Journal of Medicinal Chemistry

Article

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Development of a Plate-Based Optical Biosensor Fragment Screening Methodology to Identify Phosphodiesterase 10A Inhibitors

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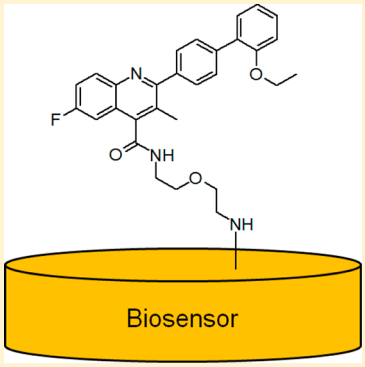
Supporting Information

ABSTRACT: We describe the development of a novel fragment screening methodology employing a plate-based optical biosensor system that can operate in a 384-well format. The method is based on the "inhibition in solution assay" (ISA) approach using an immobilized target definition compound (TDC) that has been specifically designed for this purpose by making use of available structural information. We demonstrate that this method is robust and is sufficiently sensitive to detect fragment hits as weak as KD 500 μM when confirmed in a conventional surface plasmon resonance approach. The application of the plate-based screen, the identification of fragment inhibitors of PDE10A, and their structural characterization are all discussed in a forthcoming paper.



INTRODUCTION

Evolving Applications (and Challenges) in Fragment Screening. Fragment-based drug design (FBDD) has become widely used as a tool for discovering new chemical leads.[1,2] In comparison to conventional high throughput screening, the alternative screening of low molecular weight, low complexity compounds (fragments) allows more efficient sampling of structure space[3] and can be effective using screening libraries as small as a few thousand compounds or less.[4] This area has been reviewed extensively.[5−7] Fragment screening requires highly sensitive, high quality assays in order to detect the typically weak binding fragments. Efficient and effective fragment screening activities are key to success in FBDD, but despite the increasing importance and applications of FBDD, currently employed screening approaches still suffer from distinct limitations including complex assays, lower throughput, and high false positive rates. A review of various methods and their advantages and disadvantages has recently appeared.[8]

Various one-dimensional and two-dimensional NMR methodologies have been developed and have been very successful in fragment screening.[9−11] However, NMR methods often have low throughput and high reagent consumption. More recently, optical biosensors and, in particular, surface plasmon resonance (SPR) technologies, are increasingly being used as alternatives to NMR screening.[12−14] These methods offer increased throughput (>1000 fragments/day) and lower reagent consumption (<100 μg target protein). However, the SPR approach has some challenges including unspecific interactions of test compounds with the biosensor chip (e.g., super-stoichiometric binding), limited assay sensitivity, and differences in dynamic range. Challenges often increase with (1) increasing molecular weight of the target protein, (2) decreasing molecular weight of the test compounds, and (3) more weakly binding potential hits; all of these contribute to limitations for the reliable detection of genuine binding events.[9]

Inhibition in Solution Assay (ISA) Screening Using Surface Plasmon Resonance (SPR) with Conventional Flow-Based Methods. For the optical biosensor approach, various methodologies have been employed to address these challenges discussed above.[8] Typically, the macromolecular protein target is immobilized on the biosensor. We have recently employed an alternative strategy where a smaller molecule probe compound is instead immobilized on the chip. We refer to this tethered probe as the target definition compound or TDC. The TDC must be immobilized to the biosensor without compromising the binding to the target protein. As illustrated in Figure 1, the TDC is immobilized on the sensor surface. Next, a solution containing the enzyme target is flowed across the sensor surface and that enzyme will recognize and bind to the TDC causing a measurable signal. That protein/TDC interaction can be modulated or disrupted by coincubation of the enzyme with an inhibitor that is in competition with the TDC for binding with the protein. In this

