JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Determination of in Vivo Enzyme Occupancy Utilizing Inhibitor Dissociation Kinetics

Dennis J. Murphy,*[,†] Yangsi Ou,[†] Danielle H. Euler,[§] Keith Wessner,[§] Sharon Adamski,[§] Bin Luo,[§] Gregg A. Wesolowski,[‡] Robert Vogel,[‡] Helmut Glantschnig,[‡] Laura S. Lubbers,[§] Stephen S. Carroll,[†] and Ming-Tain Lai*[,†]

[†]In Vitro Pharmacology, [‡]Bone Biology, and [§]In Vivo Pharmacology, Merck Research Laboratories, West Point, Pennsylvania 19486, United States

Supporting Information

ABSTRACT: During drug discovery, assessment of in vivo target occupancy by therapeutic candidates is often required for predicting clinical efficacy. Current strategies for determining target occupancy include using radiolabeled or irreversible surrogates, which can be technically challenging, and the results are often not sufficiently quantitative. We developed a straightforward method by applying slow-dissociation kinetics to quantitatively determine enzyme occupancy without using specialized reagents. We applied this method to determine occupancy of Cathepsin K inhibitors in bone tissues harvested from rabbit femurs. Tissues from dosed animals were harvested, flash frozen, lysed, then analyzed by a jump-dilution assay with substrate. The rate of substrate turnover was monitored continuously until reaching steady state and progress curves were fit with the equation $[\text{product}] = v_s t + ((v_i - v_s)/k_{obs})(1 - \exp(-k_{obs} t))$. The initial rate v_i represents the residual activity of the enzyme before inhibitor dissociation; v_s is the reaction rate after dissociation of the inhibitor. Occupancy is derived from the ratio of v_i/v_s. A significant benefit of the method is that data from both the occupied and unoccupied states are obtained in the same assay under identical conditions, which provides greater consistency between studies. The Cat K inhibitor MK-0674 (in vitro IC_{50} 1 nM) was tested in young rabbits (<6 month old) and showed a dose-dependent increase in occupancy, reaching essentially complete occupancy at 1.0 mg/kg. In addition the method enables measurement of the total Cat K in the target tissue. Results confirmed complete occupancy even as the osteoclasts responded to higher doses with increased enzyme production.

There is now significant evidence that the degree of occupancy of the target macromolecule by the drug candidate reflects therapeutic efficacy.[1] This is supported by a better correlation of efficacy with dissociation rate from the target than with binding potency.[2] In the process of drug development target occupancy is an important strategic parameter, affecting decisions on therapeutic safety and margin, dosing level and frequency,[3] understanding pharmacokinetic/pharmacodynamic (PK/PD) relationships,[4] translation to the clinic, patient segmentation, competitive position, and intensity of back-up effort.

Target occupancy is the determination of a particular bimolecular binding event in a living tissue or organism. Existing methods utilize photolabeling reagents, radiolabeled compounds (for example, ^{3}H, ^{11}C, ^{14}C, ^{123}I, ^{125}I, etc.) or imaging.[5] The labeled compound is either a close analogue of the therapeutic compound or a molecule proven to effectively and predictably compete with the therapeutic compound for target occupancy. In systems with complex receptor-mediated internalization behavior, radiolabels offer a distinct advantage,[5a] but can be complicated by nonspecific labeling.[1c] Incorporation of photoactive moiety can compromise the activity of the new molecule, and photolabeling often suffers from low labeling efficiency.

Imaging studies offer perhaps the greatest information content but with the greatest technical challenges. For PET imaging significant investment is required in organic and radiosynthesis, scanner preparation, animal handling and sedation, data acquisition, and image analysis. It yields direct assessment of in vivo occupancy, longitudinal studies are possible since the technique is noninvasive, and there is high translatability to human studies. PET imaging is also "target agnostic" in that enzymes, receptors, nucleic acids, etc., can be studied.[6] However, because PET imaging is so resource intensive it may not be feasible during the early phases of a drug discovery program. It is important to have an efficient and economical way of evaluating target occupancy for selecting promising candidates for further development.

