Development of an inhibitor screening assay for mono-ADP-ribosyl hydrolyzing macrodomains using AlphaScreen technology

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ABSTRACT

Protein mono-ADP-ribosylation is a post-translational modification involved in the regulation of several cellular signaling pathways. Cellular ADP-ribosylation is regulated by ADP-ribose hydrolases via a hydrolysis of the protein-linked ADP-ribose. Most of the ADP-ribose hydrolases share a macrodomain fold. Macrodomains have been linked to several diseases, such as cancer, but their cellular roles are mostly unknown. Currently, there are no inhibitors available targeting the mono-ADP-ribose hydrolyzing macrodomains. We have developed a robust AlphaScreen assay for screening of inhibitors against macrodomains having mono-ADP-ribose hydrolysis activity. We utilized this assay for validatory screening against human MacroD1 and identified five compounds inhibiting the macrodomain. Dose-response measurements and an orthogonal assay further validated four of these compounds as MacroD1 inhibitors. The developed assay is homogenous, easy to execute and suitable for screening of large compound libraries. The assay principle can be adapted also for other ADP-ribose hydrolyzing macrodomains, which can utilize a biotin-mono-ADP-ribosylated protein as a substrate.

KEYWORDS: AlphaScreen, macrodomain, MacroD1, ADP-ribosylhydrolase

INTRODUCTION

Macrodomains are ubiquitous protein modules recognizing and in some cases hydrolyzing mono-ADP-ribose (MAR) and poly-ADP-ribose (PAR) units attached to proteins.[1,2] During ADP-ribosylation, an ADP-ribose is transferred from NAD^+ to the target protein or to the growing ADP-ribose chain by ADP-ribosyltransferases (ARTDs or PARPs).[3] The modification regulates various signaling cascades and also serves as a docking site for other proteins, including macrodomains. ADP-ribosylation has essential roles in the regulation of cellular processes such as DNA repair, transcription and chromatin biology. In addition to hydrolysis of protein linked ADP-ribose, some macrodomains also hydrolyze *O*-acetyl ADP-ribose, a product of sirtuin-mediated lysine deacetylation.[4] *O*-acetyl ADP-ribose functions as a signaling molecule and has been linked to the regulation of gene silencing and ion channel gating.[5]

Macrodomains are found in over 5000 proteins in eukaryotes, prokaryotes and viruses (Pfam: PF01661).[6] The domain can be a part of a larger protein or a stand-alone domain (ca. 25 kDa) containing a mixed α/β fold. The overall fold and the ligand binding site are highly conserved but the binding and hydrolysis properties of MAR and PAR vary greatly between human macrodomains (Supplementary Table 1).[1,2] It is evident that even small structural differences have an impact on the domain function, although the activities of macrodomains are generally poorly known. Clear views of the various roles of macrodomains in cellular processes are missing. [1,6,7] To date, macrodomains have been proposed to regulate protein-protein interactions and enzymatic activities in various cellular pathways.[8,9] Macrodomain inhibition has been suggested to improve radiotherapy and chemotherapy in cancer treatment through inhibition of DNA repair and leading to apoptosis.[1,10] However, this hypothesis has not been tested in practice due to lack of macrodomain inhibitors.

Here we have developed an assay to screen inhibitors against MAR hydrolyzing macrodomains (Figure 1). The assay was tested with four human macrodomains (MacroD1, MacroD2, TARG1 and the third macrodomain of PARP14) and two trypanosomatid macrodomains. We optimized the assay parameters for MacroD1, screened a validatory library for inhibitors and identified compounds inhibiting MacroD1.

MATERIALS AND METHODS

Cloning and Expression Constructs

MacroD1 (GeneBank: NP_054786.2) construct (residues 58-325) cloned in pNH-TrxT vector and SRPK2 construct (residues 81-699) cloned in pNIC28-Bsa4 were from Structural Genomics Consortiums (Oxford, UK). cDNA of TARG1 (GeneBank: BC011709.2) was purchased from GenScript and cloned in pNH-TrxT vector. MacroD2 (GeneBank: NM_080676.5) DNA was a kind gift from Dr. Ahola (University of Helsinki, Finland) and the DNA of PARP14 macrodomain (residues 1207-1388; GeneBank: NG_051076.1) from Dr. Schüler (Karolinska Institut, Sweden). The construct of PARP10 catalytic domain (residues 809-1017) with a C-terminal his-tag was used as a template for cloning.[11] The trypanosomatid macrodomains from *Trypanosoma brucei* (TbMDO) and *Trypanosoma cruzi* (TcMDO) were previously cloned to pNH-Trxt vectors.[12]

MacroD2 (residues 7-243) and PARP10 (residues 819-1008) were cloned by PCR extension cloning to pNH-TrxT and pNIC28-Bsa4 vectors, respectively and they were sequenced. All the vectors contain a TEV-protease cleavage site after the N-terminal affinity/solubility tags.