Received: November 11, 2012
Published: March 19, 2013

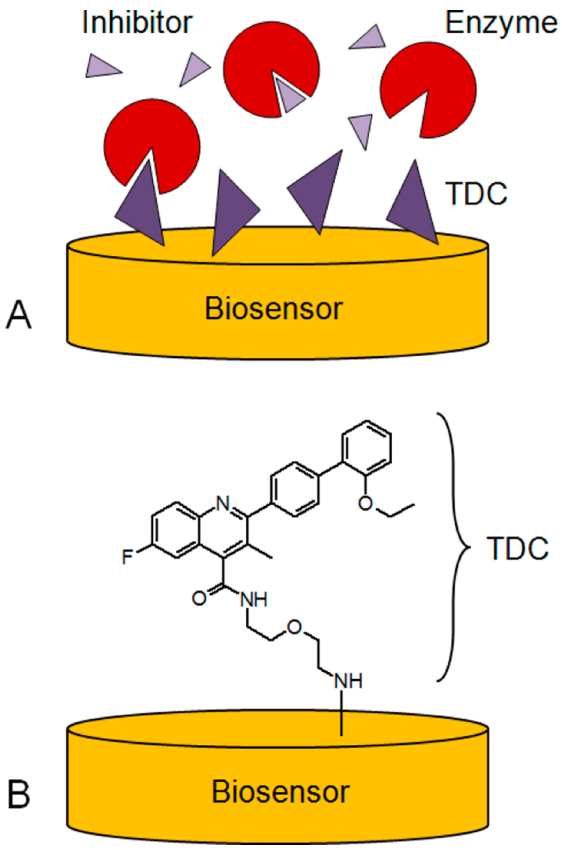


Figure 1. Schematic illustration of the ISA format used in SPR studies. (A) The ISA format employs an immobilized TDC that ideally displays high affinity to the enzyme. Competing inhibitors that are present in solution will subsequently lower the observable binding signal. (B) Illustration of the TDC (as employed in the present study) linked to the sensor surface.

manner, the inhibitor/protein interaction can be assessed qualitatively as well as quantitatively.

In general, good choices for compounds as TDCs are substrate analogues or known inhibitors. A major criteria for the design of a selective TDC is the retention of the biological activity of the parent ligand including high affinity to the target protein. The strategies for designing a suitable TDC can be very much adopted from approaches aiming to develop fluorescently labeled ligands to study ligand binding events. This is a suitable approach in cases where detailed structural information is not readily available, and one typically relies on pharmacophore information and/or structure−activity relationships for substrate analogues and/or known inhibitors for TDC design. Nevertheless, this usually requires the synthesis of a larger number of compounds that need to be tested in a trial and error fashion in order to identify suitable candidates. The potential availability of structural information furnishes the opportunity to design such TDCs much more effectively and in a rational fashion, thereby minimizing the risk of creating unsuitable reagents during assay development.

To differentiate this assay format from the more conventional direct binding assays, we termed this fragment screening protocol "inhibition in solution assay" (ISA).[15] The advantages of ISA include (1) substantially increased sensitivity, (2) generic and rapid assay development, and (3) immediate verification of competitive binding. Because this method measures precisely even small changes in the free protein concentration in the presence of a fragment, it enables the detection of fragment binding in the millimolar affinity regime and thus offers an attractive option for primary fragment screening.

Developing a New Inhibition in Solution Assay (ISA) Screening Approach Using Plate-Based OWG. The conventional flow-based methods described above have limited throughput due to the sequential application of samples onto the biosensor. The advantage of the ISA approach when employing high-throughput plate-based optical biosensor platforms has been described already for the study of inhibitor binding to human trypsin.[16] Here we describe a modification of the ISA approach adapted to a plate-based optical biosensor methodology to provide a robust and sensitive assay to screen fragments as binders/inhibitors of PDE10A. A forthcoming report will describe the identification, characterization, and structural elucidation of those fragment hits.

Inhibition in Solution Assay (ISA) Screening Methodology Using Optical Waveguide Grating (OWG). A range of alternative and complementing biosensor platforms have lately been developed to enable the study of protein−ligand interactions with even higher throughput. Evanescent field sensing provided by optical waveguides (also referred to as optical waveguide grating or OWG) has emerged as a viable complement to SPR platforms and have recently been commercialized.[17,18] Similar advantages and disadvantages as compared to those already mentioned for SPR are also applicable to these plate-based biosensor platforms; in particular, they share the sensitivity limits associated with working with immobilized target protein. Therefore, we sought to adapt in ISA methodology to be used with an OWG system. Such a system should provide the advantages of conventional ISA/SPR technology but with substantially increased speed. This approach is illustrated in Figure 2.