We describe here the design and application of a straightforward method that can be employed in determining enzyme occupancy without modification of the molecule of interest or the preparation of new reagents. Through the use of a sensitive, continuous assay and optimized preparation of ex vivo samples, enzyme occupancy can be determined with high accuracy by applying the equation used for monitoring slow-binding kinetics.

To validate the method we investigated occupancy of the enzyme cathepsin K (Cat K) by a selective inhibitor in rabbit bone tissue. Cat K is a lysosomal cysteine protease involved in bone remodeling. It plays a key role in bone resorption and has

Received: June 23, 2015
Published: August 24, 2015

DOI: 10.1021/jacs.5b06518
J. Am. Chem. Soc. 2015, 137, 11230−11233

been shown to be a promising biological target for the treatment of osteoporosis.[7] Although Cat K is expressed abundantly in osteoclasts, the relative scarcity of these cells and the nature of the bone matrix itself make the assessment of target engagement with novel inhibitors challenging in bone tissue. Rabbit Cat K shares 96% sequence identity and 99% similarity with the human Cat K, making rabbit a useful preclinical model for the development of Cat K inhibitors.[8]

The Cat K inhibitor chosen for the occupancy studies, MK-0674, is a structural analogue of odanacatib (a Cat K inhibitor; Phase III trials completed) with desirable pharmacokinetic properties, shows similar potency against Cat K to odanacatib, and is at least 240-fold selective versus cathepsins B, F, L, and S.[9] In vivo enzyme occupancy in the distal femur by MK-0674 was determined by analysis of Cat K inhibition kinetics utilizing standard in vitro assays with sample preparation from bone tissues at low temperature. This method is more straightforward and efficient compared to other approaches and provides a useful alternative in the determination of the target occupancy.

$$\mathrm{E{\cdot}I} \underset{k_4}{\overset{k_3[\mathrm{I}]}{\rightleftharpoons}} \mathrm{E} \underset{k_2}{\overset{k_1[\mathrm{S}]}{\rightleftharpoons}} \mathrm{E{\cdot}S} \xrightarrow{k_{cat}} \mathrm{E} + \mathrm{P}$$

Theory. The kinetic treatment of slow-binding enzyme inhibitors is well established and was thoroughly described by Morrison and Walsh.[10] The treatment is also utilized for analysis of the dissociation kinetics an enzyme/inhibitor complex.

In a "jump-dilution" experiment a preformed enzyme–inhibitor complex (E·I) is allowed to reach equilibrium in the absence of substrate (predilution conditions).[11] The reaction is started by a large (30- to >1000-fold) dilution into a solution containing substrate, yielding the progress curve shown in Figure 1A. The catalytic reaction (S → P) is considered a reporter of the fraction of free enzyme present. Product formation versus time is analyzed by eq 1.[10]

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

The interpretation of the parameters is as follows: the initial rate (v_i) is the rate of reaction before inhibitor has dissociated. k_{obs} is the rate at which the inhibitor establishes its new equilibrium. Due to the dilution the inhibitor concentration is now low and $k_3[\mathrm{I}]$ becomes small relative to k_4. k_{obs} thus becomes a measure of k_4. In the simple case shown above, k_4 is the "off-rate" of the inhibitor from the target and is in fact the parameter that sets the lower limit on the in vivo residency time. v_s is the rate at the new equilibrium. After a large dilution, little inhibitor is bound, and v_s approximates v_0 (v_0 is the rate in the absence of inhibitor).

An illustration of jump-dilution and analysis of the kinetic parameters for determining enzyme occupancy is shown in Figure 1B. The predilution reaction is at equilibrium, shown, for example, at 99% occupancy. The predilution reaction models the in vivo situation. Immediately after dilution into the reaction ($t = 0$) the initial velocity v_i still reflects the residual enzymatic activity of the predilution equilibrium position of 1% E and 99% E·I. At $t = 240$ min with ample dilution, only a small fraction of inhibitor is still bound, and the rate (v_s) reflects the 99% of the total enzyme present. In vivo occupancy is thus $1 - (v_i/v_s)$; in this example, 99%.

