Published in final edited form as:

Anal Bioanal Chem. 2011 September ; 401(5): 1589–1595. doi:10.1007/s00216-011-5225-7.

Screening a fragment cocktail library using ultrafiltration

Sayaka Shibata^{1,3}, Zhongsheng Zhang¹, Konstantin V. Korotkov¹, Jaclyn Delarosa¹, Alberto Napuli², Angela M. Kelley², Natasha Mueller², Jennifer Ross¹, Frank H. Zucker¹, Frederick S. Buckner², Ethan A. Merritt¹, Christophe L. M. J. Verlinde¹, Wesley C. Van Voorhis², Wim G. J. Hol¹, and Erkang Fan^{1,*}

¹Department of Biochemistry, University of Washington, Seattle, WA 98195, USA

²Department of Medicine, University of Washington, Seattle, WA 98195, USA

³Department of Chemistry, University of Washington, Seattle, WA 98195, USA

Abstract

Ultrafiltration provides a generic method to discover ligands for protein drug targets with millimolar to micromolar K_d , the typical range of fragment-based drug discovery. This method was tailored to a 96-well format, and cocktails of fragment-sized molecules, with molecular masses between 150 and 300 Da, were screened against medical structural genomics target proteins. The validity of the method was confirmed through competitive binding assays in the presence of ligands known to bind the target proteins.

Keywords

Ultrafiltration; Screening; Fragment-based drug discovery; Compound library

Introduction

Fragment-based drug discovery has attracted much attention in the past decade because it allows rapid identification of small chemical scaffolds (molecular mass 150–300 Da) with high “ligand efficiency” [1–6]. In theory, the vast chemical diversity space is sampled more efficiently by fragments than by the larger compounds of conventional chemical libraries because the smaller the molecule, the greater the chance that it may be complementary to some portion of the binding pocket [4,7]. Another advantage afforded by fragment-based drug discovery is that it leaves a large molecular weight window for fine-tuning absorption, distribution, metabolism, and excretion (ADME) properties of the initial hits during the evolution into a drug lead [3,4,8]. These appealing properties led the Medical Structural Genomics of Pathogenic Protozoa (MSGPP) consortium to take a fragment-based approach to identify novel leads for protein targets that may serve as springboards for future drug design [6]. Although protein crystallography is featured in our structural genomics effort to identify fragment-based hits, the low-throughput and labor-intensive nature of this technique prompted us to search for alternative and complementary screening methods. Since multiple protein targets are under investigation at any given time, we wanted to develop a moderate-throughput screening method that would be universal to all proteins of interest. In addition, we set out to design a screening method that would be sufficiently sensitive to identify fragments that tend to have low affinities (approximately micromolar to millimolar K_d) for target proteins.

*Corresponding author: Erkang Fan, University of Washington, Box 357742, Seattle, WA, 98195, USA. Telephone: 206-685-7048, Fax: 206-685-7002, erkang@uw.edu.

There are, in general, two different types of fragment-based screening methods: function-based and affinity-based screening [4,9]. An example of a function-based screening method is the use of fluorophore-tagged fragments to identify protease substrates [10]. Hits are identified by changes in the fluorescence signal due to the release of the fluorophore from a fragment by protease-catalyzed amide bond hydrolysis. Such function-based screening methods can detect ligands that bind with low affinity. However, these methods are not ideal for use in a genomics setting because screening conditions often must be optimized for each target protein of different function.

Several affinity-based screening methods have been reported [3,4,7,11–13]. These include X-ray crystallography [3,6,14], NMR-based techniques [1,13,15–19], surface plasmon resonance (SPR) [20–22], isothermal titration calorimetry [13,23], size-exclusion chromatography coupled with mass spectrometry [24], and ultrafiltration-based methods [25–28]. Many of these techniques have been applied to screening fragment-based libraries and each has its own strengths and weaknesses, particularly when multiple proteins need to be screened. For example, one of the advantages of both fragment cocktail crystallography and structure–activity relationship NMR spectroscopy is that they provide information about the binding modes of weakly interacting ligands (approximately millimolar K_d) to the target protein. However, it is often demanding to produce crystals suitable for fragment cocktail crystallography and to produce labeled proteins for structure–activity relationship NMR spectroscopy. SPR and saturation transfer difference NMR spectroscopy are also often used for fragment-based library screening efforts. Both are able to analyze weakly binding ligands (approximately millimolar K_d) with moderate throughput. Low protein consumption is an attractive feature of the SPR method, although it requires protein immobilization. One disadvantage of saturation transfer difference NMR spectroscopy is the requirement for relatively high concentrations (approximately 0.5 mM) of compounds, which may cause solubility problems [7].

