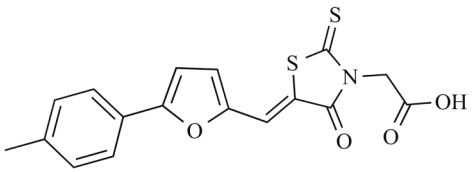
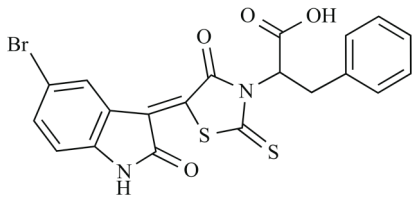
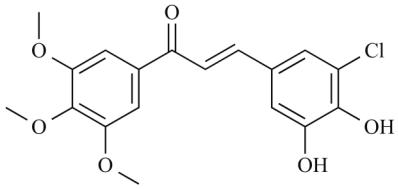
Table 1. Hill Slopes, IC50s, and Maximum Inhibition were Calculated for each of the 3 Reproducible Hits in the Presence of Full-Length AMPK and the Regulatory Fragment (Data in Parentheses).

Positive hit	Vendor, Catalog Number	Structure	Hill Slope	IC50, μM
1	Vitas-M Laboratory, Ltd., STK740822		1.4 (1.4)	2.9 (1.6)
2	Vitas-M Laboratory, Ltd., STL035166		1.3 (1.4)	2.7 (1.5)
3	Enamine, Ltd., Z64358107		1.1 (2.6)	0.3 (0.2)

To identify molecules that bind the regulatory region of AMPK, we designed a fluorescence-based assay using readily purified His-tagged protein and commercially available MANT-ADP, an analog of AMPK's primary regulatory nucleotide [1]. Binding of MANT-labeled analogs to nucleotide-binding sites on protein can be easily detected by an increase in fluorescence [6]. No crystal structures of AMPK with MANT-ADP have been published, but structures of the regulatory fragment soaked with MANT-AMP indicate binding at Sites 1 and 3 [14]. Because full-length AMPK provided a slightly larger assay window in preliminary experiments, we decided to use full-length AMPK for our primary screen and the regulatory fragment for our secondary screen. Although full-length AMPK has three available nucleotide-binding sites (one on AMPK-α and two on AMPK-γ), fluorescence studies have shown that MANT-ATP binds only two sites on both the regulatory fragment and full-length AMPK [6]. Because our assay measures MANT-ADP fluorescence and not enzyme activity, the assay could identify positive hits that may be overlooked by peptide phosphorylation kinase assays. Kinase assays, such as the ones used to identify the drugs Compound C and A-592107, are biased toward the identification of ATP-competitive inhibitors that bind AMPK-α and AMP-mimetics that increase the activity of p-AMPK, respectively [4, 26, 27]. (A-592107 was later used as a structural template for the optimized synthetic analog A-769662, but this drug was shown to be ineffective against AMPK heterotrimers containing AMPK-β_2 [27, 28]). Because purified protein phosphatases are not included in kinase activity assays, these assays are biased away from the identification of protective ADP-mimetics [4]. Our fluorescence-based assay, on the other hand, can identify molecules that displace MANT-ADP. Orthogonal follow-up assays, including *in vitro* assays in the presence of phosphatases, can then determine if positive hits are allosteric inhibitors, allosteric activators, dephosphorylation inhibitors, or candidates for structure-activity relationship (SAR) optimization.

To optimize MANT-ADP's protein-bound fluorescent signal, we tested 16 unique assay mixtures in a full-factorial screening design of experiments study (Fig. **2A**). We subsequently decided to minimize our assay's ionic strength to increase the protein-bound signal of MANT-ADP. Our full-factorial data agrees with Saiu's and Xiao *et al.*'s data, which show an inverse relationship between salt concentration and nucleotide binding affinities for AMPK-γ [6, 14]. Published nucleotide binding studies have, in fact, been performed in low ionic strength conditions [6]. Further characterization of positive hits from this assay should, however, be completed under more physiological conditions.