RESULTS AND DISCUSSION

TDC Development. In prior work, our colleagues identified a series of high affinity PDE10A inhibitors.[19,20] The binding

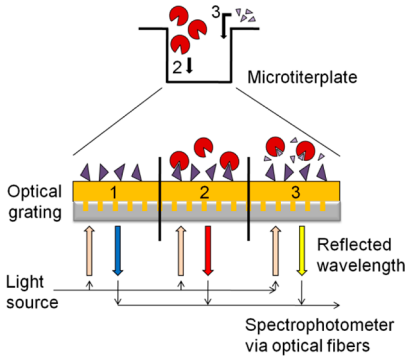


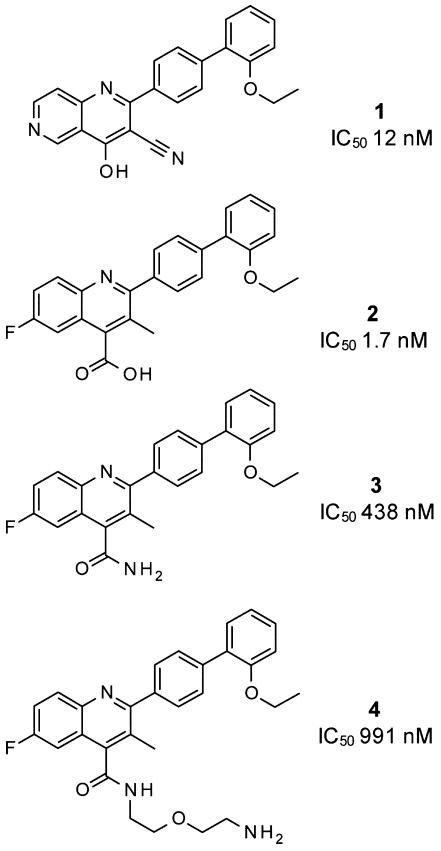
Figure 2. Schematic illustration of the ISA format used in the present study applying a plate-based OWG system. (1) In preparation for screening, a TDC is covalently attached to the functionalized biosensor surface. The incident light is reflected at a characteristic wavelength. (2) Upon addition of the protein and its subsequent interaction with the TDC, the resulting mass-change at the sensor surface is leading to a wavelength increase of the reflected light (typically 0.5−2 nm dependent on protein concentration). (3) The addition of a competitive compound results in a negative mass-change due to protein dissociation from the sensor surface and is manifested in wavelength decrease of the reflected light.

dx.doi.org/10.1021/jm301665y | J. Med. Chem. 2013, 56, 3228−3234

mode was analyzed by X-ray crystallography for selected representatives such as **1**. From this structural information it was evident that the position occupied by the hydroxyl pointed out toward the solvent and could serve as the region to attach a linker that could then be used to attach to the biosensor chip. Next, we employed **2**, which is a member of a related chemotype[21] that also had high affinity and a carboxylic acid as an easily functionizable synthetic handle. The amide analogue **3** lost about 250-fold activity relative to **2**; however, it still retained sufficient potency to indicate that analogues containing more extended linkers could be useful as tethered probes. Molecular modeling of **3** (based on the structural information for **1**) indicated that a linker with length of about eight atoms would be suitable to attach to the biosensor chip and still allow subsequent binding of PDE10A to the probe. Accordingly, we prepared **4**, which had IC_{50} 991 nM in our enzyme inhibition assay and was thus judged to be suitable as the TDC. The reduction in affinity is actually advantageous in order to enable low-affinity binding fragments to effectively compete for the same binding site. This comes at the cost of increasing protein concentration in the assay in order to obtain a good signal/noise-ratio. Thus it is important to find an optimal balance between those two parameters that still enables cost-effective screening within an affinity window that still allows the detection of low-affinity fragment binding. The attachment of **4** to the biosensor chip is shown schematically in Figure 1B.

Validation of 4 as a TDC Using Surface Plasmon Resonance (SPR). Several steps were taken during the development of a sensitive inhibition in solution assay (ISA) for the detection of fragment binding to PDE10A employing an OWG platform using the SRU BIND system. The suitability of TDC **4** for a fragment screening assay was validated by tethering it to the dextran-matrix of an SPR Biosensor. The experiments (data not shown) indicated a satisfactory immobilization level (typically 450−600 RU) of **4** onto the biosensor. Injection of a PDE10A solution demonstrated that the immobilized compound can interact specifically with PDE10A, as reflected by a large binding signal. Injecting different concentrations of PDE10A and making use of the mass transport limitations that was intentionally designed through the experimental setup (high immobilization density and low flow rate), we could show that the observed binding signal was solely determined by the mass transfer of the protein to the sensor surface and resulted in a concentration-dependent response. This setup could be used later on for the validation of the initial fragment hits as well as for their affinity determination in a similar fashion as reported earlier.[8,15]

Optical Waveguide Grating (OWG) Assay. In principle, the above SPR methodology could have been used to screen the fragment library. However, on the basis of previous experience that some compounds/fragments can also bind promiscuously to the immobilized TDC (thus impacting negatively on subsequent ligand binding experiments), we wanted to make use of the ability to measure a single compound/fragment in a designated well of a 384-well plate in a parallel fashion using a plate-based OWG platform. The simultaneous investigation of 384 individual interactions is a vast increase in throughput in comparison to iterative measurements using flow-based SPR systems. Furthermore, we wanted to conduct the screening as efficiently as possible without exposing the protein to the fragments during extended time periods to prevent time-dependent precipitation due to solubility problems or promiscuous binding.