Protein Expression and Purification

SRPK2, TbMDO and TcMDO were expressed and purified as described previously.[11,12] MacroD1, MacroD2, PARP10 and PARP14 were expressed in *E. coli* Rosetta 2 (DE3) cells using Terrific broth

auto-induction media (Formedium) supplemented with 8 g/l glycerol, 50 µg/ml of kanamycin and 34 µg/ml of chloramphenicol. TARG1 was expressed in *E. coli* BL21 (DE3) cells using Terrific broth auto-induction media (Formedium) supplemented with 8 g/l glycerol and 50 µg/ml of kanamycin. The cells were grown until OD_{600} reached 1.0 and the temperature was lowered to 18 °C for protein expression. After 16 hours of incubation, the cells were collected by centrifugation, suspended in lysis buffer (50 mM HEPES pH 7.5, 0.5 M NaCl, 10 % glycerol, 10 mM imidazole, 0.5 mM TCEP) and stored at -20 °C.

The proteins were purified with immobilized metal ion affinity chromatography (IMAC) and gel filtration. Briefly, the cell suspension was supplemented by 0.1 mM Pefabloc SC (Sigma-Aldrich), 20 µg/ml DNaseI (Roche) and 0.5 mg/ml lysozyme (Sigma-Aldrich). The cells were broken by sonication and the lysate was centrifuged at 31000×*g* at 4 °C for 45 min to remove the insoluble material. The supernatant was filtered through 0.45 µm filter and loaded onto a 1 ml HisTrap HP column (GE Healthcare). The column was washed with lysis buffer followed by wash buffer with 25 mM imidazole. The proteins were eluted by increasing the buffer imidazole concentration to 350 mM. The fusion tags were cleaved by incubating with TEV protease at 4 °C overnight.

The proteins were further purified with gel filtration using HiLoad 16/600 Superdex 75 pre-equilibrated with 20 mM HEPES pH 7.5, 0.3 M NaCl, 0.5 mM TCEP. Finally the proteins were polished by passing them through the HisTrap HP column and collecting the proteins in the flowthrough. The proteins were flash frozen in small aliquots in liquid N_2 and stored at -70 °C.

Production of mono-ADP-ribosylated SRPK2

10 µM of His-tagged SRPK2 was ADP-ribosylated with 5 µM PARP10 using 10 µM biotinylated NAD^+ as a substrate (Trevigen). The reaction was incubated in 50 mM Tris-HCl pH 7.2 at room temperature for 3.5 hours and purified using Ni-NTA Superflow (Qiagen) resin. The resin was incubated with the reaction solution for 20 minutes and washed three times using 50 mM Tris-HCl

pH 7.2, 300 mM NaCl. The bound modified SRPK2 was eluted with 50 mM Tris-HCl pH 7.2, 300 mM NaCl, 500 mM imidazole and dialyzed against 50 mM HEPES pH 7, 300 mM NaCl at 4 °C for overnight. The preparation was flash frozen in small aliquots in liquid N_2 and stored at -70 °C.

Assay Development

Activity Assay

We have previously discovered that SRPK2, mono-ADP-ribosylated by PARP10, can serve as a substrate in macrodomain catalyzed hydrolysis of protein-linked ADP-ribosyl.[11] This principle was transformed to AlphaScreen format using His-tagged SRPK2 ADP-ribosylated by PARP10 with biotinylated NAD^+. The assay measures the proximity generated luminescence using the AlphaScreen technology. SRPK2 modified with biotinylated NAD^+ binds to the nickel chelate acceptor beads via poly-His-tag and to the streptavidin donor beads via biotin. Signal decrease is achieved through hydrolyzing the biotinylated ADP-ribose from SRPK2 with MAR-hydrolyzing macrodomain.

The reactions were carried out in 384-well plates (Alphaplate, PerkinElmer) in a total volume of 25 µl. The reactions consisted of SRPK2, macrodomain (assay was optimized for MacroD1) and nickel chelate acceptor and streptavidin donor beads (PerkinElmer) in assay buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 1 mg/ml BSA). The plates were incubated at room temperature and protected from light after bead addition, and luminescence was read with Tecan Infinite M1000 Pro plate reader using the AlphaScreen detection module. Macrodomain activity was read as a decrease in total luminescence with respect to control wells (buffer instead of macrodomain).

