A Novel Method for Screening Adenosine Receptor Specific Agonists for Use in Adenosine Drug Development

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Agonists that target the A1, A2A, A2B and A3 adenosine receptors have potential to be potent treatment options for a number of diseases, including autoimmune diseases, cardiovascular disease and cancer. Because each of these adenosine receptors plays a distinct role throughout the body, obtaining highly specific receptor agonists is essential. Of these receptors, the adenosine A2AR and A2BR share many sequence and structural similarities but highly differ in their responses to inflammatory stimuli. Our laboratory, using a combination of specially developed cell lines and calcium release analysis hardware, has created a new and faster method for determining specificity of synthetic adenosine agonist compounds for the A2AR and A2BR receptors in human cells. A2A receptor expression was effectively removed from K562 cells, resulting in the development of a distinct null line. Using HIV-lentivector and plasmid DNA transfection, we also developed A2A and A2B receptor over-expressing lines. As adenosine is known to cause changes in intracellular calcium levels upon addition to cell culture, calcium release can be determined in these cell lines upon compound addition, providing a functional readout of receptor activation and allowing us to isolate the most specific adenosine agonist compounds.

Adenosine receptors play a regulatory role throughout the body, having been shown to be active in virtually every organ system studied. Extracellular adenosine accumulated in response to metabolic stress and/or cell damage has one of four effects on tissue protection and repair: (a) increasing oxygen supply to damaged tissues; (b) protection against ischemic damage due to cell conditioning; (c) countering inflammatory responses; (d) and promoting angiogenesis1–4. Due to adenosine's essential role in modulating tissue homeostasis, it is also important in a number of diseases, including neurological disorders, cardiovascular disease and autoimmune responses in the body5–7.

Adenosine modulates these different effects through binding to one or more of its four G protein coupled receptors (A1, A2A, A2B and A3). Adenosine binding with the A1 receptor decreases heart rate and neuronal activity, while all adenosine receptors have roles in many types of cancer8–12. In general, anti-inflammatory aspects of adenosine are associated with its binding to the A2AR receptor (A2AR)13–15; alternatively, adenosine binding to the A2BR receptor (A2BR) has been associated with pro-inflammatory actions of adenosine16–18. The adenosine A2AR and A2BR share many sequence and structural similarities (Fig. 1) but have opposing immunological responses to inflammatory stimuli, indicating that therapies targeting either the A2AR or A2BR need to be very specific to achieve the desired therapeutic effect19,20.

Current methods of determining receptor activity and binding of adenosine analogues for therapeutic use against specific receptors are ponderous and time-consuming. These methods often involve the use of radio-ligand binding assays followed by competition and saturation binding experiments. This is typically followed by toxicity assays in cell culture and animal models to assess safety and efficacy. Alternatively, analysis of analogues for receptor affinity by hit identification using computational software is faster and safer, but less

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reliable for true biological effects of the analogue molecule in the cell. Further, computational analysis must be followed by in vitro and in vivo toxicity experiments. Thus development of a new method for quick and safe characterization of novel adenosine analogues is needed to streamline adenosine drug development.

Our laboratory has designed a new method of A2AR and A2BR agonist analogue characterization utilizing engineered cell lines and calcium flux assays, a method that can be expanded to incorporate additional adenosine receptors. Using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology for targeted genome editing, A2AR expression was knocked out in the K562 cell line. Additionally, because of the homology of the A2AR and A2BR and the potential for adenosine analogues to bind indiscriminately between the two, A2AR and A2BR over-expressing K562 lines were created utilizing HIV-lentivector. As adenosine is known to cause changes in intracellular calcium levels upon addition to cell culture, calcium flux assays were employed with these cell lines to determine specificity21–24. Using a microplate reader, we were able to read changes in intracellular free calcium levels upon compound addition to both our knockout and over-expressing lines, allowing us to determine off-target binding of A2AR and A2BR agonists of interest. Those compounds found to be specific to the A2AR are then screened for efficacy using human naïve T-cells, evaluating them on their ability to induce differentiation of FoxP3+ regulatory T-cells, a cell type associated with immune tolerance and anti-inflammatory responses14. Overall, this new method will allow for faster characterization of adenosine receptor binding partners and will accelerate drug discovery.

