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Article

Use of BODIPY-Labeled ATP Analogues in the Development and Validation of a Fluorescence Polarization-Based Assay for Screening of Kinase Inhibitors

Bernardo Pereira Moreira, Tom Armstrong, Izabella Cristina Andrade Batista, Naiara Clemente Tavares, Camilla Valente Pires, Marina de Moraes Mourão, Franco H. Falcone,* and Lodewijk V. Dekker

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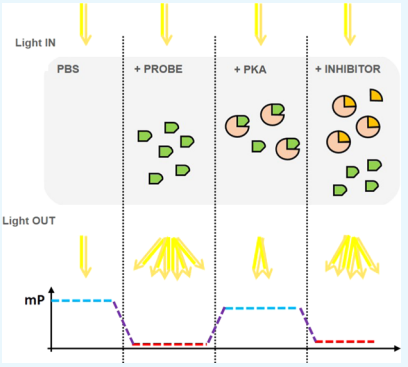
ABSTRACT: The screening of compound libraries to identify small-molecule modulators of specific biological targets is crucial in the process for the discovery of novel therapeutics and molecular probes. Considering the need for simple single-tool assay technologies with which one could monitor "all" kinases, we developed a fluorescence polarization (FP)-based assay to monitor the binding capabilities of protein kinases to ATP. We used BODIPY ATP-y-S as a probe to measure the shift in the polarization of a light beam when passed through the sample. We were able to optimize the assay using commercial Protein Kinase A (PKA) and H7 efficiently inhibited the binding of the probe when added to the reaction. Furthermore, we were able to employ the assay in a high-throughput fashion and validate the screening of a set of small molecules predicted to dock into the ATP-binding site of PKA. This will be useful to screen larger libraries of compounds that may target protein kinases by blocking ATP binding.



INTRODUCTION

A very first step on the path for the discovery of novel therapeutics is the screening of compound libraries in the search for new small-molecule modulators of biological targets. A wide range of robust assay technologies are currently available and, although no single technology is broad enough to address all of the needs in the drug discovery field, most of them are suitable for high-throughput screening (HTS). Nevertheless, the selection of an appropriate primary assay technology can greatly increase the chances of initial hit identification. One applicable technology, fluorescence polarization (FP) is a powerful approach by which alterations in the apparent molecular weight of a fluorescent probe in solution are indicated by changes in the polarization of the sample's emitted light.[1] Since FP was first applied to screening, newly advanced methods have substantially boosted this technology in the field. Advantages of FP assays include the use of an all-in-one (homogeneous) format fitted to study molecular processes in solution, comparatively low cost, availability of time-course analysis, and relatively insensitivity to some type of assay interferences such as inner filter effects.[1−3]

One major application of FP assays relies on the interrogation of biologically relevant molecular interactions, either due to direct binding of a fluorescent probe (tracer) or through competition with an unlabeled species.[2] We recently described a fluorescent tool based on the nonspecific kinase inhibitor staurosporine. The tool was highly suitable for FP applications and allowed monitoring the ATP-binding site of a large number of kinases and in this way enabled identification of inhibitory substances.[4] Although the FP technique is easily adapted for HTS applications, a significant number of kinases could not be measured using this tool. With the emergence of the new therapeutic areas for kinase drug discovery and considering the still considerably large orphan kinase family in, for example, oncology applications, the need arises for simple universal assay technologies with which one could monitor most kinases. Such tools would also allow parallel development of single assay formats for multiple different kinases, which would allow easy side-by-side screening and analysis, as in selectivity screening experiments, for example.

With this in mind, we sought to develop a FP-based system suitable for HTS using solely ATP-γ-S, a nonhydrolyzable derivative of ATP-containing BODIPY FL as the fluorophore chemosensor.[5] Being an ATP derivative, this probe is expected to bind to all kinases, including kinases for which no ready high-throughput assay system exists. Additionally, BODIPY has unique photophysical and photochemistry properties compared to fluorophores such as fluorescein.[6] This probe was originally used in studies of synthesis and transport of