For the development of the ISA on the OWG platform, we found it necessary to optimize two key parameters in order to define an optimal dynamic window for the detection of fragment binding: (1) the density of the TDC on the biosensor surface and (2) the protein concentration. For this, we evaluated a small matrix of different immobilization densities as defined by the concentration of the ligand (1 mM to 50 μM) during immobilization versus different protein concentrations (10 μM to 100 nM); this was greatly facilitated by the plate-based format of the OWG platform. This matrix was tested against a range of 20 PDE10A inhibitors that are simpler analogues of **5** (see Supporting Information) at a concentration of 20 μM spanning different molecular weights from 200 to 400 Da and affinities from K_D = 40 nM to 500 μM (as been previously determined by employing the SPR ISA format). We found that a high immobilization density of the TDC generally led to increased unspecific binding of the tested inhibitors to the TDC and that the displacement effects became less pronounced for inhibitors with weak affinity, irrespective of the protein concentration. As expected, the protein concentration plays an essential role in defining the dynamic window of the assay, as the readable signal usually gradually increases with increasing protein concentration. However, in analogy to the density of the TDC, the displacement effects at relatively high protein concentrations of 5−10 μM became less pronounced in conjunction with a diminished increase of the protein binding signal. To address this issue, we chose not to work with the maximal attainable protein binding signal but rather to reduce the protein concentration used in the assay as well as the concentration of the TDC during immobilization. By applying this matrix, we found an optimal combination of TDC density (100 μM immobilization concentration for 30 min leading to approximately 1500 ΔPWV immobilization signal) and protein

dx.doi.org/10.1021/jm301665y | J. Med. Chem. 2013, 56, 3228−3234

concentration (2 μM) that enabled the detection of fragment binding with very low affinities (close to millimolar) using a screening concentration of only 100 μM. Using these optimized screening conditions, we compared the capacity of the already mentioned PDE10A inhibitors to displace the protein from the TDC-modified biosensor surface with their affinity as determined via SPR ISA. As shown in Figure 3B, we could confirm a good correlation between the reduction in PDE10A binding to the biosensor with increasing K_D values, indicating the possibility to conduct an reliable affinity ranking that is solely based on the reduction in PDE10A binding to the TDC-modified biosensor. To assess the binding specificity in this setup, we employed **1** using a concentration that leads to full occupancy of the binding site (20 μM). We could observe almost full displacement (>98%) of PDE10A from the Biosensor surface (data not shown), indicating a highly specific binding of PDE10A to the immobilized TDC.