We sought to develop a 96-well format ultrafiltration-based screening approach to identify fragments that interact with our target proteins. Most screening protocols with ultrafiltration devices address single compound–protein interactions in the micromolar to millimolar K_d range [12,29]. Ultrafiltration methods for screening small molecule libraries to find high-affinity ligands (micromolar K_d) have been reported [28,30,31]. Typically, either a mass spectrometer or a UV detector coupled to a high-performance chromatography (HPLC) system is employed [32]. The latter combination is widely used for other purposes; hence, many laboratories could perform the ultrafiltration technique without new instrumentation. The novelty of our approach is the adaptation of the ultrafiltration technique coupled with UV detection to the needs of screening low-affinity fragment-based compound libraries against multiple proteins.

Experimental

Materials

Putative riboflavin kinase (RFK) from *Trypanosoma brucei* (TriTrypDB ID: Tb09.211.3420 and Protein Data Bank ID: 3BNW) and putative methionine aminopeptidase 1 (MetAP1) from *Plasmodium yoelii* (PlasmoDB ID: PY04617) were produced by the MSGPP consortium. The *Escherichia coli* heat-labile enterotoxin B pentamer (LTB₅) was expressed and purified as described elsewhere [33]. These proteins were flash-frozen and stored at –20 or –80°C [34]. All chemicals, including 243 fragments, were purchased from multiple chemical companies. Information on the fragment libraries is given elsewhere [6].

Cocktail preparations

Individual fragments were dissolved in dimethyl sulfoxide (DMSO) or deionized water. The retention times of individual UV active fragments were obtained by HPLC (1100 series liquid chromatography system, Agilent technologies) with reversed-phase columns, Zorbax SB-C18 (Agilent Technologies) or Onyx monolithic C₁₈ analytical column (Phenomenex), following the HPLC methods described in “Fragment cocktail sample analysis by HPLC.” For ease of identification, compounds were grouped in cocktails such that each cocktail only contained compounds with different retention times. To avoid systematic errors in the percent UV signal reduction of fragments due to precipitation of fragments in the incubation solvent (standard buffer with 6.5% DMSO), the solubility of compounds in all cocktails was analyzed in two solvent systems, 100% DMSO or standard buffer with 6.5% DMSO. The fragment cocktails dissolved in these two different solvents were analyzed by HPLC, and area under the curve (AUC) changes resulting from a difference in solvent were recorded for further fragment selections.

A cocktail targeting LTB₅ contained the known ligand *m*-nitrophenyl- α -D-galactoside (MNPG) and two internal standards. Twenty-three cocktails (136 compounds, five to nine compounds per cocktail) and 30 cocktails (243 compounds, seven to ten compounds per cocktail) were prepared for RFK and MetAP1, respectively.

Protein buffer exchange

RFK and MetAP1 were buffer-exchanged to standard buffer (500 mM NaCl, 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid, 5% glycerol, 0.025% sodium azide, pH 7.25) with 1 mM tris(2-carboxyethyl)phosphine (TCEP) by an Econo-Pac 10 DG column (Bio-Rad), according to the manufacturer's minimal dilution protocol. Proteins were reconcentrated using Microcon YM-10 centrifugal filter units (Millipore) and concentrations were measured at 280 nm with a UV-1601 spectrometer (Shimadzu).

Ultrafiltration method

Cocktail incubations with protein

Cocktails (1.3 μ L, 1.5 mM per compound) were slowly added and mixed with RFK or MetAP1 protein solution (18.7 μ L, 215 μ M) in microtubes on ice. Controls were prepared in the same manner as the protein samples except that the same volume of standard buffer with 1 mM tris(2-carboxyethyl)phosphine was added instead of the protein solution. The incubation mixtures were transferred to a prerinsed multiscreen filter plate with an Ultracel-10 membrane (Millipore) and were centrifuged at 2500 g for 30 min at 4°C. Each filtrate (approximately 10 μ L) was transferred to 0.1-mL polypropylene microinserts (Agilent Technologies) for HPLC analysis. In the case of the LTB₅–MNPG binding experiment, the assay buffer contained 50 mM tris(hydroxymethyl)aminomethane–HCl, 1 mM EDTA, 3 mM NaN₃, and 200 mM NaCl at pH 7.4, and Microcon YM-10 centrifugal filter units were used instead of a plate. The centrifugation was performed at 25 and 4°C, respectively.