**Results**

**Development of a CRISPR/Cas9 Vector for Knockout of the A2AR in K562 Cells.** Recently, CRISPR/Cas9 has emerged as an efficient and reliable way to perform targeted genome editing25. Delivery of the Cas9 nuclease and appropriate gRNAs into a cell allows the cell's genome to be cut at a specific location, inactivating existing genes or adding new ones. The human hematopoietic tumor line K562 is a non-adherent easily maintained cell line convenient for our studies because it natively expresses low levels of the A2AR and also has almost no expression of the A2BR. To create a line that strongly expresses the A2AR we developed a CRISPR/Cas9 strategy to engineer a K562 line in which A2AR expression was fully knocked out.

The A2AR gene is located on chromosome 22q11.23 and is approximately 2.6 kbp long (Fig. 2a). Several gRNA sequences for CRISPR/Cas9 mediated knockout were found for A2AR with varying quality scores (Fig. 2b, left). gRNA #1 (5′-CTCCACCGTGATGTACACCG-3′) was selected as it has a quality score of 91, indicating minimal off-target sites (Fig. 2b, upper right). BamHI cut sites were added to gRNA #1 for insertion into the plentiCRISPR loxP vector plasmid DNA (Fig. 2b, bottom right). Figure 2c shows the plentiCRISPR loxP construct and cut site on the A2AR gene. Cas9 cutting is targeted to exon 1 of the A2AR gene (black letters) rather than exon 2 (blue letters) to ensure efficient knockout of the gene. Using the T7 E1 enzyme for insertion and deletion (INDEL) assay, an indicator of CRISPR targeting efficiency, we observed 82% of transduced K562 cells with the modified A2AR locus, indicating high targeting efficiency of the plentiCRISPR loxP A2AR virus (Fig. 2d).

**Creation of a Stable A2A Receptor Knockout Line in K562 Cells.** The plentiCRISPR loxP A2AR virus was transduced into K562 cells to produce a stable A2AR knockout line. Flow cytometry on transduced cells for efficiency of transduction showed 96% of cells successfully transduced after puromycin selection (Fig. 3a). After selection, the A2AR knockout K562 cells were transfected with the pcDNA3.1 ZEO T2A CRE-GFP plasmid to remove the loxP cassette. The proviral vector cassette was successfully removed from 32% of transfected cells as

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**Figure 1.** Sequence homology between the four adenosine receptors. The A2A and A2B receptors show the most sequence homology among the adenosine receptors, with these two receptors having a 61% identity.
shown by flow cytometry (Fig. 3b). Cells were then selected by zeocin for isolation of cells without cassettes. Using RT-PCR, copy number analysis of selected subclones indicated that clones 2, 3, 6, 8, 9, 11 and 12 had total excision of the loxP cassette (Fig. 3c, left). Additional sequencing analysis confirmed that clone 12 contains a knockout of A2AR on both alleles, with one base (T) insertion on one allele and an 18 base deletion on the other (Fig. 3c, right). Clone 12 was selected for line continuation (A2AR KO).

**Development of Stable K562 Cell Lines Overexpressing A2A and A2B Receptors.** Because native K562 cells have very little endogenous expression of the A2B receptor as determined by calcium flux assay (Fig. 4), we did not first knock out the A2AR to create the K562 line overexpressing the A2A receptor. The pCL20i4r-EF1a-hgcOPT vector plasmid DNA. BsmBI cut sites were added to gRNA #1 to facilitate insertion. (c) Top, Cas9 cut site in the A2AR gene. Bottom, map of the plentiCRISPR construct. (d) T7 E1 INDEL assay for CRISPR targeting efficiency. Lanes 1 and 5, universal marker. Lane 2, PCR product. Lanes 3 and 4, PCR product after denaturing and renaturing, without (3) and with (4) T7 E1 enzyme. Arrowheads indicate T7 E1 cutting.