This theoretical treatment assumes no dissociation of inhibitor during sample preparation prior to dilution, and that

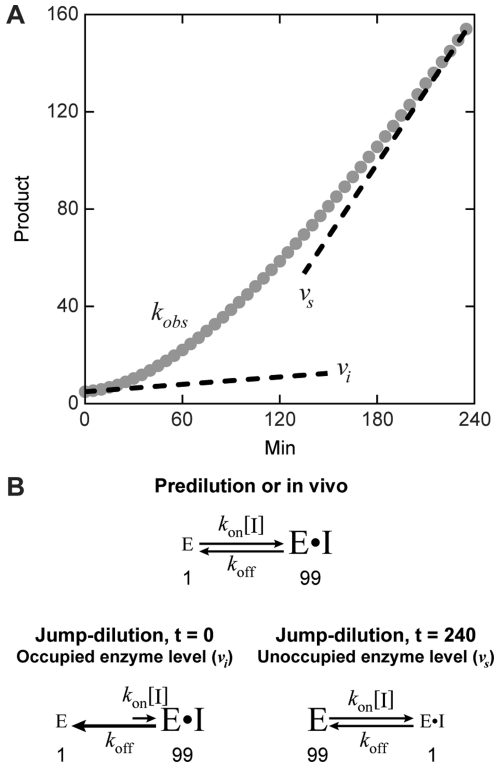

Figure 1. (A) Progress curve for jump-dilution assay. (B) Illustration of the meaning of v_i and v_s from the relative proportions of free and inhibitor-bound enzyme at the initial and final phase of the timecourse. Occupancy ≈ $1 - (v_i/v_s)$.

the dilution factor is large enough to avoid "carry-over" inhibition. In practice, minimizing inhibitor dissociation during sample preparation is a significant experimental challenge of this technique. However, as described below it can be mitigated with rapid freezing of the harvested samples and efficient lysis and dilution with cold reagents.

In Vitro Validation. First, enzyme occupancy was determined using purified protein with 8.0 μM CBZ-leu-arg-AMC as substrate at pH 5.5. A serial dilution of the inhibitor systematically varies the enzyme occupancy. Occupancy as determined in a typical "IC_{50}" experiment should match the occupancy determined by v_i versus [I] after jump-dilution. Table 1 shows the results of this experiment with several Cat K concentrations where $K_{i\text{-}app}$ of MK-0674 was determined in the two assay formats. As shown in Table 1, there is good agreement between the two assay formats, confirming that the theoretical framework is applicable in this system.

A second important parameter that was tested with purified enzyme was the off rate of MK-0674 from the rabbit Cat K. The half-life at ambient temperature was determined to be 4.1 ± 0.5 min. As discussed below, a rate this fast puts significant demands on the preparation of the ex vivo samples. Based on the Arrhenius-like temperature dependence of the inhibitor off rate[5d,12] the half-life was significantly longer at 8 °C, yielding a value of 65.5 ± 4.5 min for the purified enzyme. This was absolutely critical for the success of the study since it enabled a workup consisting of tissue excision, flash-freezing, thawing into cold lysis buffer, and enzymatic assay at 8 °C with minimal dissociation of the inhibitor. The half-life for dissociation of the ex vivo samples was 27 ± 4 min. Given the sensitivity of the dissociation rate to temperature seen in the in vitro study, the

DOI: 10.1021/jacs.5b06518
J. Am. Chem. Soc. 2015, 137, 11230–11233

Table 1. In Vitro Validation of Enzyme Occupancy Methodology[a]

	IC_{50} assay				jump-dilution assay			
Cat K (nM)	2	1	0.5	0.25	0.04	0.02	0.01	0.005
$K_{i\text{-app}}$ (nM)	1.47 ± 0.01	1.2 ± 0.1	0.93 ± 0.02	0.91 ± 0.07	0.76 ± 0.05	1.1 ± 0.1	0.9 ± 0.8	0.8 ± 0.1
v_0 (RFU/s)	927 ± 48	541 ± 4	273 ± 1	134 ± 8	21 ± 2	8.6 ± 0.6	4.12 ± 0.02	2.14 ± 0.02

[a]Mean ± standard error of the mean.

short half-life observed for the ex vivo sample could reflect a small increase in temperature.