Fragment cocktail sample analysis by HPLC

Fragment cocktails were analyzed sequentially by HPLC using an autosampler and were separated by a Zorbax SB-C18 column at a flow rate of 1.0 mL/min or an Onyx monolithic C₁₈ analytical column at a flow rate of 1.0 or 2.0 mL/min.

Calculations of percent UV signal reduction of fragments

The signals of all peaks were measured at 10-nm intervals between 220 and 260 nm, and the AUCs were calculated using ChemStation (Agilent Technologies). The AUC of the fragment in the presence of the protein sample was normalized to the maximally possible AUC, obtained in the absence of protein. To correct HPLC injection variations of protein and control samples, a volume correction was applied such that the AUCs of nonbinding fragments between the protein sample and the control are the same. Percent UV signal reductions were calculated on the basis of the normalized AUC values by using Eq. 1.

Hit fragment confirmation and competitive binding assays

Follow-up hit fragment cocktails were prepared as follows. Each hit fragment identified by initial cocktail screenings was mixed with the protein in the presence of two nonbinding fragments as internal standards and also a known ligand for competitive binding assays. Nonbinding ligands were selected on the basis of the initial cocktail screening results. Incubation and analysis protocols were as already described.

Results and discussion

Ultrafiltration methods separate free ligand from ligand–protein complexes using a semipermeable membrane [12,26,27,31]. All compounds in our fragment libraries are UV-active compounds; hence, binding can be easily followed with a UV detector by comparing UV signal reductions of each free compound incubated in the presence and the absence of the target protein. To obtain a significant reduction in UV signal, we calculated the initial concentrations of ligand and protein needed to detect weakly binding ligands with K_d up to approximately 1 mM, assuming a 1:1 binding ratio of ligand to protein. The equations governing these calculations are as follows:

$$\%UV \text{ signal reduction} = (1 - \text{Vol. correction} \frac{[L]_{\text{protein}}}{[L]_{\text{control}}}) \times 100 \quad \text{eq. 1}$$

$[L]_{\text{protein}}$: Concentration of free ligand in the filtrate in the presence of protein

$[L]_{\text{control}}$: Concentration of free ligand in the filtrate in the absence of protein

$$\%UV \text{ signal reduction} = (1 - \frac{[L]}{[L]_0}) \times 100 \quad \text{eq. 2}$$

$[L] = [L]_{\text{Protein}}$ and $[L]_0 = [L]_{\text{control}}$

$$K_d = \frac{[L][P]}{[P * L]} \quad \text{eq. 3}$$

$$[L]_0 = [L] + [P * L] \quad \text{eq. 4}$$

$$[P]_0 = [P] + [P * L] \quad \text{eq. 5}$$

$$[L] = \frac{-([P]_0 - [L]_0 + K_d) + \sqrt{([P]_0 - [L]_0 + K_d)^2 + 4K_d [L]_0}}{2} \quad \text{eq. 6}$$

How the UV signal reduction varies theoretically as a function of initial protein concentration is shown in Fig. 1 for various initial ligand concentrations assuming a K_d value of 1 mM. The calculations suggest that to observe weakly binding ligands ($K_d \sim 1$ mM) with at least 15% signal reduction under the constraint of a minimum consumption level of proteins and ligands, the optimal initial concentrations of protein and ligand need to be 0.2 mM and 0.1 mM, respectively. The choice of the minimum of 15% signal reduction is discussed later. Figure 2 illustrates the theoretical percent UV signal reductions of ligands when the K_d values of ligands are in the range from 0.001 mM to 10 mM at the initial concentrations of 0.1 mM ligand and 0.2 mM protein.