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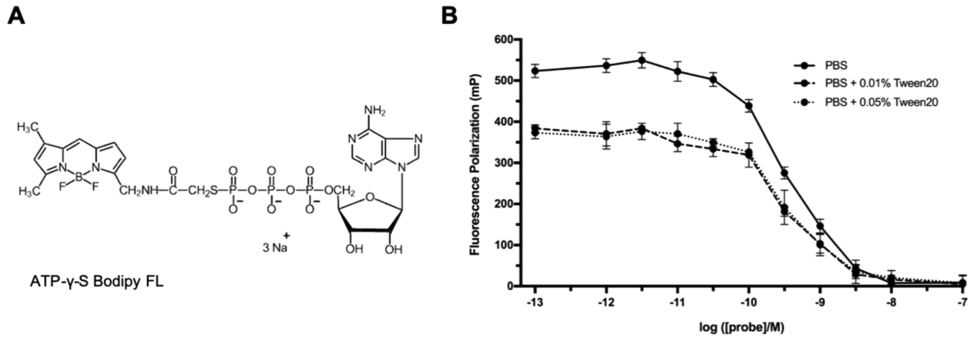

Figure 1. Use of the ATP derivative, ATP-y-S BODIPY FL (SIGMA) in fluorescence polarization assay. (A) Structure of the probe. (B) Fluorescence polarization value in response to increasing amounts of the probe in three different conditions regarding the presence or absence of the detergent (Tween20, Merck) in the buffer. Each point represents the mean ± SD (n = 3). Fluorescence polarization was measured as described in the Methods section.

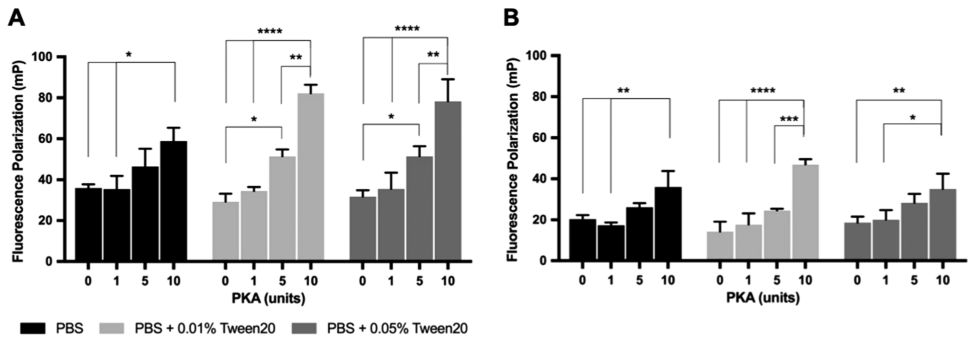

Figure 2. Binding of the probe to the enzyme PKA (Protein Kinase A, Merck) visualized by fluorescence polarization in three different conditions. Two concentrations of the probe (A) 5×10^{-9} M or (B) 1×10^{-8} M were used and increasing amounts of PKA were added to the reaction. Fluorescence polarization was measured as described in the Methods section. Each column represents the mean + SD (n = 2). Data was analyzed using two-way ANOVA ($p < 0.0001$) and Tukey's multiple comparisons test (p-value 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****)).

sphingolipids,[7] though it is currently being used in many fields, from clinical diagnostics and biotechnology to molecular biology and biochemistry. Yet, several applications include the use of BODIPY derivatives, such as sensitizers for living cells, cationic and anionic chemical sensors, medical applications, and electroluminescent agents.[8−10]

To characterize the probe and obtain proof-of-principle for the assay, we employed purified cAMP-dependent protein kinase (PKA), which is well-known and widely available for commercial purposes. Thus, in addition to establishing inhibition by known PKA inhibitors, we also employed the assay in the HTS format to validate an in silico screening of a library of small molecules predicted to dock into the ATP-binding site of PKA.

RESULTS

Kinase Binding Assay. First, we performed an in silico docking prediction of the ATP and the BODIPY FL ATP-γ-S (adenosine 5′-O-(3-thiotriphosphate), BODIPY FL) probe into the nucleotide-binding pocket (G-loop) of the protein kinase A (PKA). In silico docking of ATP recreated the experimentally observed ATP-binding poses with a good deal of accuracy (root-mean-square deviation, RMSD = 1.07 Å) (Figures 1A and S1A,B).[11] Although the probe, in comparison to the natural kinase ligand ATP, contains a relatively large additional fluorophore, in silico docking analysis predicts that it protrudes out of the ATP binding into the solvent-accessible space and does not interact with any part of the protein, apart from a hydrogen bond between the amide NH_2 and T51, hence making this docking very similar to ATP itself and quite stable (Figure S1C).