Figure 2. CRISPR vector design for knockout of A2AR in K562 Cells. (a) Map of human chromosomes, with chromosome 22q11.23 highlighted, showing the location of the A2AR gene. Additional arrowheads indicate genes with similar regions to the A2AR gene, including the A2BR gene on chromosome 17. (b) Left, guide-RNAs found for the A2AR gene. Right, guide sequence #1 was selected for insertion into the plentiCRISPR loxP vector plasmid DNA. BsmBI cut sites were added to gRNA #1 to facilitate insertion. (c) Top, Cas9 cut site in the A2AR gene. Bottom, map of the plentiCRISPR construct. (d) T7 E1 INDEL assay for CRISPR targeting efficiency. Lanes 1 and 5, universal marker. Lane 2, PCR product. Lanes 3 and 4, PCR product after denaturing and renaturing, without (3) and with (4) T7 E1 enzyme. Arrowheads indicate T7 E1 cutting.
Figure 3. Development of a stable A2AR knockout cell line in K562 cells using CRISPR. (a) Flow cytometry for Cas9 expression in K562 cells before and after transduction with the plentiCRISPR loxP A2AR. After puromycin selection to remove non-transduced cells, 96% of cells were successfully transduced. (b) Flow cytometry for removal of the loxP cassette using the pcDNA3.1 ZEO T2A CRE-GFP plasmid. 32% of cells express GFP, indicating successful removal of the proviral vector cassette. These cells were further selected using zeocin for isolation of cassette free cells. (c) Left, RT-PCR of clones selected from loxP cassette-free A2AR knockout K562 cells. Right, sequencing of clone 12 reveals a one base (T) insertion on one allele and an 18 base deletion on the other for homologous knockout of the A2AR gene.

Figure 4. Calcium flux assay for the BAY60 A3BR agonist compound shows significant specificity to the A3BR receptor. A2AR KO, naïve and A2BR OE cells were treated with BAY60 and release of calcium was measured as relative fluorescence units (RFU). Cells with vehicle addition were used for baseline reads.
vector (constructed as described in the Methods) was transduced into K562 naïve cells. Flow cytometry analysis of the stably transduced A2AR overexpressing K562 line (A2AR90) shows high expression of A2AR in over 97% of transfected cells (Fig. 5a).

To create the A2BR overexpressing K562 line (A2BR OE), we transfected our A2AR knockout line (created as described above) with the pEYFP-N1-A2BR plasmid, which also expresses YFP upon successful transfection of the A2BR. After transfection and neomycin selection (100 μg/ml), the stable A2BR overexpressing line showed 95.5% of YFP (Fig. 5b). Since we did not have an A2BR antibody, we used the calcium flux assay (described below) to show response to a known A2BR agonist as evidence that A2BR is being expressed.

Engineered adenosine cell lines coupled with calcium flux assay can quickly determine specificity of A2A and A2B receptor agonists. To test the efficiency of these cell lines to detect specificity of A2BR agonists, we used the well studied A2B receptor agonist BAY60–6583 (BAY60), which is highly selective for the A2BR over A1, A2A and A3 receptors26. A2AR knockout, naïve and A2BR overexpressing cells were plated and subjected to calcium flux assay during BAY60 addition. Using a microplate reader, changes in intracellular free calcium levels upon compound addition were recorded as relative fluorescence units (RFU) over time. No response was seen from naïve or A2AR KO cell lines, while there was significant increase in RFU from A2BR overexpressing cells treated with BAY60 (Fig. 4). This experiment confirms that the A2BR OE line overexpresses functional A2BR, allowing demonstration of specificity for A2BR agonists.

Our laboratory obtained several newly developed A2AR agonists from Lewis and Clark Pharmaceuticals Inc. for testing using our cell lines27. Compound LNC-3047 was tested for A2AR specificity using calcium release detection. LNC-3047 shows specificity, as calcium release increases proportionally to A2AR expression (Fig. 6a). The A2AR90 cell line showed significantly higher release of calcium over the naïve K562 cells treated with LNC-3047. In contrast, the knockout line showed no increase in calcium release over baseline upon addition of compound, indicating that LNC-3047 is highly active in stimulating A2AR (Fig. 6a, Top). When the A2AR OE line was treated with LNC-3047, there was no calcium release in cells expressing high levels of A2AR, indicating that LNC-3047 is very specific to the A2A receptor and does not bind to the A2B receptor (Fig. 6a, Bottom).

A second compound tested, LNC-3015, was shown to be non-specific for the A2AR and confirms the specificity, efficiency and utility of our method for distinguishing agonists that are highly specific for A2AR versus those with residual A2B activity. LNC-3015, while showing calcium release in both the A2AR90 line and the naïve line, also shows release in the A2AR knockout line, indicating non-specific binding to additional receptors (Fig. 6b, Top). When this compound was further screened using the A2AR overexpressing line, it can be seen that LNC-3015 causes calcium release in this line as well, indicating that LNC-3015 is also able to bind to the A2B receptor in addition to the A2A receptor (Fig. 6b, Bottom). Overall, these results show that our method using these engineered K562 lines is efficient and fast in identifying non-specific binding of agonists to adenosine receptors.

