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An experimental check of backscattering interferometry

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Abstract

Backscattering interferometry (BSI) was used to determine the association constants for four well-known biomolecular interactions: protein A + IgG, trypsin + antitrypsin, trypsin + *p*-aminobenzamidine, and antithrombin + heparin. Each gave well-defined binding curves and K_d values in close agreement with published findings obtained using other techniques. These results stand in direct contrast to the claims in a 2015 publication in this journal (Discussion of “Back Scattering Interferometry revisited—a theoretical and experimental investigation” Jørgensen, T.M.; Jepsen, S.T.; Sørensen, H.S.; di Gennaro, A.K.; Kristensen, S.R. *Sensors and Actuators B* **2015**, 220, 1328–1337, doi: 10.1016/j.snb.2015.06.121), thus invalidating the claim that BSI is unable to make measurements of this kind. The experimental details are discussed, and several potential sources of error in the previous publication are identified. No comments are made here on the discussion of the theoretical aspects of the BSI technique.

Keywords

backscattering interferometry; label-free; binding constants; protein-ligand interactions; experimental methods

Introduction

A 2015 publication in this journal by T.M. Jørgensen, S.T. Jepsen, and colleagues, titled “Discussion of “Back Scattering Interferometry revisited – a theoretical and experimental investigation” discussed the use of the label-free analytical technique of backscattering interferometry (BSI) to quantitate binding interactions involving a wide variety of biomolecules [1]. In addition to challenging its theory and terminology, the authors tested the use of BSI on three well-known binding interactions: trypsin + antitrypsin, trypsin + *p*-aminobenzamidine (*p*-AB), and antithrombin + heparin. In a previous published critique from the same group [2], the binding of protein A + IgG was also tested. In these publications it was claimed that three of the four experiments did not yield meaningful signals using BSI, and that the fourth case (trypsin + antitrypsin) generated a linear and very large increase in signal through the point at which binding would expected to be saturated,

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rendering the measurement useless for determination of a binding constant. Thus, these authors claim to have tested BSI and found it unable to measure intermolecular interactions under conditions reported previously by several laboratories. One of those laboratories is ours, therefore we deemed it important to test these claims. We describe here the same analyses performed on our BSI instrument, which provided results in sharp contrast to those published by Jørgensen, et al.

The BSI technique was originally described by Bornhop and colleagues in 2007 [3], and has been the subject of numerous publications from that laboratory and others [4–16]. The instrumentation is not yet commercially available, but can be readily constructed from standard optical parts. It uses a specially designed microfluidic channel; all design details have been published, with a recent report [17] detailing potential pitfalls and solutions. BSI detects changes in bulk refractive index (RI) produced by binding events in solution; a recent theoretical and experimental treatment from the Bornhop laboratory has proposed that this signal results primarily from changes in hydration, polarizability, and other solute properties that contribute to bulk RI of any solution [17]. Thus, while BSI can be employed in many different media including organic solvents [12], it has so far been most widely used for species in aqueous solutions, consistent with the unique features of water as a medium and solvent.

Materials and methods

Materials

Antibodies (human IgG1 and mouse IgM) were purchased from Santa Cruz Biotechnologies and kept frozen at -80°C until use. Heparin was purchased from Sigma-Aldrich and kept at 4°C until use. *p*-aminobenzamidine was purchased from VWR and stored at 4°C until use. Trypsin was purchased from VWR as a lyophilized powder, stored at 4°C , and reconstituted with DPBS prior to use. Alpha-1-antitrypsin was purchased from Sigma-Aldrich as a lyophilized powder and reconstituted with DPBS prior to use. Antithrombin III (human) was purchased from ThermoFisher as a lyophilized powder stored at 4°C , and reconstituted with water prior to use. Protein A was purchased from ThermoFisher as a lyophilized powder and reconstituted in DPBS prior to use. Bovine serum albumin was purchased from ThermoFisher Scientific in pre-diluted (1mg/mL) ampoules and stored at 4°C prior to use. 1X DPBS was purchased from Corning and stored at 4°C prior to use. Microfluidic chips were purchased from Micronit Microfluidics under a joint licensing agreement between Vanderbilt University and the Georgia Institute of Technology.