To optimize the probe concentration to be used in the assay, FP was measured for several concentrations of the probe, ranging from 10^{-13} to 10^{-7} M in three different conditions (Figure 1B). Low amounts of Tween20 (0.01 or 0.05%) were used to reduce (or remove) nonspecific binding of the probe, hence providing a more realistic value of the free/bound probe in the sample. As shown in Figure 1B, around 10^{-10} M of the probe was capable of causing depolarization of the light, with this effect reaching saturation around 10^{-8} M. The presence of the detergent lowered the polarized fluorescence values by roughly 30% when compared to the probe alone (Figure 1B). For application in the assay, we have selected the optimal concentration of the probe that causes a full depolarization signal without saturation of it, thus the chosen optimal concentration corresponded to a range of 5×10^{-9} to 1×10^{-8} M (−8.5 to −8.0 on the x-axis, Figure 1B).

It is known that ATP is the natural ligand of protein kinases; hence if the analogous ATP probe can bind to a protein kinase, the light depolarization previously observed could be reversed. Purified PKA was used as a model kinase to bind to the probe, since it is a standard known kinase largely and commercially available. The probe concentrations corresponding to the optimal depolarization values (5×10^{-9} and 1×10^{-8} M) were chosen to evaluate the binding of PKA in a dose-dependent manner. PKA was able to bind to the probe and cause a significant increase in FP, specifically in the presence of 0.01% Tween20 detergent as from 5 units of the PKA enzyme (Figure

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2). Higher amounts of the enzyme (10 units) caused an increase in polarization values either in the presence or absence of the detergent (Figure 2). The lower values observed when using 1×10^{-8} M of the probe indicate that at this concentration, there might have been some irreversible saturation of the signal caused by the free probe (Figure 2B).

These data indicate that we could use the kinetics between the PKA and the probe to identify compounds that can block that same interaction. To confirm this, we used H7 (Figure 3A), a known ATP-binding site blocker, to validate the present

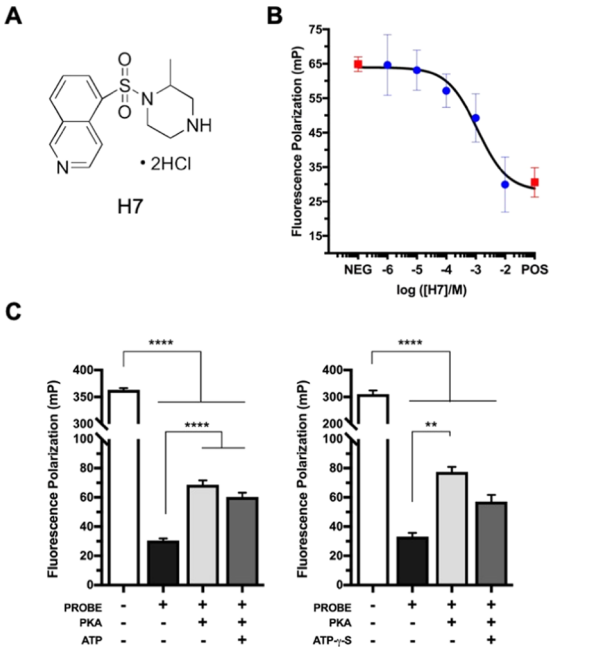

Figure 3. Competition for the nucleotide-binding site of PKA. (A) Structure of H7 inhibitor. (B) Blocking of the binding between the probe and PKA by H7 inhibitor. The probe (5×10^{-9} M) was incubated with 5 units of PKA in the presence of increasing concentrations of H7, and specific binding was measured as described in the Methods section (mean ± SD, n = 3). Data were fitted in GraphPad Prism log(inhibitor) vs response, variable slope (four parameters). (C) Effect of the natural ligand ATP (left) and the ATP-γ-S (right) on the probe−PKA interaction. Data is represented by mean ± SEM from several experiments (n = 8) (left). Fluorescence polarization was measured as described in the Methods section and Tukey's multiple comparison test (p-value <0.0001 (****)) was used for statistical analysis.