Assay sensitivity was first tested to establish optimal SRPK2 concentration and a maximal signal (hook point).[13] Modified SRPK2 (15 µl) and nickel chelate acceptor beads (5 µl) were added to the well, and after 30 min incubation streptavidin donor beads (5 µl) were added followed by 60 min of incubation. Final concentrations were 0-500 nM modified SRPK2 and 15 µg/ml acceptor and donor beads (Figure 2A). To establish whether MacroD1 is able to hydrolyze the biotinylated substrate a concentration series of MacroD1 was titrated against SRPK2 and ADP-ribosyl hydrolysis activity was measured by setting the conversion of the control wells (wells with SRPK2 only) to 0%. Modified SRPK2 (7.5 µl) and MacroD1 (7.5 µl) were added to the wells and incubated at room temperature for 180 min. Next nickel chelate acceptor beads (5 µl) were added to the well, and after 30 min incubation streptavidin donor beads (5 µl) were added followed by 60 min of incubation. Concentrations of modified SRPK2 and MacroD1 were 50 nM and 0-600 nM, respectively (before bead addition) (Figure 2B).

Assay optimization

Once we had demonstrated that the assay principle works, we began to optimize various assay parameters. We first optimized the addition order of the AlphaScreen beads using three sequences (Figure 2C): (a) Modified SRPK2 was incubated with MacroD1 for 180 min followed by the addition of both beads and 90 min incubation; (b) Modified SRPK2 was incubated with MacroD1 for 180 min followed by the addition of acceptor beads and 45 min incubation, and then addition of donor beads and 45 min incubation; (c) Modified SRPK2 was incubated with MacroD1 for 180 min followed by the addition of donor beads and 45 min incubation, and then addition of acceptor beads and 45 min incubation.

We then optimized the AlphaScreen bead concentration (12.5, 10, 7.5, and 5 µg/ml) (Figure 2D) and bead incubation time (Figure 2E). Once we had established the optimal parameters for the beads, we optimized the time and concentration dependency of the MacroD1 catalytic reaction. Finally, we measured the DMSO tolerance of the assay with 0-10 % DMSO concentrations.

Assay was validated by measuring repeatability of the maximal and minimal signals between different wells, plates, and days (Table 1). Altogether, five plates containing maximal and minimal signals were measured during three days, one plate on days one and two, and three plates on day three. 40 maximal and minimal signal points each were included in each of the plates and well-to-well, plate-to-plate and day-to-day variations were calculated as coefficients of variations (CVs). The quality of the assay was measured with common statistical parameters: signal-to-noise ratio (S/N), signal-to-background ratio (S/B), and screening window coefficient (Z′).[14,15]

Library Screening

MacroD1 was screened for inhibition against MicroSource Spectrum collection. 2000 compounds were screened at a single concentration (100 µM). The compounds (0.15 µl) were transferred to the assay plates with Echo acoustic dispenser (Labcyte). Then 7.5 µl of MacroD1 and SRPK2 (final concentrations 800 nM and 50 nM, respectively) were added to the assay plates. The plates were incubated for 1 hour 20 min at room temperature followed by the addition of the Acceptor and Donor bead mixture (10 µl, final concentration 5 µg/ml) and additional incubation of 3 hours. Each screening plate contained blank wells (AlphaScreen beads only), negative controls (0% inhibition) with no inhibitor and positive controls (100% inhibition) with no MacroD1.

Potency measurements

Dose-response curves for hit compounds were measured in quadruplicates from 320 µM 3.2 nM using half-logarithmic dilutions. The compounds were transferred to the assay plates with Echo, followed by the addition of 7.5 µl of both MacroD1 (final concentration 800 nM) and SRPK2 (final concentration 50 nM). The plates were incubated for 40 min at room temperature followed by the addition of the Acceptor and Donor bead mixture (10 µl, final concentration 5 µg/ml) and additional incubation of 3 hours. The dose-response curves were fitted using a 4-parameter nonlinear regression analysis (sigmoidal dose-response fitting with variable slope) with GraphPad Prism version 5.03 for Windows (GraphPad Software, La Jolla, CA).