Detection of Ligand Binding: Ligand-analyte binding was accomplished by incubating a fixed amount of one ligand (antithrombin, IgG1, trypsin) with varying concentrations of cognate ligands (heparin, protein A, *p*-aminobenzamidine, and antitrypsin) for 40 min at 20°C . Solutions were drawn via gentle vacuum suction into the channels of a microfluidic chip for analysis using a backscattering interferometer. Prior to analysis, microfluidic chip surfaces were treated with brief (2 min) incubation with piranha etch, followed by incubation (12 h) with 1mg/mL 1,1,2,2-perfluorooctylsilatrane in methanol. Following incubation, microfluidic chips were rinsed extensively with methanol and water prior to use. Chip surfaces were regenerated in between experiments by extensive rinsing with buffer.

The microfluidic chips were maintained at 25°C using a feedback-controlled peltier system. The high-contrast fringes produced by each sample were generated using a fiber-coupled, frequency-stabilized HeNe laser as a light source and recorded on a CCD camera. Measurements were analyzed using a combination of in-house software, Microsoft Excel, and OriginPro.

Data analysis

Spatial changes in interference fringes were measured in near real-time using a high-resolution camera and a fast Fourier transform. In the general case, a Fourier transform is defined as

$$A(x) = F[a(y)] = \int a(y) e^{-i2\pi xy} dy \quad (1)$$

Where $A(x)$ is the complex Fourier transform of the function $a(x)$, $F[x]$ denotes the Fourier transform operation, y is a spatial variable, and f is a spatial frequency in the Fourier domain. For a particular set of characteristic frequencies, it is possible to calculate the observed phase change of the fringe pattern by evaluating the real and imaginary parts of the Fourier transform at a given frequency. As a result, differences in phase changes can be calculated for different ligand-receptor binding pairs. It was observed that only cognate ligand-binding interactions created substantial differences in phase when compared to binding-nonbinding pairs. For example, the maximal binding phase change for a particular sample was typically of the magnitude ~0.01 Rad, while non-binding phase changes are approximately 10 times lower.

Typically, the equilibrium dissociation constant K_D was determined by plotting phase changes as a function of varying ligand concentration, compared to those for a non-binding control, and fitting the resulting points to a simple single site binding model given by

$$y = \frac{y_{\max}(x)}{K_D + (x)} \quad (2)$$

where y is the observed, calculated phase change, $y_{\max}$ is the observed, maximal, calculated phase change, x is the ligand concentration, and K_D is the equilibrium dissociation constant. By incubating samples for long time periods (tens of minutes to hours) and not removing excess ligand, it was assumed that the observed binding systems were in a state of dynamic equilibrium.

Results and discussion

Figure 1 shows BSI data obtained on our instrument for the four interactions reported by Jørgensen, et al. to give no clear signal. All experiments were performed using endpoint (equilibrium) measurements in which one of the two components of interest was held at a fixed concentration near the literature K_d value and the other component was added sequentially to cover an appropriate concentration range. The data collection was done in

oversampled mode, in which a large number of data points were collected involving relatively small changes in concentration, with each data point being measured once. Each resulting binding curve was fit to the complete data set using the standard Langmuir binding isotherm. As shown in Table 1, all K_d values ($= 1/K_a$) measured with BSI were in good agreement with those reported in the literature from other techniques.

A discussion of problems that appear to plague the approach reported by Jørgensen, et al. is given below, followed by remarks on each individual experimental case. We find four main areas in which the experiments were performed in a manner inconsistent with standard descriptions of the technique in the literature and standard practices of binding constant measurement in general.

1) Instrument setup

In section 2.2 of the report in question [1], the authors state “The BSI setup used for this work is described in detail in our previous work [18].” Unfortunately, the experimental setup described in that previous work (reference [2] here) is flawed, and differs in important ways from the instrumentation that they purported to replicate, the one described by Bornhop and coworkers in 2007 [3]. In short, Jørgensen et al. appear to have constructed a different instrument than described by Bornhop [13], and used by us and others.