To validate the conclusions from the calculations, the ultrafiltration method was tested with LTB₅ protein, for which we had a known ligand, MNPG ($K_d = 175 \mu\text{M}$ at 25°C) [35]. When initial concentrations of 0.1 mM ligand and 0.2 mM protein were used, $51.0 \pm 0.5\%$ UV signal reduction was obtained. This value is in good agreement with the theoretical signal reduction of 46.7% (Fig. 2). Subsequently, we repeated the LTB₅–MNPG ultrafiltration assay at 4°C because protein samples are sometimes prone to degradation at room temperature. Under these conditions the UV signal reduction was $73.0 \pm 0.1\%$, which suggested a slightly tighter binding of MNPG to LTB₅ at 4°C than at 25°C. Thus, we screened MSGPP proteins with a 96-well-format ultrafiltration plate at 4°C.

During the screening of fragment cocktails against multiple proteins, we found that nonbinders showed an average UV signal reduction of $0.2 \pm 4.6\%$. Therefore, to avoid a large number of false positives, we set the threshold for accepting a compound as a hit to approximately 3 times the standard error or 15% UV signal reduction.

The ultrafiltration method using the defined parameters was first applied to the recombinant RFK. A total number of 134 fragments in 23 cocktails were screened against RFK. The cocktail screening resulted in four initial hits. Subsequent individual affinity measurements for these initial hits confirmed three hit fragments (Table 1), whereas one initial hit did not generate a reproducible UV signal reduction. To verify these three hits and to gain knowledge of the nature of the ligand–protein interaction, we performed competitive binding assays in the presence of ADP and Mg(II) (ADP•Mg) or flavin mononucleotide (FMN) (Fig. 3). FMN and ADP are products of the enzymatic reaction and Mg(II) is a cofactor [36,37]. In the presence of FMN, the detected concentrations of the three hits were approximately 60% higher than without FMN, indicating binding at the FMN recognition site. Interestingly, the affinity of the three hits fragment increased by approximately 50% in the presence of ADP•Mg relative to the fragments alone, indicating that ADP•Mg had synergistic effects on the affinity of the hits fragment. This observation agrees with the previously reported stabilization of the riboflavin binding site in the presence of ADP•Mg [38,39]. The enhanced binding of fragments in the presence of ADP•Mg also raised the question of whether synergistic effects may be observed among fragments in the cocktails. We believe that, in principle, our cocktail screening method is capable of detecting synergistic effects if a hit is found to have significantly smaller UV signal reduction in hit validation as compared with the cocktail screening experiment. On the other hand, if antagonistic effects exist among fragments, our screening method may miss the hits as false negatives.

To further validate the method, we applied the ultrafiltration technique to another potential drug target, the *Plasmodium yoelii* MetAP1 protein. A total number of 243 fragments in 30 fragment cocktails were used in this screen. The cocktail screening resulted in ten initial hit fragments with approximately 30% to approximately 90% UV signal reduction. The individual affinity measurements for these hits confirmed nine hit fragments, whereas one initially hit did not generate a reproducible UV signal reduction. Among these nine hit fragments, the top six hits with the highest binding affinities were investigated in further experiments that tested competitive binding with methionine (Table 2). These experiments showed that methionine was able to compete against lower-affinity ligands more efficiently than higher-affinity ligands (Fig. 4). All six fragments appeared to be directed toward the recognition site of methionine, which is a product of the enzymatic reaction [40]. Although the fragment cocktail library contained compounds with diverse chemical structures, these MetAP1 hit fragments share the common features of a fused ring system and/or a carboxylate substituent. Interestingly, similar moieties can be found in known inhibitors of MetAP1 from various organisms. These known MetAP1 inhibitors coordinate the metal ions that are in close proximity to the methionine recognition site [41–44]. In summary, the ultrafiltration screen of 243 total fragments against MetAP1 yielded nine hits on the basis of the cutoff parameters and six of these were validated as being competitors with methionine for binding of MetAP1.