Enzyme Occupancy in Rabbit Bone Tissue. To apply the method to determine in vivo occupancy of a Cat K inhibitor we used tissues from New Zealand White rabbits that were dosed with vehicle or MK-0674. Distal femurs were excised at 24 h postdosing, flash frozen, and pulverized. For the progress curve analysis frozen tissue was resuspended in cold CS lysis buffer with vortexing at an initial dilution of 1:250. Further dilution into assay buffer and then into the assay buffer containing substrate gave a final dilution of 1:50 000. The procedure was performed in less than 2 min for six tissue samples. In addition to the jump-dilution experiments, a parallel set of reactions was run with a saturating amount of inhibitor (100 nM) to serve as background for subtraction of the low level of non-Cat K dependent hydrolysis of the fluorescent substrate. Representative curves are shown in Figure 2.The variation in occupancy with dose is shown in Table 2. As expected with a potent, orally bioavailable inhibitor, dose-dependent occupancy was seen, reaching complete occupancy at 1 mpk. These results have significant impact on drug discovery strategy for Cat K inhibitors by directing medicinal chemistry back-up efforts toward improving the safety and selectivity of the inhibitors instead of improving occupancy.

Comparing the steady state slopes at the different doses revealed that there was also a dose-dependent increase in the amount of active enzyme in the tissue (Table 2). This phenomenon has been observed in a previous study where inhibition of Cat K activity by inhibitor treatment of osteoclasts led to significant increases of the ratio of prepro-Cat K as well as the mature enzyme. Leung et al. saw an increase in cat K expression by immunostaining in optical microscopy that was quantitated by Western blot at 6-fold for the prepro form and 2-fold for the mature enzyme.[13] The 5-fold increase seen here could simply reflect species differences but could also reflect increased dynamic range of expression of Cat K in young versus aged animals (L. Lubbers, unpublished observations). Nevertheless complete inhibition was still achieved even as the osteoclasts responded to higher doses with increased enzyme production.

Obtaining data simultaneously on both the occupied and unoccupied target represents a significant benefit of the method since both are obtained in the same assay under identical conditions. Comparable data with other techniques would require comparing dosed versus vehicle-treated samples, with all of the attendant variability. This approach offers direct translation from in vitro to in vivo occupancy via a straightforward application of slow-dissociation kinetics after jump-dilution. Several factors are important for success of the method. First the off-rate of the nascent therapeutic has to be slow enough to allow the time required for sample preparation. To the extent that the residency time hypothesis is borne out, this will be the rule rather than the exception. Decreasing the temperature can significantly prolong the dissociation half-life to enable the study. Again a sufficiently long half-life is essential