Two of the most notable differences are: (a) lack of attention to proper temperature control and (b) the use of a glass capillary instead of a microfluidic chip. It appears from the information provided by Jørgensen et al. that the sample capillary rests on the surface of a heating block, which would not provide even temperature throughout the sample. The optical configuration is not as reported in the collective work on BSI as it applies to the determination of molecular interactions. In addition, the authors do not describe treatment of the glass capillary, either by rinsing between injections, passivation of the glass surface (transiently via a blocking reagent or permanently via silanization or a similar technique), or using large injection volumes as a rinsing agent (or a combination of all three techniques). This is a critical error, for the need to properly treat and wash the sample channel is essential to success and is emphasized in every detailed experimental description of BSI from the Bornhop laboratory and ours.

The ray tracing diagrams presented by Jørgensen, et al. represent another feature of concern. While these appear to be reasonable at first glance, their relevance is questionable given the complexity of the optical train and the simplicity of the modeling approach. The four-beam ray-trace offered represent too few rays to fully sample the optical train, and comparison of the fringe patterns published by the Bornhop group for both the capillary and the chip [24, 25] display significantly different intensity profiles from those presented by Jørgensen. This is likely due to alignment differences (angle of impinging laser, etc.), dissimilar capillary dimension, and the difference in capillary material and structure – soda-glass capillary vs. the polyimide coated fused silica chip used by Bornhop. (The polyimide coating is an active refractive index interface that will impact the final fringe pattern.) Furthermore, no consideration appears to be provided for the contribution to the fringe pattern from reflections emanating from the capillary holder. Thus, Jørgensen and colleagues may have confused backscattering interferometry with a different publication describing absolute

refractive index measurement [26], in which interferometric patterns are captured at angles other than near 0° .

The Jørgensen system and our Bornhop-design system also differ in the nature of the chip. While Jørgensen and colleagues model an object somewhat similar to our chip with isotropically etched channels, predicting poorer sensitivity than a capillary, their model is misleading for at least two reasons. It uses only two beams, thereby undersampling the object, and ignores the contribution of the glass cover-plate and the channel substrate (thickness) to the beam profile, both of which have been previously shown to be critical for sensitivity optimization [27].

2) Sample referencing

BSI is a universal sensor, and so its assays must be performed as relative measurements in order to extract the binding event from the other non-specific interactions and solution composition changes. Jørgensen and colleagues did not perform sample referencing in an appropriate manner, either using the ligand (the binding partner that changes in concentration) in the absence of the receptor (the partner held at constant concentration), or with a non-binding analogue to the ligand. The need for referencing against a nonbinding control has been noted in multiple publications by us [4, 9, 10, 28] and Bornhop [11, 15] using the terms “reference” or “control” for this type of correction procedure. This measurement is not a “blank,” which is what Jørgensen and colleagues apparently used. While the original publication describing BSI [3] did not describe a sample reference for each measurement, it showed (for the cases studied, not generally) that signal changes arising from nonspecific interactions were small in magnitude relative to the binding-related signal. We later decided that reference/control measurements should be done in every case as a best practice. The omission of sample/reference measurements by Jørgensen and coworkers may account for their erroneous report that BSI does not detect binding in the cases they describe, for without proper referencing, signals obtained due to nonspecific interactions may eclipse those associated with specific binding.

3) Data treatment

Several factors in the Jørgensen paper reflect poor or inconsistent representation or analysis of the data. (a) Although the authors report that they were unable to determine a binding affinity for trypsin to p-AB using BSI, no zero point can be found on the graph. This limitation makes any attempt to elicit binding information impossible. (b) Graphs of data are not scaled correctly, and non-linear effects (binding isotherms) are all treated with linear fitting methods that are inappropriate because the data plotted is not linearized. (c) The use of error bars in the representation of BSI data is inconsistent. In the case of the heparin-antithrombin measurements, this makes it quite difficult to determine whether their experiment failed due to use of too much heparin (see below) or the measurement was simply noisy.

4) Inappropriate concentration ranges

In two cases described below, Jørgensen and coworkers used concentrations of the interacting species far higher than K_d , dramatically diminishing the potential dynamic range

of any measurement. As with any analytical technique, when using BSI to analyze the interactions of species with unknown binding affinities we first probe a range of concentrations to focus subsequent attention on concentrations approximating K_d . This standard practice was apparently ignored by Jørgensen and colleagues.