In conclusion, the ultrafiltration method is a generic affinity-based screening technique that is capable of detecting weakly binding ligands from fragment-based compound libraries. This technique requires no modifications of compounds or proteins, such as isotopic labeling or immobilizations. This method has moderate throughput when implemented in a 96-well format. Furthermore, the method requires only an HPLC system with a UV detector, which is widely available in many laboratories. Currently, our screening protocol is limited to UV-active compounds. For non-UV-active fragment libraries, this limitation may be overcome by performing competitive binding ultrafiltration assays similar to the hit validation for MetAP1 using methionine, in which a signal change of a known hydrophobic and UV-active ligand is monitored. Another possible solution is the use of an alternative detector such as a mass spectrometer. We use the ultrafiltration method in our MSGPP consortium [45] as a complement to the generic differential scanning fluorimetry technique to screen fragment cocktail libraries [46]. Both methods are generic but are useful in different affinity ranges: ultrafiltration for millimolar to micromolar K_d values, and differential scanning fluorimetry for low-micromolar to sub-nanomolar K_d values. To the best of our knowledge, this is the first time that ultrafiltration has been successfully demonstrated as a method for fragment-based cocktail screening.

Acknowledgments

We thank Eric T. Larson for his critical review of the manuscript. This work was supported by National Institutes of Health grants P50GM64655 (SGPP), P01AI067921 (MSGPP), and AI34501.

References

1. Shuker SB, Hajduk PJ, Meadows RP, Fesik SW. Science. 1996; 274 (5292):1531–1534. [PubMed: 8929414]
2. Oprea TI, Davis AM, Teague SJ, Leeson PD. J Chem Inf Comput Sci. 2001; 41 (5):1308–1315. [PubMed: 11604031]
3. Carr R, Jhoti H. Drug Discovery Today. 2002; 7 (9):522–527. [PubMed: 11983569]
4. Erlanson DA, McDowell RS, O'Brien T. J Med Chem. 2004; 47 (14):3463–3482. [PubMed: 15214773]
5. Hajduk PJ, Greer J. Nature Reviews Drug Discovery. 2007; 6 (3):211–219.