Verification of specificity of A2AR agonist compounds in cell culture. After determining specificity of A2AR agonist compounds with calcium flux, these compounds were tested for efficacy in human T-cells. The
adenosine 2A receptor has been shown to play a part in the induction of regulatory T-cells from naïve T-cells, a cell type shown to be important for immune tolerance. Using naïve human CD4+CD25− T-cells, LNC-3047 and LNC-3015 were tested for their ability to convert naïve T-cells to FoxP3+ regulatory T-cells. Using flow cytometry and gating on viable CD4+CD25+ cells, untreated cells were found to have 16.6% CD4+CD25+FoxP3+ cells (Fig. 7). Overall, LNC-3047 showed a 2-fold increase in FoxP3 expression (p = 0.0079), while LNC-3015 was around a 1.5 fold increase (p = 0.0317). The reduced efficacy of LNC-3015 may be due to activation of inflammatory pathways by binding of LNC-3015 to the A2B receptor, reducing the differentiation of regulatory T-cells in preference of cytotoxic CD8+ T-cells.

Binding data in the form of Ki values obtained from Lewis and Clark Pharmaceuticals Inc. for both LNC-3015 and LNC-3047 confirmed our results (Supplementary Table S1). Ki values are commonly used to predict clinically relevant drug interactions, with smaller Ki values indicating a greater binding affinity of the compound for the receptor28–30. Both LNC-3015 and LNC-3047 have much smaller Ki values for the human A2AR than CGS-21680 and Regadenoson (Lexiscan), two commonly used A2AR agonists that have been shown to be highly specific to the A2AR. The selectivity of LNC-3047 for the A2A receptor over both the A1 and A3 receptors is much higher than that of LNC-3015, supporting our calcium flux and cell culture findings. Together, both the cell culture expression of FoxP3 and Ki binding values validate our calcium flux method for identifying A2A receptor specific agonists.

Discussion
The use of adenosine agonists has become more prevalent in recent years and is a growing field for many areas of treatment, including cancer, cardiovascular issues and autoimmune diseases14,31,32. Production of agonists specific to individual receptors remains a slow and challenging process and requires the use of expensive and hazardous radioactive reagents. With the development of our new method, we hope to speed up and streamline this process to decrease the time from bench to bedside.

To accomplish this goal, we first developed three distinct new K562 cell lines. Because A2A and A2B receptors share many structural and sequence similarities, we focused on these receptors. We created A2A receptor knockout cells as well as an A2A and A2B overexpressing line. Using these lines, and in conjunction with calcium release, we were able to determine the specificity of several compounds. This method of determining binding specificity of agonists is much quicker, safer and more efficient than the current radio-ligand, binding and competition assays currently used.
The first compound tested, BAY60, is a known A2BR agonist. We found that our cell lines were sufficient to determine specificity of this compound, with a significant increase in calcium release from A2BR overexpressing cells, but no release over baseline for naïve and A2AR knockout cells. Two additional compounds, LNC-3047 and LNC-3015, were expected to be specific to the A2AR in human cells. While we did find that LNC-3047 is very specific, LNC-3015 was found to partially bind to the A2BR, as seen by RFU increase in A2BR overexpressing cells treated with LNC-3015. When these two compounds were tested for efficacy in promoting FoxP3 expression, it was found that LNC-3047 was more efficient at inducing regulatory T-cell differentiation. FoxP3 expression in human cells, while significant, was lessened in cells treated with LNC-3015, most likely due to activation of inflammatory pathways through its binding to A2BR.

Our data have shown that our novel method can quickly and efficiently determine receptor-binding specificity for both the A2AR and the A2BR. In addition, this technology can easily be expanded in the future to test both the A1 and A3 receptors. Using CRISPR technology, several knockout lines can be generated in K562, jurkat or any other cell type to test a range of compounds. Overall, this method allows for faster and more sensitive testing of new adenosine agonist compounds.

**Methods**

**Vectors and Plasmids.** HIV-lentiviral vectors were produced by transient transfection of DNA from four separate plasmids into 293 T cells as follows: 20 μg of vector plasmid DNA, 12 μg of pCAG-GP, 4 μg of pCAG-RTR, and 4 μg of pVSV-G plasmid DNA were transfected using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA).
purity DNA, gRNA targeting the A2AR region was amplified using Dream Taq Green PCR Master Mix (2X) (Thermo Devices, Sunnyvale, CA, USA). Cells were transduced with 1X virus and 5 μg/ml of protamine sulfate, followed by spinning at 2,500 rpm for 30 minutes.