Unfractionated heparin (UFH) + antithrombin (Figure 1A)—Our experiment employed antithrombin (or BSA control) at 17 nM, treated with variable concentrations of unfractionated heparin (approximate MW = 15,000; 150 IU/mg) from 0 to 1 μ M, with most measurements in the 0–100 nM range. This is the appropriate concentration range for an interaction with a K_d of approximately 10 nM [18]. As illustrated in Figure 1, our binding curves show robust, quantifiable signal, providing a K_d value that is in excellent agreement with the known affinity. A much higher antithrombin concentration (1.6 μ M) was used by Jørgensen and colleagues.

Protein A + IgG (Figure 1B)—The K_d determined in our laboratory by BSI was in between values observed in the literature (0.83 ± 0.13 nM, vs. 0.1–1.62 nM). It is possible that this reflects the true binding affinity versus a surface-immobilized antibody (~ 0.1 nM as obtained by Bio-Rad using SPR analysis) or includes interactions of protein A with other regions of IgG that are not accurately compensated for using IgM as reference.

Trypsin binding to *p*-aminobenzamidine (Figure 1C)—The binding curve shown in Figure 1 stands in stark contrast to the noisy BSI data shown by Jørgensen, et al., which also omits a zero-point value. Our result gave a slightly higher affinity than the data obtained in previous studies [29].

Trypsin + antitrypsin (Figure 1D)—Jørgensen and colleagues performed this experiment with 2 μ M trypsin and increasing concentrations of antitrypsin, from 0 to 10 μ M, showing a linear increase in BSI signal over this range. Given that the known K_d of this interaction is below 5 nM [23], the use of low-micromolar concentrations of the binding partners invalidates the measurement, since the binding interaction would be saturated for all data points. Furthermore, these investigators kept a significant amount of *p*-aminobenzamidine in solution for unexplained reasons. In contrast, we used trypsin (or BSA reference) at 1 nM, treated with variable concentrations of antitrypsin from 0 to 120 nM, giving clean results consistent with established affinity values.

Conclusion

In their published critique, Jørgensen, et al. criticized both the theoretical underpinning of BSI and its experimental practice. We focus here only on the experimental results because they are directly testable in an incontrovertible manner. By not commenting on the sections of the Jørgensen publication discussing background and theory, we do not endorse those sections of their paper. The molecular interactions selected by Jørgensen and colleagues were measured correctly in our laboratory by properly configured and performed BSI measurement. We therefore find the experimental claims of Jørgensen, et al., that BSI cannot detect these binding events, to be without merit.

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Biographies

Michael M. Baksh received his B.A. (Molecular and Cell Biology) and Ph.D. (Biophysics) degrees from the University of California Berkeley in 2001 and 2006, respectively. He continued his research efforts as a postdoctoral fellow at UC Berkeley and The Scripps Research Institute, and is now Research Scientist at the Georgia Institute of Technology. His work has focused on the development of a variety of analytical techniques and applications, including scalable membrane-based assays, backscattering interferometry, and functional studies of integral membrane-bound proteins, HIV protease, and cellular uptake mechanisms.

M.G. Finn received his B.Sc. and Ph.D. degrees from the California Institute of Technology and the Massachusetts Institute of Technology in 1980 and 1986, respectively. His postdoctoral research (Stanford University) was followed by academic appointments at the University of Virginia (1988–1998), The Scripps Research Institute (1998–2013), and the Georgia Institute of Technology (2013-present). His current scientific interests include the use of virus particles as molecular and catalytic building blocks for vaccine and functional materials development, the development of click chemistry for organic and materials synthesis, polyvalent interactions and advanced linker technologies in drug targeting, and the use of evolution for the discovery of molecular function.