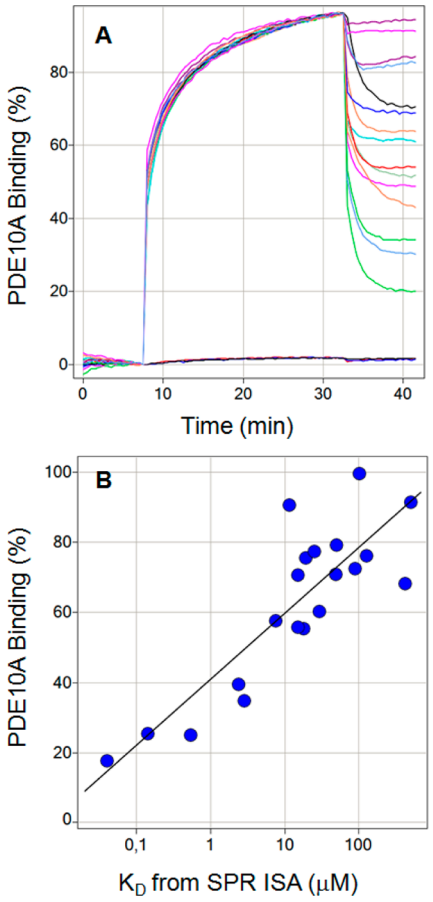


Figure 3. Representative results from the OWG ISA. (A) Shown are typical sensorgrams (example of 14 compounds displaying different potencies at 20 μM and highlighted by different colors) illustrating the experimental setup of the OWG ISA. After buffer equilibration, the addition of PDE10A to TDC-containing wells at 7 min results in specific binding to the biosensor surface. Addition of protein to unmodified wells is used as control and to assess specificity. The addition of compounds after equilibration at 33 min results in a displacement of PDE10A from the biosensor surface due to specific competition with the TDC. The magnitude of the displacement reflects the potency of the compounds. (B) Correlation of compound activity in the OWG ISA and compound affinity determined via SPR ISA in the test set of 20 PDE10A inhibitors (details in Supporting Information). In (A) and (B), the binding responses are shown as PDE10A binding %, i.e., the binding of PDE10A to the TDC-modified biosensor in the presence of fragment in relation to the controls containing only PDE10A protein. PDE10A binding percentage is defined by the OWG signal prior to addition of PDE10A (defined as 0% binding) in relation to the equilibrated OWG signal after addition of PDE10A at the given concentration (defined as 100% binding). The magnitude of the displacement correlates well with the affinity of the compounds (linear regression line depicted in black) and can thus be used for selection of compounds and preliminary affinity ranking.

Fragment Screening. To illustrate the experimental setup, the time-course of the signals from several wells of a 384-well plate are shown in Figure 3A. After the equilibration of all the wells, a PDE10A solution is added and the specific interaction of the TDC with PDE10A is reflected in a significant increase of the signal which reaches a new equilibrium signal that is arbitrarily set to 100%. The subsequent addition of compounds that are competing with the PDE10A−TDC interaction influences the equilibrium and is reflected in a significant decrease of the binding signal corresponding to the affinity of the compounds (Figure 3B).

CONCLUSIONS

In summary, we have described a new in-solution assay (ISA) approach making use of plate-based optical biosensors that offers an attractive option to SPR. The methodology is based on derivatizing the sensor chip with a tool compound or TDC that selectively recognizes and binds to the target protein at a well-defined site. By making use of structural information, we could drive rational design and synthetic modification of a tool compound by optimal positioning of a suitable functional group and linker to allow for the controlled immobilization of the compound while retaining its high binding affinity to the target; PDE10A. This methodology greatly simplifies assay development and improves robustness. The displacement of a large molecule from the biosensor surface in the presence of a fragment offers an increased dynamic range that allows to reliably detect millimolar binding events at concentrations that are an order of magnitude lower. This in combination with the high-throughput capability of OWG systems appears to be an attractive alternative for primary fragment screening. The use of this system to identify fragment hits for PDE10A, as well as their further characterization and structural elucidation, will be described in a forthcoming report.

EXPERIMENTAL SECTION

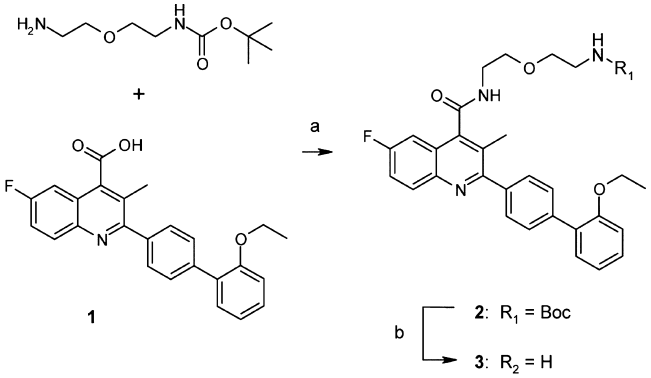
General Synthetic Methods. All reagents and solvents were purchased from commercial sources and used without further purification. Nonaqueous reactions were performed under a nitrogen atmosphere. Reactions conducted in aqueous media were run under an ambient atmosphere unless otherwise noted. Microwave irradiation was carried out in a Personal Chemistry Emrys Optimizer microwave. Flash column chromatography was performed using silica gel (MP, 32−63 μm, 60 Å) or using silica gel cartridges (GraceResolv or RediSep normal phase disposable flash columns) on an ISCO Companion. Reverse phase preparative HPLC purification was performed with an Agilent 1200 LC using a Gemini C18 column (250 mm × 21.2 mm, 10 μm particle diameter). Conditions: mobile phase A was 2.5 mM aqueous ammonium bicarbonate solution, and mobile phase B was acetonitrile. A gradient was run from 30 to 90% B over 16 min at a column flow rate of 65 mL/min. UV detection was recorded at 254 and 220 mm. All final compounds were determined to be ≥95% purity by LCMS and ^{1}H NMR unless specifically mentioned. Proton NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer or a Bruker DRX 300 MHz spectrometer. Chemical shifts are reported in ppm relative to tetramethylsilane as an internal standard. Apparent peak multiplicities are described as s (singlet), br s