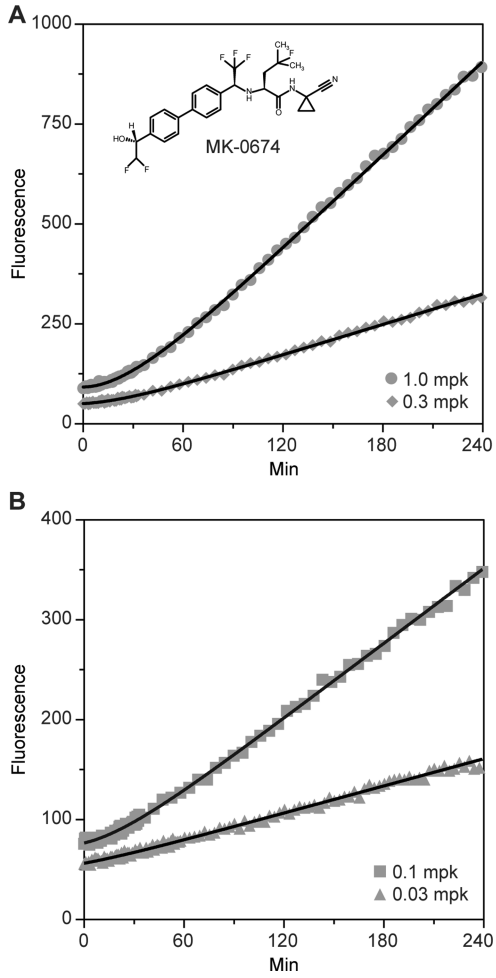

Figure 2. Representative progress curves at 8 °C showing regain of Cat K activity after jump-dilution of excised femoral bone tissue from animals dosed with MK-0674; (A) higher and (B) lower doses. Every third point is shown; the upper curve in each graph has been offset for clarity. The solid line shows the nonlinear regression fit of fluorescence vs time to the fit of eq 1 to obtain v_i and v_s.

to the success of the method. Second, a highly sensitive assay is required to achieve a high dilution to minimize carry-over inhibition. Third, the substrate should be relatively specific to the target enzyme; a high level of substrate turnover by off-target enzymes complicates the analysis. In the case here there was a low level of noncathepsin K activity that could be determined and subtracted out via the use of a saturating level of inhibitor.

Our approach is described based on a single-step binding mechanism. However, with a two-step binding mechanism (EI isomerizing to EI*),[10] our approach may underestimate occupancy since the EI complex may rapidly dissociate to free enzyme upon jump-dilution.

DOI: 10.1021/jacs.5b06518
J. Am. Chem. Soc. 2015, 137, 11230−11233

Table 2. Cat K Occupancy and Relative Activity of v_s in Rabbit Distal Femur after Dosing with MK-0674

	dose (mg/kg)							
	veh.	0.01	0.03	0.1	0.3	1	3	10
occupancy (%)	NA	34 ± 4	49 ± 4	89 ± 1	95 ± 2	101 ± 2	108 ± 7	107 ± 4
activity	1.0 ± 0.2	0.95 ± 0.21	1.2 ± 0.2	1.9 ± 0.2	2.1 ± 0.3	5.1 ± 1.2	3.4 ± 0.9	4.9 ± 1.1
n	10	2	6	2	2	10	8	8

Given that the enzyme occupancy is determined by the ratio of v_i over v_s, if enzyme occupancy is less than 35%, the results may not be as robust because there is not enough difference between v_i and v_s, for the curve fitting to work well. However, for a drug candidate to be efficacious, it generally requires a high level of occupancy, where this method can be applied reliably.

In summary the method provides a novel approach to determine enzyme occupancy by applying a traditional kinetic analysis with a standard enzymatic assay, which should be able to facilitate selecting promising compounds with optimal target occupancy for further development as therapeutic agents.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b06518.

Materials and Methods for in vitro and ex vivo cathepsin K sample preparation and assays (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*dennis.murphy@merck.com

*mingtain_lai@merck.com

Notes

The authors declare the following competing financial interest(s): Employees of Merck, Inc.

■ ACKNOWLEDGMENTS

The authors would like to thank Mike Klimas and Eric Hostetler for insights into imaging technology.

■ REFERENCES

(1) (a) Le, S.; Gruner, J. A.; Mathiasen, J. R.; Marino, M. J.; Schaffhauser, H. *J. Pharmacol. Exp. Ther.* **2008**, *325*, 902. (b) Lindstrom, E.; von Mentzer, B.; Pahlman, I.; Ahlstedt, I.; Uvebrant, A.; Kristensson, E.; Martinsson, R.; Noven, A.; de Verdier, J.; Vauquelin, G. *J. Pharmacol. Exp. Ther.* **2007**, *322*, 1286. (c) Methot, N.; Guay, D.; Rubin, J.; Ethier, D.; Ortega, K.; Wong, S.; Normandin, D.; Beaulieu, C.; Reddy, T. J.; Riendeau, D.; Percival, M. D. *Mol. Pharmacol.* **2008**, *73*, 1857.

(2) (a) Lu, H.; Tonge, P. J. *Curr. Opin. Chem. Biol.* **2010**, *14*, 467. (b) Copeland, R. A.; Pompliano, D. L.; Meek, T. D. *Nat. Rev. Drug Discovery* **2006**, *5*, 730. (c) Copeland, R. A. *Expert Opin. Drug Discovery* **2010**, *5*, 305.