FP binding assay. As shown in Figure 3B, H7 inhibited the binding of the probe to PKA in a dose-dependent manner with an IC_{50} value of approximately 1.13 mM. Additionally, a ligand competition experiment was performed by adding unlabeled ATP to assess if it could bind preferentially to the enzyme, displacing the probe and causing the polarization values to decrease. As shown in Figure 3C (left), although ATP appeared to slightly reverse the effect caused by the binding of the probe to PKA, this was not considered statistically significant. The same behavior was observed in the presence of increased PKA (10 units) and when the order of reagent addition was varied (results not shown). Then, we used the same ATP analogous that is present in the BODIPY probe, ATP-γ-S. As observed in Figure 3C (right), the ATP slightly reverses the polarization observed by the ligation probe−PKA.

Since the natural ligand ATP could not preferentially bind to PKA in the presence of the probe, we hypothesized that both molecules would have a high score of affinity to dock in the same site in PKA. Thus, ATP is not an efficient competitor against the probe to bind to PKA. Indeed, predicted docking scores were high for both the ATP and the probe (144.14 and 70.73, respectively) when analyzed by the ChemPLP Score function.

In Silico Docking Analysis of PKA and In Vitro Validation. First, a docking analysis was carried out to identify potential inhibitors of the human PKA. Initial docking was performed on a subset of 10 000 compounds from the University of Nottingham Managed Chemical Compound Collection (MCCC) using both the ChemScore Kinase and ChemPLP Score scoring functions from the Genetic Optimization for Ligand Docking (GOLD) platform.[12] It was noticed that the range of score values obtained for the same 10 000 compounds was significantly different between the two scoring functions (Figure S2A). In each case, the ChemScore Kinase scoring function provided significantly lower scores than the ChemPLP function. Additionally, both functions showed a broad divergence with regards to the highest-scoring compounds as there were only 28 (18.1%) matching compounds in the top 100 and 12 (15.2%) in the top 50 between the two scoring functions (Figure S2B and Tables S1 and S2). Next, we selected the 12 common compounds with the highest score in both scoring functions that matched on the top 50 set (Table 1 and Figure S2B), as well as 5 negatively scored predicted compounds to serve as negative controls (Table 1).

The selected compounds were tested for their inhibitory activity against the PKA−probe binding. As shown, 2 out of 12 compounds significantly blocked PKA−probe binding thus lending support to the in silico prediction (Figure 4A).

Table 1. Comparison of the Scores for 17 Matching Compounds between ChemScore Kinase and ChemPLP Functions[a]

ID	MCCC sample ID	ChemScore Kinase	ChemPLP Score
Positive			
B1	NCC-00066504	62.79	100.77
B2	NCC-00069179	60.16	108.41
B3	NCC-00068966	56.95	99.12
B4	NCC-00063353	55.62	101.23
B5	NCC-00027360	53.02	93.79
B6	NCC-00073056	52.97	92.98
B7	NCC-00071565	52.31	99.53
B8	NCC-00067960	52.13	93.54
B9	NCC-00062976	51.55	97.02
B10	NCC-00063592	51.45	95.70
B11	NCC-00070778	48.47	91.73
B12	NCC-00066895	48.07	95.12
Negative			
N1	NCC-00000100	−28.25	−17.98
N2	NCC-00000176	−5.98	−10.25
N3	NCC-00000179	−5.27	−48.39
N4	NCC-00000267	18.91	−82.98
N5	NCC-00006655	15.14	−29.74

[a]Twelve positively scored compounds were detected in the top 50 list and five negatively scored compounds were selected as negative controls.

