Determination of in Vivo Enzyme Occupancy Utilizing Inhibitor Dissociation Kinetics

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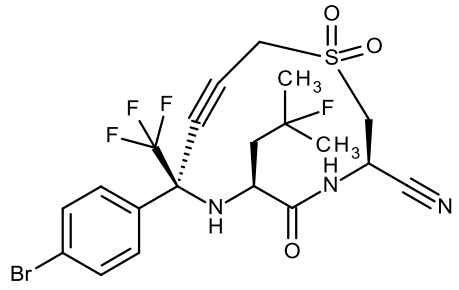
SUPPORTING INFORMATION

MATERIALS AND METHODS

Reagents

Rabbit Cathepsin K[1] was expressed and purified by Biotechnology Research Institute using standard techniques. The enzyme was activated by incubation for 18 h at 4^{o}C in 50 mM NaOAc, pH 4.1, 1.0 M NaCl, 2.0 mM EDTA, and 5.0 mM DTT. Enzyme concentration was determined by active site titration with compound 1 [Patent WO08116302] and fitting to Equation 1.[2]

$$RFU = v_0(1-(((((E_0+I+(K_{i\text{-}app}))-(((E_0+I+(K_{i\text{-}app}))\text{^}2)-4E_0I)\text{^}0.5))/(2E_0))))+bkgnd \quad (1)$$



Compound **1**

CBZ-leucine-argine-amimomethylcoumarin (CBZ-leu-arg-AMC, Bachem I-1960) was dissolved to 25 mM in 100% DMSO and aliquots were stored at -80^{o}C until use. Ten mM

solutions of MK-0674[3] were prepared from solid in 100% DMSO and aliquots were stored at -80^{o}C until use.

In Vitro Validation

Reactions were run in 50 mM MES buffer at pH 5.5 with 2.5 mM EDTA, 2.5 mM DTT, and 0.01% P-20 detergent. A set of pre-incubation reactions were prepared with rabbit Cat K and a serial dilution of inhibitor MK-0674. An aliquot of the pre-incubation reaction was assayed by the addition of 25X substrate ('direct assay'). A second aliquot was assayed by 50X dilution into substrate ('jump-dilution assay'). The pre-incubation reactions contained 2, 1, 0.5 or 0.25 nM enzyme and a 1.66X, 14 point serial dilution from 21.6 nM to 0.028 nM. Additional reactions with 100 nM inhibitor and no inhibitor were also run. Serial dilutions of the inhibitor were made in 100% DMSO. The pre-incubation reactions contained one of four concentrations of enzyme (see below) and a 1.66X, 14 point serial dilution of inhibitor, and were incubated at RT for 30 to 60 min. For the direct assay 48 µL of the pre-incubation reaction was added with mixing to 2 µL of 25X substrate in 100% DMSO yielding final concentrations of 2.0, 1.0, 0.50 or 0.25 nM enzyme, 8.0 µM substrate, and a 1.66X, 14 point serial dilution of inhibitor from 21.6 nM to 0.028 nM, 10% total DMSO. Additional reactions with 100 nM inhibitor and no inhibitor were also run. For the jump-dilution reaction 1 µL of the pre-incubation reaction was added with mixing to 49 µL of substrate buffer yielding final concentrations of 40, 20, 10 or 5 pM enzyme, 8.0 µM substrate, and inhibitor serial dilution from 432 pM to 0.56 pM, 10% total DMSO. Substrate hydrolysis was monitored by following the fluorescence increase from the liberated coumarin on a Perkin Elmer Envision Fluorometer in a black 384 well Optiplate; X380 M460 filters with a 400 nm dichroic mirror. $K_{i\text{-}app}$ for the direct assay was determined by fitting the initial slope (0-30s) vs. [MK-0674] to eq 1 for tight-binding inhibitors.[4] E_0 was fixed based on the previous active-site titration since E_0 is poorly determined when E_0 is at or below $K_{i\text{-}app}$.[2] For the jump-dilution experiment progress curves were fit to eq 2[4] (eq 1, main text) to determine v_i, k_{obs}, and v_s. v_i vs.[MK-0674] was fit to eq 1 to determine $K_{i\text{-}app}$.

$$[P] = v_s t + ((v_i - v_s)/k_{obs})(1 - \exp(-k_{obs}t)) + bkgnd \quad (2)$$

For experiments run at 8^{o}C, a BMG FluoStar Plate-reading fluorometer was placed in a cold room. Excitation and emission wavelengths were set to 380 and 460 nm, respectively. Pre-dilution plates were prepared with cold buffer; enzyme and MK-0674 were incubated prior to dilution for 1 h prior to 64X dilution into 8.0 µM substrate and immediate assay in cold buffer.

Ex Vivo Assays

All studies were reviewed and approved by the Institutional Animal Care and Use Committee prior to study start. Animals were allowed free access to food and water. Six-month old, ovariectomized New Zealand White rabbits (Covance, Denver PA) were dosed orally with vehicle consisting of water, 0.5% methocel and 0.25% SDS or a Cat K inhibitor MK-0674 at doses ranging from 0.01 mg/kg to 10 mg/kg (n = 2-10/dose group). Twenty-four hours after dosing, animals were euthanized. The right and left distal femurs were excised and the distal portions of the bones were removed and then flushed with ice cold PBS. Bone tissue was flash frozen in liquid nitrogen. The frozen tissue was maintained on dry ice and pulverized to a powder with a Cellcrusher (Cellcrusher Limited, Cork Ireland). Cellcrushers were cleaned with 95% ethanol in between samples. Individual bone powder samples were transferred to 1.5 mL LoBind vials (Catalog #022431081, Eppendorf International) and stored at -80^{o}C prior to assay.

Assay plates, the BMG Plate-reader and other equipment were equilibrated at least 24 h in a cold room. Buffers were kept on ice. Tissue samples were kept on dry ice and rapidly weighed into 15 mL tubes for processing. CS buffer contained 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Na_2 pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4 along with protease inhibitors 40 µg/mL Bestatin, 1 mg/mL Pefabloc, and 0.7 µg/mL Pepstatin. Approximately 20 mg of frozen tissue was re-suspended in 250 µL ice cold CS buffer per mg of tissue and immediately vortexed for 15 s. After two 1:10 serial dilutions into assay buffer, the sample was assayed by 1:1 dilution into16 µM substrate for a final dilution of

1:50 000. Data was recorded for 250 *x* 14 s followed by 250 *x* 40 s (or more frequently, e.g. 250 *x* 6 s) on the FluoStar fluorometer to optimize data collection for both the dissociation and steady-state phases of the reaction. The background (*e.g.* substrate hydrolytic activity not derived from Cat K) reaction was determined for each sample by measuring the rate in the presence of 100 nM final MK-0674. The time courses from the Cat K-dependent reaction were fit to eq 2 with the added constraint that k_{obs} <0.035 min^{-1} ($t_{1/2}$ >20 min). The slope of the corresponding background reaction was subtracted to obtain the reported values of v_i and v_s. Six samples (12 assays), from addition of CS buffer to frozen tissue to start of the kinetic read, were processed in approximately 2 minutes.

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