References

1. Jørgensen TM, Jepsen ST, Sørensen HS, Di Gennaro AK, Kristensen SR. Back Scattering Interferometry revisited – a theoretical and experimental investigation. *Sensors and Actuators B*. 2015; 220:1328–37. DOI: 10.1016/j.snb.2015.06.121
2. Jepsen ST, Jørgensen TM, Zong W, Trydal T, Kristensen SR, Sørensen HS. Evaluation of back scatter interferometry, a method for detecting protein binding in solution. *Analyst*. 2015; doi: 10.1039/c4an01129e
3. Bornhop DJ, Latham JC, Kussrow A, Markov DA, Jones RD, Sorensen HS. Free-Solution, Label-Free Molecular Interactions Studied by Back-Scattering Interferometry. *Science*. 2007; 317:1732–6. [PubMed: 17885132]
4. Kussrow A, Kaltgrad E, Wolfenden ML, Cloninger MJ, Finn MG, Bornhop DJ. Measurement of Monovalent and Polyvalent Carbohydrate-Lectin Binding by Back-Scattering Interferometry. *Anal Chem*. 2009; 81:4889–97. [PubMed: 19462965]
5. Latham JC, Stein RA, Bornhop DJ, Mchaourab HS. Free-Solution Label-Free Detection of alpha-Crystallin Chaperone Interactions by Back-Scattering Interferometry. *Anal Chem*. 2009; 81:1865–71. [PubMed: 19178288]
6. Kussrow AK, Enders CS, Castro AR, Cox DL, Ballard RC, Bornhop DJ. The potential of backscattering interferometry as an in vitro clinical diagnostic tool for the serological diagnosis of infectious disease. *Analyst*. 2010; 135:1535–7. [PubMed: 20414494]
7. Pesciotta EN, Bornhop DJ, Flowers RA II. Back-Scattering Interferometry: A Versatile Platform for the Study of Free-Solution versus Surface-Immobilized Hybridization. *Chem Asian J*. 2010; 6:70–3.
8. Sétif P, Harris N, Lagoutte B, Dotson S, Weinberger SR. Detection of the Photosystem I:Ferredoxin Complex by Backscattering Interferometry. *J Am Chem Soc*. 2010; 132:10620–2. [PubMed: 20681677]

9. Baksh MM, Kussrow A, Mileni M, Finn MG, Bornhop DJ. Label-Free Quantification of Membrane-Ligand Interactions Using Backscattering Interferometry. *Nature Biotechnol.* 2011; 29:357–60. [PubMed: 21399645]
10. Kussrow A, Baksh MM, Bornhop DJ, Finn MG. Universal Sensing by Transduction of Antibody Binding using Backscattering Interferometry. *ChemBioChem.* 2011; 12:367–70. [PubMed: 21290534]
11. Olmsted IR, Xiao Y, Cho M, Csordas A, Sheehan JH, Meiler J, et al. Measurement of Aptamer-Protein Interactions with Back-Scattering Interferometry. *Anal Chem.* 2011; 83:8867–70. [PubMed: 22032342]
12. Pesciotta EN, Bornhop DJ, Flowers RA II. Backscattering Interferometry: An Alternative Approach for the Study of Hydrogen Bonding Interactions in Organic Solvents. *Org Lett.* 2011; 13:2654–7. [PubMed: 21510617]
13. Kussrow A, Enders CS, Bornhop DJ. Interferometric Methods for Label-Free Molecular Interaction Studies. *Anal Chem.* 2012; 84:779–92. [PubMed: 22060037]
14. Tiefenbrunn T, Forli S, Baksh MM, Chang MW, Happer M, Lin YC, et al. Small Molecule Regulation of Protein Conformation by Binding in the Flap of HIV Protease. *ACS Chem Biol.* 2013; 8:1223–31. [PubMed: 23540839]
15. Kammer MN, Olmsted IR, Kussrow AK, Morris MJ, Jackson GW, Bornhop DJ. Characterizing aptamer small molecule interactions with backscattering interferometry. *Analyst.* 2014; 139:5879–84. [PubMed: 25229067]
16. Saetear P, Perrin AJ, Bartholdson SJ, Wanaguru M, Kussrow A, Bornhop DJ, et al. Quantification of Plasmodium-host protein interactions on intact, unmodified erythrocytes by back-scattering interferometry. *Malaria J.* 2015; 14:88.
17. Bornhop DJ, Kammer MN, Kussrow A, Flowers RA II, Meiler J. Origin and Prediction of Free-Solution Interaction Studies Performed Label-Free. *Proc Natl Acad Sci USA.* 2016; 113:E1595–E604. [PubMed: 26960999]
18. Olson ST, Björk I, Sheffer R, Craig PA, Shore JD, Choay J. Role of the Antithrombin-binding Pentasaccharide in Heparin Acceleration of Antithrombin-Proteinase Reactions. *J Biol Chem.* 1992; 267:12528–38. [PubMed: 1618758]
19. Vered Bronner, MT., Bravman, Tsafirir. Rapid Screening and Selection of Optimal Antibody Capturing Agents Using the ProteOn XPR36 Protein Interaction Array System. Bio-Rad Laboratories Inc; 2009.
20. Yang L, Biswas ME, Chen P. Study of binding between protein a and Immunoglobulin G using a surface tension probe. *Biophys J.* 2003; 84:509–22. [PubMed: 12524303]
21. Evans SA, Olson ST, Shore JD. Para-Aminobenzamidine as a Fluorescent-Probe for the Active-Site of Serine Proteases. *J Biol Chem.* 1982; 257:3014–7. [PubMed: 7037776]
22. Malánková M, Turková J. Determination of dissociation constants of complexes of trypsin and its low molecular weight inhibitors by affinity chromatography in zonal and frontal analysis arrangement. *J Solid-Phase Biochem.* 1977; 2:237–49.
23. Cohen AB. Interaction of Alpha-1-Antitrypsin with Chymotrypsin, Trypsin and Elastase. *Biochim Biophys Acta.* 1975; 391:193–200. [PubMed: 1079736]
24. Bornhop DJ. Microvolume Index of Refraction Determinations by Interferometric Backscatter. *Appl Optics.* 1995; 34:3234–9.
25. Tarigan HJ, Neill P, Kenmore CK, Bornhop DJ. Capillary-scale refractive index detection by interferometric backscatter. *Anal Chem.* 1996; 68:1762–70.
26. Sorensen HS, Pranov H, Larsen NB, Bornhop DJ, Andersen PE. Absolute refractive index determination by microinterferometric backscatter detection. *Anal Chem.* 2003; 75:1946–53. [PubMed: 12713055]
27. Swinney K, Markov D, Bornhop DJ. Chip-scale universal detection based on backscatter interferometry. *Anal Chem.* 2000; 72:2690–5. [PubMed: 10905294]
28. Lau JL, Baksh MM, Fiedler JD, Brown SD, Kussrow A, Bornhop DJ, et al. Evolution and Protein Packaging of Small Molecule RNA Aptamers. *ACS Nano.* 2011; 5:7722–9. [PubMed: 21899290]