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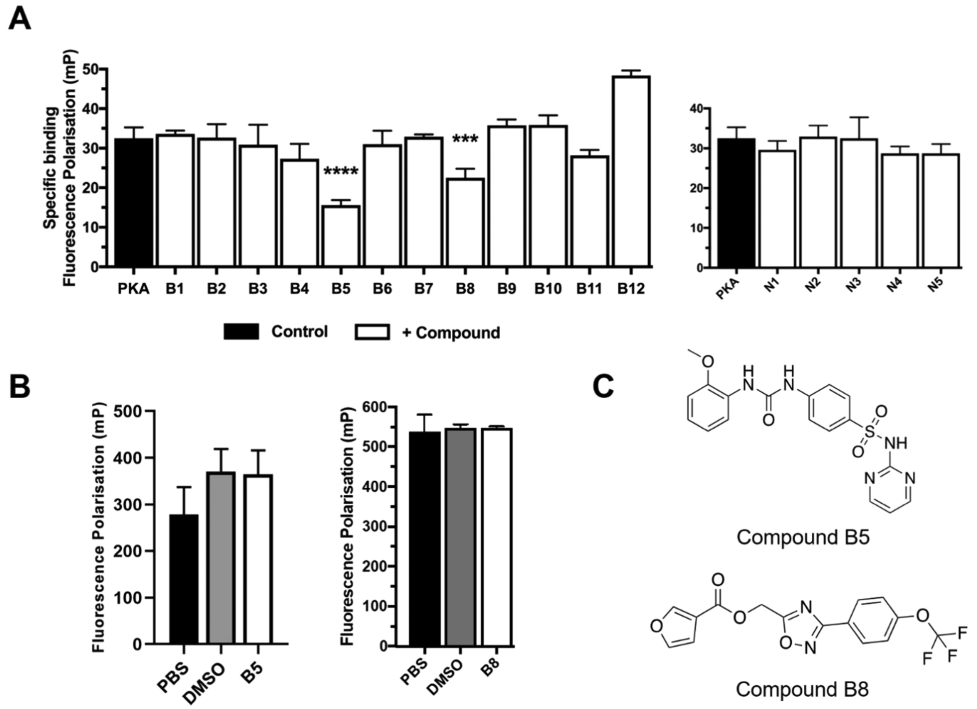

Figure 4. Screening to evaluate the inhibitory activity of several compounds against the binding of the probe to commercial PKA. (A) ATP-γ-S BODIPY probe (5×10^{-9} M) was incubated with 5 units of PKA in the presence of different compounds (10 μM) predicted by both score functions (B1−B12, positive; N1−N5, negative). Values of FP (mP) represent specific binding and it was measured as described in the Methods section (mean ± SD, $n = 3$). Data was analyzed using one-way ANOVA ($p < 0.0001$) and Tukey's multiple comparisons test (p-value 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****)). (B) Fluorescence polarization values for the positive compounds B5 and B8 incubated in phosphate-buffered saline (PBS)/dimethyl sulfoxide (DMSO). Bars represent the average of three replicates. Compounds were tested in 10 μM final concentration. (C) Structure of compounds B5 and B8.

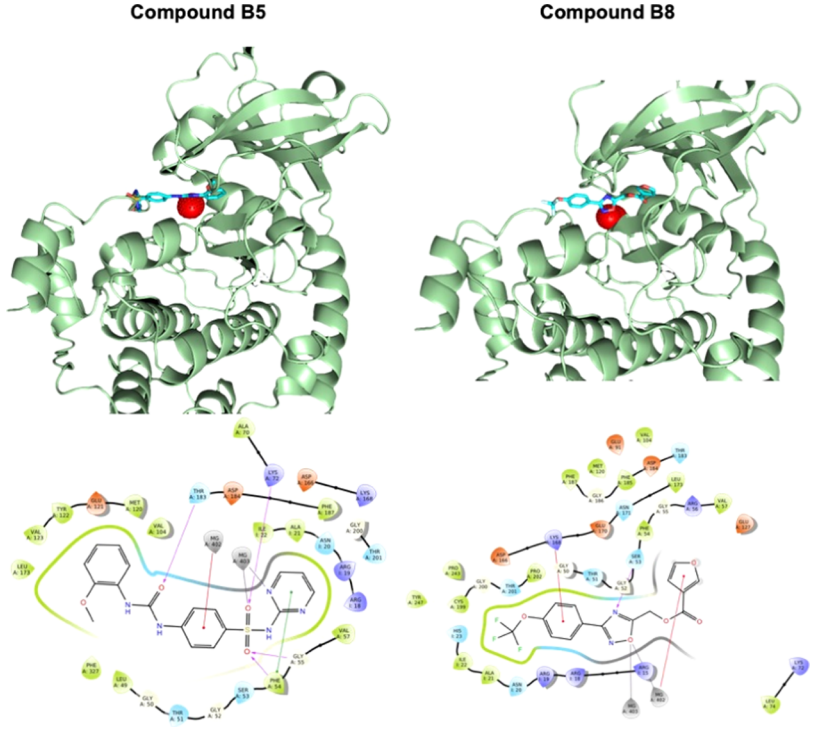

Figure 5. Predicted binding pose of compounds B5 (left) and B8 (right) in the crystal structure of PKA (PDB: 4WB5), showing the similar interactions as seen for the natural ligand ATP. Reference structure: carbon = cyan; oxygen = red; nitrogen = dark blue; fluoride = light blue; sulfur = orange; Mg^{2+} is represented by rounded red shapes. Bottom schematic maps represent the interactions of the compounds B5 (left) and B8 (right) inside the ATP-binding pocket. Amino acids are represented as a three-letter code with its corresponding position in the PKA amino acid sequence. MG = magnesium cation (Mg^{2+}).