dx.doi.org/10.1021/jm301665y | J. Med. Chem. 2013, 56, 3228−3234

(broad singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), or m (multiplet). Coupling constants (*J*) are reported in hertz (Hz). High resolution LCMS analyses were conducted on an Agilent TOF 6210 MS using a Sorbax SB-C8 column (2.1 mm × 30 mm, 1.8 μm particle diameter) at 60 °C (Agilent 1200 LC). Conditions: mobile phase A was water:acetonitrile:formic acid (98:2:0.1 v/v/v), and mobile phase B was water:aceteonitrile:formic acid (2:98:0.05 v/v/v). A linear gradient was run from 5 to 95% B over 1.5 min and held at 95% for 0.4 min. The column flow rate was 1.2 mL/min. Diode array UV detection was an averaged signal recorded from 210 to 320 nm. Retention time (t_R) was reported in minutes (min). All final compounds that were tested in biological assays had purity >95% as determined by analytical HPLC using at least two different sets of conditions.

2-(2′-Ethoxybiphenyl-4-yl)-6-fluoro-3-methylquinoline-4-carboxylic Acid[1] *(**2**).* **2** was prepared from 5-fluoroindoline-2,3-dione and 1-(4-bromophenyl)propan-1-one using the Pfitzinger reaction[2] according to literature methods. Compound **2** was coupled with *N*-Boc-2,2′-oxybis(ethylamine) (**3a**) and then deprotected to afford **4** (Scheme 1).

Scheme 1[a]



[a]Reagents and conditions: (a) TBTU, HOBT, DIPEA, DMF, RT, 66%; (b) HCl, 89%.

*tert-Butyl 2-(2-(2-(2′-Ethoxybiphenyl-4-yl)-6-fluoro-3-methylquinoline-4-carboxamido)ethoxy)ethylcarbamate (**2**).* A mixture of 2-(2′-ethoxybiphenyl-4-yl)-6-fluoro-3-methylquinoline-4-carboxylic acid[1] (**1**) (0.075 g, 0.19 mmol), *tert*-butyl 2-(2-aminoethoxy)-ethylcarbamate[3] (0.064 g, 0.31 mmol), and HOBT hydrate (0.036 g, 0.24 mmol) in DMF (2.5 mL) was treated with TBTU (0.076 g, 0.24 mmol) and diisopropylethylamine (0.100 mL, 0.57 mmol) and then stirred at room temperature overnight. Additional TBTU (0.035 g) was added, and stirring continued for 2 h. DMF was removed under vacuum. The concentrate was partitioned between aqueous potassium carbonate solution and ethyl acetate. The organic portion was washed (water, brine), dried (magnesium sulfate), filtered, and evaporated. The residue was purified by flash column chromatography on silica gel using 0−50% ethyl acetate in hexane as eluent to provide the desired product as an oil (0.073 g, 66%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.20−1.35 (m, 9 H), 1.37 (t, *J* = 7.0 Hz, 3 H), 2.51 (s, 3 H), 3.27−3.36 (m, 2 H), 3.58 (t, *J* = 5.2 Hz, 2 H), 3.71−3.79 (m, 2 H), 3.78−3.86 (m, 2 H), 4.08 (q, *J* = 7.0 Hz, 2 H), 4.75 (br s, 1 H), 6.58 (br s, 1 H), 6.97−7.09 (m, 2 H), 7.27−7.35 (m, 1 H), 7.38 (dd, *J* = 7.5, 1.7 Hz, 1 H), 7.41−7.52 (m, 2 H), 7.58 (d, *J* = 8.2 Hz, 2 H), 7.69 (d, *J* = 8.2 Hz, 2 H), 8.13 (dd, *J* = 9.0, 5.3 Hz, 1 H). MS *m*/*z* (ES^+), $(M + H)^+$, HRMS (calcd) for $C_{34}H_{38}FN_3O_5$ = 588.28683; HRMS (obsd) = 588.28583. HPLC t_R = 1.68 min.

