase HPLC using a Kinetex 2.6µ C18 100Å column (3.0 mm x 30 mm, Phenomenex). The slope of the ln(%remaining) *vs.* time point line was used to calculate $t_{1/2}$ according to the following formula:

Half-life ($t_{1/2}$) = – ln(2) / Slope

Human liver microsomal stability

Microsome stability was evaluated by incubating test compound (1 µM final concentration) with 1 mg/mL hepatic microsomes at pH 7.4. The reaction was initiated by adding NADPH (1 mM final concentration). The mixtures were vortexed gently at 37 ˚C. At each time point of 0, 5, 15, 30 and 60 minutes, an aliquot was removed from

each tube. Terfenadine/tolbutamide in ACN/MeOH (1:1, v/v) was added to quench and precipitate the microsomal incubations. Samples were capped and vigorously vortexed and then centrifuged at 4 ˚C. An aliquot of each supernatant was transferred for LC-MS/MSC analysis. The MS detection was performed using a SCIEX API 4000 Q trap instrument. Each compound was analyzed by reverse phase HPLC using a Kinetex 2.6µ C18 100Å column (3.0 mm x 30 mm, Phenomenex).

Caco-2 permeability

The Caco-2 cell monolayers were used for transport studies 21 days post-seeding after measuring the TEER values (>600 Ohms/cm^2). The donor working solution was prepared by diluting DMSO stock of test articles with transport media to 10 µM. For A → B directional permeability, the donor test article working solution was added to the apical (A) compartment, and the transport media added to the basolateral (B) compartment. For B → A directional permeability, the test article working solution was added to the basolateral (B) compartment, and transport media added to the apical (A) compartment. The cells were incubated in a humidified atmosphere of 5% CO_2 at 37 ˚C for 90 minutes. At the end of the incubation, samples were taken from both donor and receiver compartments and transferred into 96-well assay plates containing internal standard solution (IS) in each well. After centrifugation, the supernatant solutions were analyzed by LC-MS/MS. The MS detection was performed using a Sciex API 4000 instrument. Each compound was analyzed by reversed phase HPLC. The parameters P_{app} (apparent permeability) and efflux ratio were calculated as follows:

$P_{app} = (dQ/dt) \times (1/C_0) \times (1/A)$

Efflux ratio = P_{app} [B → A] / P_{app} [A → B]

where dQ/dt is the permeability rate, C_0 is the initial concentration in the donor compartment, and A is the surface area of the cell monolayer (0.33 cm^2). The P_{app} value is a rate measured in cm/s.

Drug accumulation in cells

Overview

The aim of this protocol is to measure the cellular and extracellular concentration of dosed drugs in MOLT4 cells. The procedure used was similar to that previously reported.[3, 4] Compounds were screened in duplicate at 0.5 µM concentration at 15 minutes, 1 hr, 2 hr, 4 hr and 8 hr timepoints using 500,000 cells/well.

Day 1

Prepare 5 2 mL deep-well blocks for the time course experiment. Prepare Compound Plate in clear round bottomed 96-well plate at WAC (working assay concentration) of 125 μM: 2 μL of 10 mM Compound DMSO stock + 158 μL of DMSO (compounds are analyzed in duplicate). Transfer 2 μL of Compound Plate to 96 deep well plates. Harvest cells, resuspend at 1 x 10^6 cells per mL. Add 500 μL of cells (1 x 10^6 cells/mL) to each deep well plate well containing 2 μL of compound. Incubate for desired time.

At each timepoint: spin down cells (300 *g*, 3 minutes); remove 100 μL of supernatant, transfer to Greiner round bottom 96-well plate, seal with adhesive foil and store at -80 ˚C; wash remaining cells with cold PBS using plate washer (link function); spin cells (300 *g*, 3 min); remove PBS using aspirate "10" function on plate washer. Seal plate with adhesive foil and store at -80 ˚C. Prepare 5-10 mln cell pellet in a 40 mL Falcon tube for analytical matrix matching.

Day2

Media Processing

Defrost media and mix well. Take 20 μL of media and add it to 180 μL of pure MeCN. Mix well and centrifuge at 3000 *g* for 3 minutes (1 in 10 dilution). Dilute the media further for LC-MS analysis by taking 100 μL of supernatant and adding 100 μL of pure water. Mix well (1 in 2 dilution). Prepare matrix matched media solution: 2 mL [RPMI + 10% FBS] + 18 mL MeCN + 20 mL 50:50 MeCN/H_2O. Analyze the samples on UPLC-MS/MS.