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Moreover, the detectable change in the mP value represented the competitive binding of the compound to PKA rather than interference from the compound itself with the probe signal (Figure 4B). The compounds' structure (Figure 4C) and their docking prediction (Figure 5) indicate that they have a strong specific binding to the ATP pocket of PKA. Compound B5 mimics similar interactions as the ATP probe, the urea group interacts with the Mg^{2+} ions, and the methoxy benzene ring occupies a similar space to the adenosine ring in ATP. Compound B8 also interacts with the Mg^{2+} ions through the five-membered ring and the trifluoromethoxy group occupies a hydrophobic region of the binding site (Figure 5).

Additionally, a total of 31 of the top-scoring compounds from each function were also tested in the same conditions previously used for the H7 inhibitor (Figure S3A,B). A total of 7 out of 62 selected compounds significantly inhibited the binding of the probe to PKA. From those, only one presented minor signal interference. The mP value for the compound itself was 21% lower when compared to that of PBS, indicating that this compound itself was able to depolarize the light (Figure S3C).

DISCUSSION

As characterized first for PKA, typical protein kinases are nucleotide-binding proteins.[11] Therefore, the kinase must bind an ATP molecule for the enzyme to undergo a conformational change and switch to an active state, required for substrate binding and catalytic core functioning.[13,14] Thus, molecules that are able to block the docking of ATP into its pocket, either by direct competition or by allosteric regulation, are considered promising protein kinase inhibitors and drug candidates.[15−18] Indeed, the ATP-binding pocket is considered the main focus for inhibitor design,[19] and although it is generally well conserved between kinases, small differences exist in the lining of the binding area that can be exploited to introduce kinase selectivity, as demonstrated by the high level of selectivity now achieved for kinase inhibitors.[20,21]

Here, we describe the development of an ATP-kinase binding evaluation assay using an analogous ATP-containing fluorophore chemosensor, named BODIPY FL ATP-γ-S (adenosine 5′-O-(3-thiotriphosphate), BODIPY FL, sodium salt). This probe is an ATP molecule conjugated with the fluorescent dye known as BODIPY FL (4-4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacine-3-yl).[5] For instance, ATP derivatives of this probe have been used as an indicator to measure ATP influx through the outer membrane of the mitochondria[22] as well as a label for histidine kinases in bacterial two-component systems.[23] Here, we used the probe to measure the shift in the fluorescence polarization caused by the addition of inhibitors that block the ATP binding to protein kinases, specifically to PKA.

Our docking analysis shows that the probe sits at the ATP-binding site of PKA in a similar manner as described for the natural ligand ATP.[21,24] However, the fluorophore protrudes out of the pocket and does not impair the binding of the probe to the enzyme, thus making the use of the probe compatible for this kind of assay. Additionally, we were able to determine the right amount of probe (5×10^{-9} M) to use in order to provide a steady reading value for depolarized light without saturating the signal, similar to what is observed for the theoretical value for free fluorescein (27 mP).[25]

All experiments in this study were carried out in the presence of 0.01% Tween20 in the buffer. Nonionic detergents are generally used as blocking agents, which can help avoid nonspecific binding of the probe and decrease the background signal just as they do in immunoblotting assays.[26] This is in agreement with our data showing that the presence of the detergent promotes specific binding of the probe to PKA.

When the natural ligand was used in competition with the probe, we noticed a slight preference for the ATP rather than the probe. This preference was even higher when ATP-γ-S was used as the competitor. In addition, the same behavior was observed for higher concentrations of ATP, 10 and 50 mM (data not shown). Also, the fact that the known H7 inhibitor was able to efficiently block the binding of the probe indicates that the probe is specifically binding to PKA into the ATP-binding pocket. It is possible, however, that in addition to the ATP-binding site, the BODIPY probe also binds to alternative sites on the PKA molecule, resulting in incomplete displacement by ATP-γ-S, which would only displace the probe bound to the ATP-binding site.