*N-(2-(2-Aminoethoxy)ethyl)-2-(2′-ethoxybiphenyl-4-yl)-6-fluoro-3-methylquinoline-4-carboxamide (**3**).* A solution of *tert*-butyl 2-(2-(2-(2′-ethoxybiphenyl-4-yl)-6-fluoro-3-methylquinoline-4-carboxamido)ethoxy)ethylcarbamate (**2**) (0.058 g, 0.10 mmol) in methanol (3.0 mL) was treated with 3N aqueous hydrochloric acid (3.0 mL, 9.0 mmol) and concentrated hydrochloric acid (0.5 mL, 6.0 mmol). After stirring at room temperature for 1 h, the reaction mixture was concentrated under reduced pressure. The resulting aqueous material was further concentrated with additional methanol (3×) to provide a yellow oil that solidified when placed under vacuum. The solid hydrochloride salt was taken up in methanol (5 mL), treated with MP-carbonate (0.5 g, Aldrich, typical loading 2.5−3.5 mmol N/g)), and stirred at room temperature for 30 min. The suspension was filtered and then concentrated under reduced pressure to an oil. The crude material was purified by flash column chromatography on silica gel using a gradient mixture of 0−10% solvent A in solvent B, where solvent A was a 2 M solution of ammonia in methanol and solvent B was dichloromethane, to provide the product as an oil. The oil was taken up in 1:1 acetonitrile/water (2 mL) and lyophilized to provide the desired product as a solid (0.043 g, 89%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.37 (t, *J* = 7.0 Hz, 3 H), 1.52 (br s, 2 H), 2.50 (s, 3 H), 2.87 (br s, 2 H), 3.56 (t, *J* = 5.2 Hz, 2 H), 3.70−3.89 (m, 4 H), 4.08 (q, *J* = 7.0 Hz, 2 H), 6.66 (t, *J* = 5.3 Hz, 1 H), 6.94−7.10 (m, 2 H), 7.28−7.36 (m, 1 H), 7.38 (dd, *J* = 7.5, 1.7 Hz, 1 H), 7.41−7.53 (m, 2 H), 7.58 (d, *J* = 8.2 Hz, 2 H), 7.69 (d, *J* = 8.2 Hz, 2 H), 8.13 (dd, *J* = 9.2, 5.2 Hz, 1 H). MS *m*/*z* (ES^+), $(M + H)^+$, HRMS (calcd) for $C_{29}H_{30}FN_3O_3$ = 488.23440; HRMS (obs) = 488.23447. HPLC t_R = 1.37 min.

Cloning and Expression of PDE10A 449−789. The pET21MDX7/hPDE10A clone was cotransformed with protein chaperon vector pKJE8 (TaKaRa Bio Inc.) into BL21-Gold(DE3) cells. Coexpression was done following the protocols provided by TaKaRa Bio Inc. Expression of protein chaperons was induced using 1 mg/mL arabinose and 5 ng/mL tetracycline at a culture OD600 of 0.3. Expression of target protein was induced by an addition of 0.1 mM IPTG at an OD600 of 0.6. After induction, the cell culture was grown at 16 °C overnight.

Protein Purification. Cells were harvested and resuspended into lysis buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 1 mM DTT) at the ratio of 5 mL of lysis buffer per gram of cells. Then 1 mM $MgCl_2$ and 1 mM PMSF was added to the suspension before sonication on ice (5 s on-time, 10 s off-time, total 3 min on-time). Cell lysate was centrifuged at 13000 rpm for 60 min at 4 °C. The supernatant was loaded onto a pre-equilibrated 5 mL Ni-NTA column and washed with 10 CV of lysis buffer followed by 20 CV of lysis buffer with 20 mM imidazole. The target protein was subsequently eluted with lysis buffer containing 300 mM imidazole. Protein fractions were collected and analyzed by SDS-PAGE. Fractions containing PDE10A protein were diluted six times using a 50 mM Tris-Cl (pH 7.5) buffer. $ZnCl_2$, $MgCl_2$, AMP, and β-mercaptoenthanol were added to final concentrations of 0.1 mM, 0.1 mM, 1 mM, and 10 mM, respectively. The protein sample was loaded onto a 5 mL HiTrap-QP column (GE Healthcare Life Sciences) pre-equilibrated with 50 mM Tris-Cl, pH 7.5, 50 mM NaCl, and 10 mM β-mercaptoenthanol. A 0−50% gradient of 50 mM Tris-Cl, pH 7.5, 2 M NaCl, 10 mM β-mercaptoenthanol was used for elution. The flow-through was collected for further purification. Fractions containing the target protein were digested with TEV protease at 4 °C overnight while dialyzing against the lysis buffer. Completeness of His-tag cleavage was assessed by SDS-PAGE. As a last purification step, the protein sample was loaded to a pre-equilibrated Ni-NTA column. The flow-through from loading and washing with lysis buffer containing 5, 10, and 20 mM imidazole was collected. After analysis by SDS-PAGE, the purest fractions were pooled.