29. MalaníKová M, Turková J. Determination of dissociation constants of complexes of trypsin and its low molecular weight inhibitors by affinity chromatography in zonal and frontal analysis arrangement. *Journal of Solid-Phase Biochemistry*. 2:237–49.

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Highlights

- A recent paper (10.1016/j.snb.2015.06.121) incorrectly dismisses backscattering interferometry.
- Potential reasons for this error are identified, including the use of concentrations far in excess of K_d .
- BSI is a powerful technique for label-free measurement of binding interactions in solution.

Figure 1.

Representative plots of BSI signal vs. ligand concentration for the determination of binding constants for the following pairs of molecules; in each case the second was added to a fixed concentration of the first. Each measurement is plotted as a Δrad versus concentration of the ligand. All determinations were referenced against the phase change observed for the nonbinding reference pair, both at the same concentration as the fixed-concentration species (target). (A) antithrombin (17 nM) + heparin, reference = BSA (17 nM) + heparin; (B) human IgG1 (1 nM) + protein A, reference = mouse IgM (1 nM) + protein A; (C) trypsin (1 μM) + *p*-aminobenzamidine, reference = BSA (1 μM) + *p*-aminobenzamidine; (D) trypsin (1 nM) + antitrypsin, reference = BSA (1 nM) + antitrypsin.

Table 1

Binding constants determined by BSI from the plots shown in Figure 1.

Fixed-concentration binding partner	Variable-concentration ligand	K_d (BSI)	Literature value and reference
antithrombin	heparin	15 ± 9 nM	10 ± 3 [18]
IgG1	protein A	0.83 ± 0.13 nM	$<0.1 - 1.62$ nM [19, 20]
trypsin	<i>p</i> -aminobenzamidine	0.64 ± 0.26 μ M	$1.6 - 19$ μ M [21, 22]
trypsin	antitrypsin	1.2 ± 0.6 nM	<5 nM [23]