To develop the K562 derived A2AR overexpressing (A2AR90) line, the lentivector transfer plasmid pC2L014-EF1a-hA2aR was created (see Supplementary Fig. S1). This was accomplished by digestion of a previously described lentivector EF1a-hgCOPT for removal of hgc OPT DNA using the EcoRI and NolI enzymes (Thermo Fisher Scientific, Rockville, MD, USA), followed by insertion of the 1.27 kb hA2aR cDNA (derived as described below)33.

To create the A2AR knockout line (A2AR KO), a viral vector was created using the plentiCRISPR loxP plasmid (Supplementary Fig. S1), which was modified from the original plasmid plentiCRISPR V2 (Addgene plasmid #173707) by addition of a loxP site in the 3′LTR. To accomplish this, the plentiCRISPR V2 plasmid was first cut with PmeI and MluI (Thermo Fisher Scientific) to remove the WPRE (woodchuck hepatitis virus post-transcriptional regulatory element) and SIN (Self-inactivating) 3′LTR. The WPRE and loxP site from pHAGE2Ef1a-full-STEM CCA (a plasmid DNA having 4 transcription factors for iP cells) was amplified with the following primer set (forward primer; 5′-AGCTGATACGCATGCTGATGTTACGTTGAGTTCGATTGATCC-3′ and reverse primer; 5′-TCCTTTTTAGTTTTAAACTGCTAGAGATTTCCTTCC-3′) using Phusion Polymerase (New England Biolabs, Ipswich, MA, USA) and cloned into the open plentiCRISPR V2 plasmid DNA. Using the BsmBI digestion enzyme (Thermo Fisher Scientific), the plentiCRISPR loxP plasmid was linearized and eluted for insertion of the A2AR guide RNA (gRNA). The loxP cassette was removed from stable knockout cell lines using the pCDA3.1 ZEO T2A CRE-GFP vector (Supplementary Fig. S1).

Finally, for development of the stable A2BR overexpressing line (A2BR OE), we used the pEYFP-N1-A2BR plasmid, which expresses both the A2BR and the eYFP chromophore to simplify selection of transfected clones, a gift from Robert Tarran (Addgene plasmid #37202, Supplementary Fig. S1)35.

RNA Extraction, Reverse-Transcription and qRT-PCR. Human peripheral blood was collected from healthy volunteers after written informed consent under IRB approved NIH clinical protocol 05-I-0213. All methods were carried out in accordance with NIH guidelines and regulations. Human white blood cells were used for RNA extraction using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The A2AR cDNA was synthesized following the manufacturer’s protocol by the High Capacity cDNA Reverse Transcription Kit (ABIResearch, New York, NY, USA) using primers (forward primer; 5′-GCCGATCTTTGATCAGCGCTGAGGCTC-3′ and reverse primer; 5′-GGCTTTATGTTTTAAGACTTGCTAGATTTC-3′) for cloning into the pC2L014-EF1a- backbone. The copy number of the HIV-lentivector gene in DNA isolated from transfected K562 cells was determined by quantitative TaqMan PCR (Applied Biosystems, Foster City, CA, USA), using a multiplex method that allows us to monitor both the HIV-lentivector gene and the human housekeeping gene phenol sulfo transferase (STP) in the same well. The sequence of primers and probe for the HIV-lentivector were; forward primer: 5′-TGAAGGCGGAAGGAAAGCCA-3′, reverse primer: 5′-CCGTCGGCGCTTCAG-3′, probe: 5′-FAM-AGCTCCTCTCGAGCGCGAGCTGCG-3′. The sequence of primers and probe for STP gene were; forward primer: 5′-GAGCTCCTCTCGAGCGCGAGCTGCG-3′, reverse primer: 5′-GGCTTTATGTTTTAAGACTTGCTAGATTTC-3′, probe: 5′-VIC-CCCGAGGAGACTTGATGGAACCTTCC-3′. TaqMan PCR conditions were: 50°C/2 min: 95°C/10 min: 40 cycles, 95°C/15 sec, and 60°C/60 sec. Single copy or two copies of HIV-lentiviral vector in K562 cells confirmed by southern blot assay was used for standard curve of transgene copy number.