6. Verlinde C, Fan EK, Shibata S, Zhang ZS, Sun ZH, Deng W, Ross J, Kim J, Xiao LR, Arakaki TL, Bosch J, Caruthers JM, Larson ET, LeTrong I, Napuli A, Kelley A, Mueller N, Zucker F, Van Voorhis WC, Buckner FS, Merritt EA, Hol WGJ. *Curr Top Med Chem*. 2009; 9 (18):167–687.
7. Carr RAE, Congreve M, Murray CW, Rees DC. *Drug Discovery Today*. 2005; 10 (14):987–992. [PubMed: 16023057]
8. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. *Adv Drug Del Rev*. 2001; 46 (1–3):3–26.
9. Deng GJ, Sanyal G. *J Pharm Biomed Anal*. 2006; 40 (3):528–538. [PubMed: 16256286]
10. Wood WJL, Patterson AW, Tsuruoka H, Jain RK, Ellman JA. *J Am Chem Soc*. 2005; 127 (44): 15521–15527. [PubMed: 16262416]
11. Makara GM, Athanasopoulos J. *Curr Opin Biotechnol*. 2005; 16 (6):666–673. [PubMed: 16257522]
12. Vuignier K, Schappler J, Veuthey JL, Carrupt PA, Martel S. *Anal Bioanal Chem*. 2010; 398 (1): 53–66. [PubMed: 20454782]
13. Murray CW, Carr MG, Callaghan O, Chessari G, Congreve M, Cowan S, Coyle JE, Downham R, Figueroa E, Frederickson M, Graham B, McMenamin R, O'Brien MA, Patel S, Phillips TR, Williams G, Woodhead AJ, Woolford AJA. *J Med Chem*. 2010; 53 (16):5942–5955. [PubMed: 20718493]
14. Hartshorn MJ, Murray CW, Cleasby A, Frederickson M, Tickle IJ, Jhoti H. *J Med Chem*. 2005; 48 (2):403–413. [PubMed: 15658854]
15. Mayer M, Meyer B. *J Am Chem Soc*. 2001; 123 (25):6108–6117. [PubMed: 11414845]
16. Hajduk PJ, Meadows RP, Fesik SW. *Science*. 1997; 278(5337):497. [PubMed: 9381145]
17. Hajduk PJ, Meadows RP, Fesik SW. *Q Rev Biophys*. 1999; 32 (3):211–240. [PubMed: 11194565]
18. Hajduk PJ, Gontsyan A, Didomenico S, Cowart M, Bayburt EK, Solomon L, Severin J, Smith R, Walter K, Holzman TF, Stewart A, McGaraughty S, Jarvis MF, Kowaluk EA, Fesik SW. *J Med Chem*. 2000; 43 (25):4781–4786. [PubMed: 11123986]
19. Vanwetswinkel S, Heetebrij RJ, van Duynhoven J, Hollander JG, Filippov DV, Hajduk PJ, Siegal G. *Chem Biol*. 2005; 12 (2):207–216. [PubMed: 15734648]
20. Frostell-Karlsson A, Remaeus A, Roos H, Andersson K, Borg P, Hamalainen M, Karlsson R. *J Med Chem*. 2000; 43 (10):1986–1992. [PubMed: 10821711]
21. Rich RL, Day YSN, Morton TA, Myszka DG. *Anal Biochem*. 2001; 296 (2):197–207. [PubMed: 11554715]
22. Navratilova I, Hopkins AL. *Acs Medicinal Chemistry Letters*. 2010; 1 (1):44–48.
23. Ciulli A, Williams G, Smith AG, Blundell TL, Abell C. *J Med Chem*. 2006; 49 (16):4992–5000. [PubMed: 16884311]
24. Flarakos J, Morand KL, Vouros P. *Anal Chem*. 2005; 77 (5):1345–1353. [PubMed: 15732917]
25. Wieboldt R, Zweigenbaum J, Henion J. *Anal Chem*. 1997; 69 (9):1683–1691. [PubMed: 21639291]
26. Chen CJ, Chen S, Woodbury CP, Venton DL. *Anal Biochem*. 1998; 261 (2):164–182. [PubMed: 9716419]
27. Johnson BM, Nikolic D, van Breemen RB. *Mass Spectrom Rev*. 2002; 21 (2):76–86. [PubMed: 12373745]
28. Kamchonwongpaisan S, Vanichthanankul J, Tarnchompoo B, Yuvaniyama J, Taweechai S, Yuthavong Y. *Anal Chem*. 2005; 77 (5):1222–1227. [PubMed: 15732900]
29. Nerli B, Romanini D, Pico G. *Chem Biol Interact*. 1997; 104 (2–3):179–202. [PubMed: 9212783]
30. Comess KM, Schurdak ME, Voorbach MJ, Coen M, Trumbull JD, Yang H, Gao L, Tang H, Cheng X, Lerner CG, McCall O, Burns DJ, Beutel BA. *J Biomol Screen*. 2006; 11 (7):743–754. [PubMed: 16973923]
31. vanBreemen RB, Huang CR, Nikolic D, Woodbury CP, Zhao YZ, Venton DL. *Anal Chem*. 1997; 69 (11):2159–2164. [PubMed: 9183179]
32. Liu H, Delgado M, Forman LJ, Eggers CM, Montoya JL. *Journal of Chromatography-Biomedical Applications*. 1993; 616 (1):105–115. [PubMed: 8376481]
33. Minke WE, Roach C, Hol WGJ, Verlinde C. *Biochemistry (Mosc)*. 1999; 38 (18):5684–5692.