Although increasing concentrations of MANT-ADP and AMPK would have produced a larger assay window, we decided to continue with low concentrations of each so that all MANT-ADP-binding sites would be saturated by positive hits when screening libraries at 5 μM or less (Fig. **2B**). Instead of maximizing the Z'-factor, we prioritized the sensitivity of the assay to small molecule detection. Despite the low signal-to-background ratio, the assay was robust and identified positive hits that dose-dependently inhibited MANT-ADP fluorescence. It is important to note that our experiments used a standard Coumarin (360 nm excitation and 460 nm emission) filter set. The greatest difference between MANT-ADP's bound and un-bound signals, however, occurs close to 440 nm (Fig. **1B**). Switching from a 460 nm to a 440 nm filter could be an alternative way to increase the assay window and/or Z'-factor without changing the biochemistry, but has the disadvantage of potentially increasing the interference of autofluorescent compounds in the near-ultraviolet range.

One substantial drawback to using MANT-ADP as a fluorescent probe is the potential for false negatives due to the high percentage of autofluorescent compounds near 460 nm [29-31]. Thus, our assay cannot distinguish between fluorescent true negatives and fluorescent false negatives that inhibit binding of MANT-ADP to AMPK. A red-shifted fluorophore, such as TNP-ADP, could reduce this problem. Substitution of 0.1 μM MANT-ADP with 0.1 μM TNP-ADP, however, failed to produce an assay window (Fig. **S2**). Furthermore, trinitrophenylation replaces both of the ribosyl hydroxyl groups on nucleotides [32]. Because these hydroxyl groups form hydrogen bonds with aspartate residues on AMPK-γ, trinitrophenylation could decrease the affinity of adenine nucleotides to AMPK-γ [9, 14]. In their discussion of a TNP-ATP-based screening assay, Guarnieri *et al.* suggested using MANT-ATP as an alternative because TNP-ATP had such a weak binding affinity to their target kinase [22]. To compensate for TNP-ATP's high K_D, the authors increased their probe, protein, and library concentrations to 150, 100, and 100 μM, respectively [22]. Indeed, we had to increase TNP-ADP to 100 μM to observe a window between the probe's protein-bound and unbound fluorescent signals (Fig. **S2**). A better probe may be a red-shifted Alexa Fluor-ADP analog that retains one of the ribosyl hydroxyls needed for binding. Alexa Fluor-ADP is not environment-sensitive, however, so one would need to use fluorescence polarization to detect binding of the probe to AMPK.

Finally, our library screen yielded a relatively low hit rate of 0.04% with a 60% confirmation rate. Multiple factors may have contributed to our low hit rate. First, nearly one-fourth of our library molecules produced fluorescent signals that were higher than the average DMSO control signal and, therefore, limited the screening efficiency, thereby lowering the hit rate. These molecules, which are shown on our scatter plot as having negative inhibition (Fig. **4**), are most likely autofluorescent and may include false negatives that inhibit binding of MANT-ADP to AMPK. It is unlikely that these molecules could be increasing fluorescence by promoting interactions between AMPK and MANT-ADP, as the protein concentration exceeds the MANT-ADP concentration by almost 5-fold. Any MANT-ADP molecules that dissociate from one AMPK heterotrimer should bind immediately to another heterotrimer. Finally, the small molecule library we screened herein includes molecules that were pre-selected based on structural similarities to known kinase inhibitors and could, therefore, bind kinase domains [16, 17]. If MANT-ADP binds only the nucleotide-binding sites on AMPK-γ, then the assay will be biased toward the discovery of small molecules that bind the regulatory region of AMPK and away from the discovery of promiscuous kinase modulators that bind the canonical, catalytic ATP-binding pocket found throughout the kinome. Our low hit rate, therefore, may reaffirm this bias and may be much lower than the hit rate for future screens of more diverse small molecule libraries.

CONCLUSION

This screen has identified small molecules that are capable of inhibiting MANT-ADP's protein-bound fluorescent signal. Small molecules that dose-dependently inhibit MANT-ADP fluorescence may include AMPK inhibitors or activators. Orthogonal follow-up studies with these molecules will demonstrate if they can also displace ADP and will be performed both biochemically *in vitro* and then in cell lines for AMPK modulation in a cell intact physiological environment. Positive hits that bind the regulatory fragment but do not regulate AMPK may be used as structural templates for SAR optimization. Ultimately, the best way to determine if these molecules actually regulate AMPK is to test them in both cell-based assays and *in vitro* kinase assays before evaluating *in vivo* function.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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