Flow Cytometry. Cells were washed and resuspended in serum free and azide free PBS (Life Technologies) and were stained with A2AR-FITC, CD4-FITC or CD25-PE for 30 minutes at room temperature. Cells transduced with lentICRISPR loxP A2AR virus were marked using anti-FLAG-FITC antibody (Sigma-Aldrich, St. Louis, MO, USA) after fixing and permeabilization. An isotype control was used for each fluorophore used. For FoxP3 staining, cells were fixed and permeabilized for 30 minutes at room temperature using the eBioscience FoxP3 staining buffer set (Affymetrix, San Diego, CA, USA), followed by staining with anti-human FoxP3-APC (Affymetrix). Samples were run on a benchtop BD FACS Canto flow cytometer (BD Biosciences, San Diego, CA, USA) and a FACScan (BD Biosciences). Analysis was performed using FlowJo FACS analysis software (FlowJo LLC, Ashland, OR, USA) and CellQuestPro (BD Biosciences).

T7 E1 Assay. Transduced or transfected cell DNA was isolated using the DNeasy blood and tissue kit (Qiagen). From purified DNA, gRNA targeting the A2AR region was amplified using Dream Taq Green PCR Master Mix (2X) (Thermo Fisher Scientific). The sequence of primers was; forward primer: 5′-AATGAGGAGCCTTCGAGGAGCGACCC-3′, reverse primer: 5′-AAGATGCGAGCCTTCGAGGAGCGACCC-3′. The PCR product was purified using the GeneJET PCR Purification kit (Thermo Fisher Scientific). The purified PCR product was denatured at 95°C for 5 minutes and then ramped down to 25°C at 5°C/min, followed by cutting with 10 units of T7 E1 enzyme (New England Biolabs) at 37°C for 1 hour.

Calcium Flux. Calcium flux assays were performed using the FLIPR Calcium 3 Assay Explorer Kit (Molecular Devices, Sunnyvale, CA, USA). 100 ul of 2.5 × 105 K562 cells were seeded per well in a 96-well plate. An equal volume of Calcium Assay 3 Dye was added to suspended cells and allowed to incubate for 1 hour at 37°C. Agonist compound solutions were prepared in a separate 96 well plate at a 10X higher concentration than desired final concentration. Plates were then loaded into the FLEXstation 3 Multi-Mode Microplate Reader (Molecular Devices) and allowed to equilibrate for 5 minutes. In conjunction with the SoftMax Pro Microplate Data Acquisition and Analysis Software (Molecular Devices), the FLEXstation added compounds and read the change in fluorescence intensity resulting from calcium release in each well.
Peripheral blood mononuclear cells (PBMCs) were first prepared from human blood by isolation using lymphocyte separation medium (MP Biomedicals, Solon, OH, USA). Human peripheral blood was collected from healthy volunteers after written informed consent under IRB approved NIH clinical protocol 05-1-0213. Possible contaminating red blood cells were lysed using ACK lysis buffer (Quality Biological, Gaithersburg, MD, USA). CD4^+ CD25^+ T-cells were isolated from whole PBMC using the human CD4^+ CD25^+ regulatory T-cell isolation kit (Miltenyi, Gaithersburg, MD, USA). Cells were cultured in RPMI (Life Technologies) with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA) and 100 μg/ml Penicillin/Streptomycin (Quality Biological), activated with CD3/CD28 human T-Activator Dynabeads (Life Technologies) and analyzed 72 hours after plating.

**Statistics.**

Statistics were performed for all cell culture experiments. Error bars are defined as mean ± SEM. Student’s T-test with Mann-Whitney was used to determine significance as all sample sizes were N = 5 and Mann-Whitney provides a more conservative p-value for small sample sizes.

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**Acknowledgements**
This work was supported by the National Institutes of Health [1-ZIA-AI000989, 1-ZIA-AI000988]. LNC-3015 and LNC-3047 were a kind gift from Lewis and Clark Pharmaceuticals Inc., Charlottesville, VA 22901 USA.

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**Additional Information**
Supplementary information accompanies this paper at http://www.nature.com/srep

**Competing Interests:** The authors declare no competing financial interests.

**How to cite this article:** Jones, K. R. et al. A Novel Method for Screening Adenosine Receptor Specific Agonists for Use in Adenosine Drug Development. *Sci. Rep.* **7**, 44816; doi: 10.1038/srep44816 (2017).

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