34. Deng JP, Davies DR, Wisedchaisri G, Wu MT, Hol WGJ, Mehlin C. *Acta Crystallographica Section D-Biological Crystallography*. 2004; 60:203–204.
35. Pickens JC, Merritt EA, Ahn M, Verlinde C, Hol WGJ, Fan EK. *Chem Biol*. 2002; 9 (2):215–224. [PubMed: 11880036]
36. Yamada Y, Merrill AH, McCormick DB. *Arch Biochem Biophys*. 1990; 278 (1):125–130. [PubMed: 2157358]
37. Efimov I, Kuusk V, Zhang XP, McIntire WS. *Biochemistry (Mosc)*. 1998; 37 (27):9716–9723.
38. Bauer S, Kemter K, Bacher A, Huber R, Fischer M, Steinbacher S. *J Mol Biol*. 2003; 326 (5): 1463–1473. [PubMed: 12595258]
39. Karthikeyan S, Zhou QX, Osterman AL, Zhang H. *Biochemistry (Mosc)*. 2003; 42 (43):12532–12538.
40. Li X, Chang YH. *Proc Natl Acad Sci U S A*. 1995; 92 (26):12357–12361. [PubMed: 8618900]
41. Lowther WT, Zhang Y, Sampson PB, Honek JF, Matthews BW. *Biochemistry (Mosc)*. 1999; 38 (45):14810–14819.
42. Kawai M, BaMaung NY, Fidanze SD, Erickson SA, Tedrow JS, Sanders WJ, Vasudevan A, Park C, Hutchins C, Comess KM, Calvin D, Wang J, Zhang Q, Lou P, Tucker-Garcia L, Bouska J, Bell RL, Lesniewski R, Henkin J, Sheppard GS. *Bioorg Med Chem Lett*. 2006; 16 (13):3574–3577. [PubMed: 16632353]
43. Lu JP, Chai SC, Ye QZ. *J Med Chem*. 2010; 53 (3):1329–1337. [PubMed: 20038112]
44. Altmeyer MA, Marschner A, Schiffmann R, Klein CD. *Bioorg Med Chem Lett*. 2010; 20 (14): 4038–4044. [PubMed: 20621724]
45. Crowther GJ, Napuli AJ, Thomas AP, Chung DJ, Kovzun KV, Leibly DJ, Castaneda LJ, Bhandari J, Damman CJ, Hui R, Hol WGJ, Buckner FS, Verlinde C, Zhang ZS, Fan EK, Van Voorhis WC. *J Biomol Screen*. 2009; 14 (6):700–707. [PubMed: 19470714]
46. Fan E, Baker D, Fields S, Gelb MH, Buckner FS, van Voorhis WC, Phizicky E, Dumont M, Mehlin C, Grayhack E, Sullivan M, Verlinde C, DeTitta G, Meldrum DR, Merritt EA, Earnest T, Soltis M, Zucker F, Myler PJ, Schoenfeld L, Kim D, Worthey L, LaCount D, Vignali M, Li J, Mondal S, Massey A, Carroll B, Gulde S, Luft J, DeSoto L, Holl M, Caruthers J, Bosch J, Robien M, Arakaki T, Holmes M, Le Trong I, Hol WGJ. *Methods Mol Biol*. 2008; 426:497. [PubMed: 18542886]

Fig. 1. Calculation of the UV signal reduction as a function of protein concentration for a compound with $K_d = 1$ mM by the ultrafiltration method.

Fig. 2. Calculation of the UV signal reduction as a function of K_d by the ultrafiltration method. Initial assay conditions were 0.1 mM ligand and 0.2 mM protein.

Fig. 3.

Competitive binding assays for riboflavin kinase (*RFK*) ultrafiltration hits. The fragments were 9-aminoacridine (*1*), benz[*cd*]indo-2(*1H*)-one (*2*), and 3-bromophenol (*3*). *Error bars* represent the standard deviation of three independent experiments. *FMN* flavin mononucleotide

Fig. 4. Competitive binding assays for methionine aminopeptidase 1 (*MetAP*) ultrafiltration hits. The fragments are 2-amino-8-hydroxyquinoline (4), isoquinoline-3-carboxylic acid (5), 2-hydroxycinnamic acid (6), quinoline-8-carboxylic acid (7), 2,3-dihydro-1,4-benzodioxine-6-carboxylic acid (8), and quinaldic acid (9). *Error bars* represent the standard deviation of three independent experiments.

Table 1

Fragments identified as ligands of riboflavin kinase by ultrafiltration.

Compound	Fragment	Structure	% UV signal reduction ^a
1	9-aminoacridine		32.5 ± 1.9
2	benz[cd]indo-2(1 <i>H</i>)-one		21.3 ± 4.4
3	3-bromophenol		14.6 ± 3.1

^a Average of three independent experiments

Table 2

Fragments identified as ligands of methionine aminopeptidase 1 by ultrafiltration.

Compound	Fragment	Structure	% UV signal reduction ^a
4	2-amino-8-hydroxyquinoline		91.5 ± 1.4
5	isoquinoline-3-carboxylic acid		91.7 ± 1.1
6	2-hydroxy-cinnamic acid		75.9 ± 0.3
7	quinoline-8-carboxylic acid		70.4 ± 2.7
8	2,3-dihydro-1,4-benzodioxine-6-carboxylic acid		51.8 ± 2.8
9	quinaldic acid		50.2 ± 0.8

^a Average of three independent experiments