(3) Gabrielsson, J.; Dolgos, H.; Gillberg, P. G.; Bredberg, U.; Benthem, B.; Duker, G. *Drug Discovery Today* **2009**, *14*, 358.

(4) Ploeger, B. A.; van der Graaf, P. H.; Danhof, M. *Drug Metab. Pharmacokinet.* **2009**, *24*, 3.

(5) (a) Laruelle, M. *J. Cereb. Blood Flow Metab.* **2000**, *20*, 423. (b) Malany, S.; Hernandez, L. M.; Smith, W. F.; Crowe, P. D.; Hoare, S. R. *J. Recept. Signal Transduction Res.* **2009**, *29*, 84. (c) Skorey, K.; Waddleton, D.; Therien, M.; LeRiche, T. *Anal. Biochem.* **2006**, *349*, 49. (d) Tillotson, B.; Slocum, K.; Coco, J.; Whitebread, N.; Thomas, B.; West, K. A.; MacDougall, J.; Ge, J.; Ali, J. A.; Palombella, V. J.; Normant, E.; Adams, J.; Fritz, C. C. *J. Biol. Chem.* **2010**, *285*, 39835.

(6) (a) Weissleder, R.; Pittet, M. J. *Nature* **2008**, *452*, 580. (b) James, M. L.; Gambhir, S. S. *Physiol. Rev.* **2012**, *92*, 897.

(7) Stoch, S. A.; Wagner, J. A. *Clin. Pharmacol. Ther.* **2008**, *83*, 172.

(8) (a) Robichaud, J.; Oballa, R.; Prasit, P.; Falgueyret, J. P.; Percival, M. D.; Wesolowski, G.; Rodan, S. B.; Kimmel, D.; Johnson, C.; Bryant, C.; Venkatraman, S.; Setti, E.; Mendonca, R.; Palmer, J. T. *J. Med. Chem.* **2003**, *46*, 3709. (b) Crane, S. N.; Black, W. C.; Palmer, J. T.; Davis, D. E.; Setti, E.; Robichaud, J.; Paquet, J.; Oballa, R. M.; Bayly, C. I.; McKay, D. J.; Somoza, J. R.; Chauret, N.; Seto, C.; Scheigetz, J.; Wesolowski, G.; Masse, F.; Desmarais, S.; Ouellet, M. *J. Med. Chem.* **2006**, *49*, 1066. (c) Desmarais, S.; Masse, F.; Percival, M. D. *Biol. Chem.* **2009**, *390*, 941.

(9) Isabel, E.; Bateman, K. P.; Chauret, N.; Cromlish, W.; Desmarais, S.; Duong, l. T.; Falgueyret, J. P.; Gauthier, J. Y.; Lamontagne, S.; Lau, C. K.; Leger, S.; LeRiche, T.; Levesque, J. F.; Li, C. S.; Masse, F.; McKay, D. J.; Mellon, C.; Nicoll-Griffith, D. A.; Oballa, R. M.; Percival, M. D.; Riendeau, D.; Robichaud, J.; Rodan, G. A.; Rodan, S. B.; Seto, C.; Therien, M.; Truong, V. L.; Wesolowski, G.; Young, R. N.; Zamboni, R.; Black, W. C. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 887.

(10) Morrison, J. F.; Walsh, C. T. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1988**, *61*, 201.

(11) Copeland, R. A.; Basavapathruni, A.; Moyer, M.; Scott, M. P. *Anal. Biochem.* **2011**, *416*, 206.

(12) Copeland, R. A. *Evaluation of Enzyme Inhibitors in Drug Discovery*, 2nd ed.; Wiley: Hoboken, NJ, 2013; p 141.

(13) Leung, P.; Pickarski, M.; Zhuo, Y.; Masarachia, P. J.; Duong, L. T. *Bone* **2011**, *49*, 623−635.

DOI: 10.1021/jacs.5b06518
J. Am. Chem. Soc. 2015, 137, 11230−11233