Additionally, in the present study, we performed an in silico analysis to obtain docking scores for a library of compounds that potentially block the ATP-binding site of protein kinases. Docking was performed using the GOLD software package, which includes a built-in kinase scoring function, ChemScore Kinase, that was initially used to score the compounds being investigated. Moreover, a recent article inspecting numerous docking platforms suggested that the ChemPLP scoring is more accurate at reproducing experimental structures than the dedicated ChemScore Kinase function.[27] In an attempt to have a robust analysis, we used both scoring functions, ChemScore Kinase and ChemPLP Score. We observed a significant difference in the scores generated by each of the two algorithms. This suggests that ChemScore Kinase produces empirically lower scores but produces similar trends as to ChemPLP Score. In each case, there were some compounds that scored uniquely high with one of the scoring functions, and biological assessment of these compounds by our assay suggested that the ChemPLP Score scoring function is superior in terms of its ability to identify potential inhibitors.

CONCLUSIONS

The universal FP-based assay developed in the present study is quick and cost effective and may be used as an HTS method to cover libraries for potential new drugs against key kinase targets of many organisms. Furthermore, this assay is not substrate-based, and it is useful mainly for screening compounds that target kinases or any other ATP-binding enzymes. It is therefore developed to be used as a tool to evaluate if a given compound can inhibit the binding of ATP to the enzyme by measuring the fluorescence polarization shift caused by the probe released from the enzyme in the presence of an inhibitor.

METHODS

Fluorescence Polarization Assays. Assays were performed in 384-well black flat-bottom plates in 50 μL final volume of 0.01 M phosphate-buffered saline (PBS), pH 7.4 containing 2 mM magnesium chloride at room temperature. Increased concentrations of BODIPY FL ATP-γ-S (SIGMA) were used to define the ideal concentration for light depolarization in serial dilutions, and it was established the use of 5×10^{-9} M of the probe, unless stated otherwise. Commercial Protein Kinase A catalytic subunit (PKA from

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bovine heart, Merck) was used as the enzyme source, after standardization using 1, 5, and 10 enzyme units, experiments were carried out using 5 units (approximately 500 μg) of enzyme per reaction, unless stated otherwise. A concentration of 0.01% nonionic detergent (Tween20, Merck) was added to the mixture unless stated otherwise. Natural ATP ligand as well as ATP-γ-S were used at 1 mM concentration, unless stated otherwise. Reading of the plates was performed using a PerkinElmer Envision 2104 Multilabel plate-reading spectrophotometer using 480 nm excitation and 535 nm emission filters, suitable for measurement of fluorescein. Fluorescence polarization was determined by measuring the parallel and perpendicular fluorescence emission intensity with respect to the polarized excitation light and is expressed in millipolarization (mP) units. Specific PKA inhibitor H7 was purchased from Merck.

In Silico Docking Analyses for PKA. The in silico docking analyses for PKA were performed using the Genetic Optimization for Ligand Docking (GOLD) platform from The Cambridge Crystallographic Data Centre (CCDC) and a selection of 10 000 compounds available within the University of Nottingham Managed Chemical Compound Library (MCCC). The structure 4WB5, PKA in complex with ATP was retrieved from the Protein Data Bank database (PDB, https://www.rcsb.org/) and prepared using the Protein Preparation Wizard Tool in Maestro (Schrödinger Release 2018-4: Maestro, Schrödinger, LLC, New York, NY, 2018). The structure was subject to H-bond optimization and energetic minimization using the OPLS3 force field. The resulting structure was then saved as a PDB file for future use. An SDF file containing all of the ligand available in the MCCC was obtained, and these structures were prepared using the LigPrep tool within Maestro. The various protonation states for each molecule were calculated between pH 7.0 ± 2.0, and the resulting structures were saved as an SDF file for future use. Docking was performed using GOLD (5.6) using the standard search efficiency settings. The active site was defined by the native ATP ligand in the 4WB5 crystal structure. The solution structures were saved in the SDF file format.