SPR Inhibition in Solution Assay for Fragment Validation and K_D Estimation. A tethered free amine ligand **3** was covalently linked to the dextran layer on the CM5 sensor chip (GE/Biacore) using the Amine Coupling Kit (GE/Biacore) and performed according to the manufacturer's recommendations. Carboxyl groups on the dextran layer were activated by injecting a 1:1 mixture of 0.5 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC) and 0.5 M *N*-hydroxysuccinimide (NHS) at 5 μL/min for 7 min. The activated carboxyl groups on the control channels were blocked by injecting a 1 M ethanolamine solution for 7 min at 5 μL/min. The tethered ligand was coupled to the biosensor surface by injecting a 100 μM solution of the compound (HBS-P buffer plus 2% DMSO) over the chip surface at 10 μL/min for 12 min. The remaining activated groups were blocked with 1 M ethanolamine. Compounds were tested at either a

dx.doi.org/10.1021/jm301665y | J. Med. Chem. 2013, 56, 3228−3234

single concentration or in a concentration response curve. For the concentration response curve, compounds were serially diluted 1:4 in DMSO to form a seven-point concentration response curve. To a 96-well plate, 1.4 μL/well compound solutions were spotted. The PDE10A catalytic domain at 170 nM was added to the plate and the mixture incubated for 15 min at RT, and then the plate was sealed and placed in the Biacore 3000 instrument. Samples were injected at 35 μL/min within 17 s. The signal generated on the control channel was subtracted from the signal of the channel with the tethered ligand. The value used to calculate percent inhibition of binding was the slope of the response measured 8 s after the start of the injection. The surface of the chip was regenerated by injecting 0.5% SDS for a period of 15 s.

OWG Inhibition in Solution Assay for Fragment Screening. The tethered free amine ligand **3** was covalently linked to the activated surface of a GA4 384-well biosensor plate (SRU Biosystems). Prior to activation, the biosensor plate was washed with deionized water. A mixture of *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) was prepared by mixing 1.9 mL of a 10 mM NHS-solution with 0.2 mL of a 650 mg/mL solution of EDC and adding 17.9 mL of deionized water. From this mixture, 20 μL was added into each well of the 384-well biosensor plate and incubated for 15 min at RT. After incubation, all liquid was quickly removed to apparent dryness by flick and tap and the tethered ligand was coupled to the biosensor surface by adding 20 μL of a 100 μM ligand solution in 10 mM Na-acetate, pH 5.60, to the individual wells. After 30 min incubation, the biosensor plate was washed with HBS-P buffer to remove excess ligand and 20 μL of 0.4 M ethanolamine was added to block remaining activated groups for 30 min. Thereafter, the plate was washed extensively with HBS-P buffer and the biosensor was finally stabilized with HBS-P plus 1% DMSO. Some wells of the plate have been selected as control wells by omitting the addition of the ligand solution after the activation step.

The fragment screening was performed by first preparing a 1 mM solution of the selected fragments in HBS-P plus 1% DMSO. The biosensor plate was equilibrated with 22.5 μL of HBS-P plus 1% DMSO, and a baseline read was obtained with a sampling rate of two data points per minute. Then 4.5 μL of a 12 μM PDE10A solution in a matching buffer was added, and a new equilibrium signal was obtained after 25−30 min, which was typically within the range of 500−600 pm. Thereafter, 3 μL of the fragment solution was added to reach a final fragment concentration of 100 μM. A new equilibrium signal was obtained after another 5−10 min. The displacement of PDE10A from the tethered compound upon addition of the fragment was reported as a percent of residual binding of PDE10A in relation to the maximum equilibrium signal. For the concentration response curve, increasing amounts of compounds were sequentially added into each individual well of the plate and the new equilibrium signal after each addition was used to calculate the percent of residual binding in order to form a four-point concentration response curve. This was used to calculate the IC_{50} values.

■ ASSOCIATED CONTENT

Supporting Information

Information and assay data for the used inhibitors for assay validation. Result of the molecular modeling of **3** based on the structural information for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

FBDD, fragment-based drug design; ISA, inhibition in solution assay; NMR, nuclear magnetic resonance; OWG, optical waveguide grating; PDE10A, phosphodiesterase 10A; PWV, peak wavelength value; SPR, surface plasmon resonance; TDC, target definition compound

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