Cell Pellet Processing

Defrost pellets and add 200 μL of pure MeCN to each well. Sonicate for 10 minutes, then centrifuge at 3000 *g* for 3 minutes. Dilute the supernatant for LC-MS analysis: 50 μL supernatant + 450 μL 40:60 MeCN/H_2O (1 in 20 dilution).

Day 3

MS data were processed using TargetLynx and Microsoft Excel to provide the intracellular and extracellular concentrations of the test compounds. The ratio of these values provides Kp (Table 1).

Intracellular unbound fraction

The protocol was identical to that described previously.[3] Figure 1 is a plot of the intracellular unbound fraction of the IMiDs and CC-220 in MOLT4 cells following 4 hours incubation (lopinavir, atorvastatin and caffeine are controls).

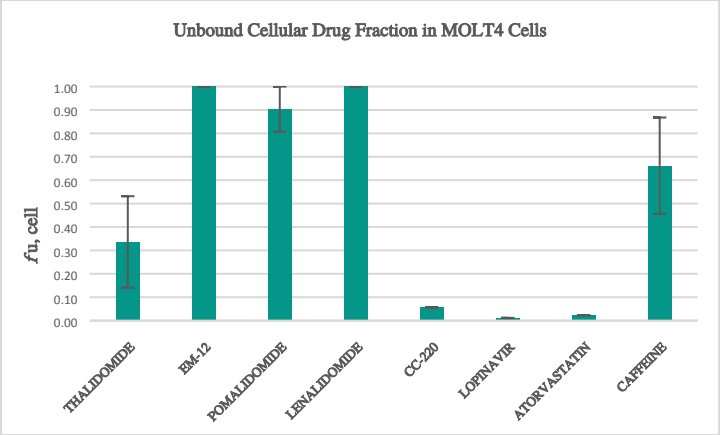

Figure S1. Intracellular unbound fractions of IMiD drugs and candidate CC-220 (iberdomide).

IKZF1 HiBiT assay

The protocol used to determine IKZF1 degradation potency was the same as that previously reported (here in MOLT4 cells rather than Jurkat).[5]

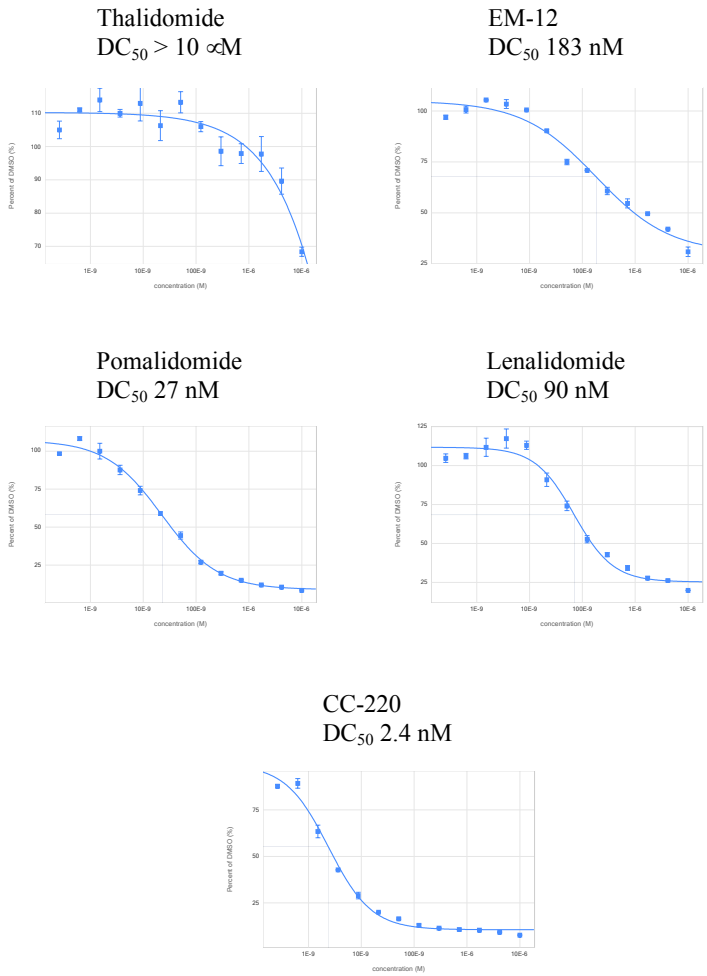

Figure S2. IKZF1 potency of IMiDs and CC-220 in the HiBiT assay system.

References

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