Compounds. Compounds were obtained from a library of small molecules from the MCCC Library provided at the Centre of Biomolecular Sciences (University of Nottingham). All compounds were dissolved in DMSO and were used at a final concentration of 10 μM. The requested compound codes are: NCC-00066504, NCC-00069179, NCC-00068966, NCC-00063353, NCC-00027360, NCC-00073056, NCC-00071565, NCC-00067960, NCC-00062976, NCC-00063592, NCC-00070778, NCC-00066895, NCC-00000100, NCC-00000176, NCC-00000179, NCC-00000267, NCC-00006655, NCC-00067772, NCC-00068009, NCC-00067155, NCC-00075821, NCC-00073101, NCC-00004123, NCC-00073207, NCC-00004578, NCC-00069395, NCC-00071160, NCC-00070853, NCC-00071708, NCC-00016314, NCC-00040680, NCC-00066365, NCC-00009799, NCC-00074851, NCC-00020178, NCC-00000041, NCC-00000037, NCC-00066879, NCC-00067308, NCC-00033008, NCC-00071549, NCC-00072265, NCC-00067150, NCC-00063994, NCC-00070051, NCC-00063034, NCC-00072272, NCC-00070760, NCC-00069625, NCC-00076211, NCC-00029407, NCC-00069711, NCC-00013234, NCC-00014721, NCC-00034842, NCC-00018391, NCC-00069640, NCC-00041365, NCC-00067733, NCC-00066507, NCC-00072319, NCC-00065651, NCC-00066826, NCC-00072585, NCC-00069782, NCC-00066588, NCC-00066671, NCC-00042920, NCC-00072060, NCC-00074246, NCC-00066984, NCC-00073991, NCC-00069672, NCC-00072007, NCC-00067319, NCC-00012226, NCC-00069605, NCC-00070551, and NCC-00066958.

Statistical Analysis. All data were analyzed using GraphPad Prism version 7.00 for Mac (GraphPad Software, La Jolla, California, www.graphpad.com), and statistical analyses are stated where appropriate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b03344.

> Predicted binding pose of the ATP and the probe into PKA; docking scores and data analysis for the compounds' in silico screening; binding activity of additional compounds; and tables with the top 50 compounds with better docking scores for each scoring function (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Franco H. Falcone − *Biomedizinisches Forschungszentrum Seltersberg, Institut für Parasitologie, Justus-Liebig-Universität Gießen, 35392 Gießen, Germany; School of Pharmacy, Division of Molecular Therapeutics and Formulation, University of Nottingham, Nottingham NG7 2RD, United Kingdom;* orcid.org/0000-0002-1732-9932; Email: franco.falcone@vetmed.uni-giessen.de

Authors

Bernardo Pereira Moreira − *Biomedizinisches Forschungszentrum Seltersberg, Institut für Parasitologie, Justus-Liebig-Universität Gießen, 35392 Gießen, Germany; Instituto de Pesquisas René Rachou, Fundação Oswaldo Cruz—FIOCRUZ, Belo Horizonte 30190-002, Minas Gerais, Brazil; School of Pharmacy, Division of Molecular Therapeutics and Formulation, University of Nottingham, Nottingham NG7 2RD, United Kingdom;* orcid.org/0000-0001-5266-5336

Tom Armstrong − *School of Chemistry, University of Nottingham, Nottingham NG7 2RD, United Kingdom*

Izabella Cristina Andrade Batista − *Instituto de Pesquisas René Rachou, Fundação Oswaldo Cruz—FIOCRUZ, Belo Horizonte 30190-002, Minas Gerais, Brazil; School of Pharmacy, Division of Molecular Therapeutics and Formulation, University of Nottingham, Nottingham NG7 2RD, United Kingdom*

Naiara Clemente Tavares − *Instituto de Pesquisas René Rachou, Fundação Oswaldo Cruz—FIOCRUZ, Belo Horizonte 30190-002, Minas Gerais, Brazil; School of Pharmacy, Division of Molecular Therapeutics and Formulation, University of Nottingham, Nottingham NG7 2RD, United Kingdom*

Camilla Valente Pires − *Instituto de Pesquisas René Rachou, Fundação Oswaldo Cruz—FIOCRUZ, Belo Horizonte 30190-002, Minas Gerais, Brazil; School of Pharmacy, Division of Molecular Therapeutics and Formulation, University of Nottingham, Nottingham NG7 2RD, United Kingdom*

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Marina de Moraes Mourão − *Instituto de Pesquisas René Rachou, Fundação Oswaldo Cruz—FIOCRUZ, Belo Horizonte 30190-002, Minas Gerais, Brazil*

Lodewijk V. Dekker − *School of Pharmacy, Division of Biomolecular Science and Medicinal Chemistry, University of Nottingham, Nottingham NG7 2RD, United Kingdom*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.9b03344

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Notes

The authors declare no competing financial interest.

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