Western Blot

Time dependency of the removal of ADP-ribose from SRPK2 catalyzed by MacroD1 was tested by incubating the modified SRPK2 (0.5 µM) with MacroD1 (4 µM) in 25 mM HEPES pH 7.5, 100 mM NaCl, 1 mg/ml BSA for 15 s – 20 min. Validation of the hit molecules as potential MacroD1 inhibitors was performed using western blot as an orthogonal assay. The modified SRPK2 (1 µM) and MacroD1 (4 µM) with a hit compound (100 µM) were incubated in 25 mM HEPES pH 7.5, 100 mM NaCl for 2 h 30 min. All the reactions were stopped by adding 2× Laemmli buffer (Bio-Rad, USA) and incubating at 95 °C for 5 min. The samples were analyzed by SDS-PAGE and transferred onto a nitrocellulose membrane (Whatman, UK). After transfer, the membrane was stained with 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid to verify equal sample loading. The membrane was blocked using 1% Casein in 1xTB (Bio-Rad, USA). The samples were visualized with 1:15000 streptavidin conjugated horseradish peroxidase (PerkinElmer, USA) using a chemiluminescent substrate (WesternBright™ ECL, Advansta Corporation, USA).

RESULTS

Activity Assay for ADP-ribosyl hydrolyzing macrodomains

No screening assays have been described for mono-ADP-ribosyl hydrolyzing macrodomain to date. Recently, however, a high –throughput screening assay for PAR degrading macrodomain, PARG was develop and utilized for inhibitor screening.[16] Here we aimed to establish an AlphaScreen –based screening assay applicable to mono-ADP-ribosyl hydrolyzing macrodomains that measures the hydrolysis of protein-linked ADP-ribosyl by macrodomains. To this end we used poly-His-tagged SRPK2, which was ADP-ribosylated by PARP10 with biotin-NAD^+. This produces SRPK2 that has both N-terminal poly-His fusion tag and biotin-ADP-ribose as a covalent modification. The modified SRPK2 interacts with nickel-chelate acceptor beads *via* the poly-His-tag and with streptavidin donor beads *via* the biotin (Figure 1). The interaction can be relieved by hydrolyzing the ADP-ribose from SRPK2 by ADP-ribosyl hydrolases (Figure 1).

Assay sensitivity was first tested to establish optimal SRPK2 concentration and maximal signal (hook point) (Figure 2A).[13] We found that maximal signal was reached between 50 to 100 nM SRPK2 concentration. We chose 50 nM SRPK2 concentration to be used in the subsequent assays. Next, we titrated 50 nM SRPK2 with MacroD1 to find optimal concentration for the assay (Figure 2B). The aim was to achieve a robust screening assay with the ability to clearly differentiate between actives and non-actives in the screening stage, so protein concentration resulting to 60% signal decrease (60% theoretical ADP-ribosyl hydrolysis) was aimed at. Based on the experiment we decided to use 400 nM MacroD1 (180 min incubation time) in following experiments.

We had added the AlphaScreen beads in the initial experiments based on the recommended protocol (PerkinElmer): after the enzymatic reaction, the acceptor beads were added followed by 30 min incubation and addition of the donor beads followed by 60 min incubation. The effect of addition order of the beads was tested to improve assay sensitivity and for ease-of-use (Figure 2C). We kept

the total incubation time in the experiments 90 min. Maximal signal was achieved when both beads were added together and this sequence also resulted in the highest Z′. Z′ was the same when either acceptor or donor beads where added first but higher signal was achieved when acceptor beads were added first. Based on these results, we decided to add both beads simultaneously and we used this sequence in subsequent experiments.

To reduce the running costs of the assay, we optimized the AlphaScreen bead concentration required for an acceptable signal window for screening. The reactions were run with four bead concentrations (Figure 2D). As expected, the AlphaScreen signal together with Z′ decreased with bead concentration. However, even the lowest concentration (5 µg/ml) resulted in an acceptable signal level and Z′, and it was selected as the bead concentration for the follow-up experiments.

The length of bead incubation time can have a significant effect on the assay sensitivity and therefore we optimized it using the 5 µg/ml bead concentration (Figure 2E). AlphaScreen signal increased almost linearly with incubation time to the highest time point (7 h) tested. To compromise between signal strength and assay time, we chose 3 hour bead incubation for the assay.

We further optimized the MacroD1 concentration and incubation time (Figure 3A). A clear MacroD1 concentration dependency in the ADP-ribosyl hydrolysis is evident. However, the hydrolysis shows poor time dependency as most of the hydrolysis takes place already at the first time point and incubation time does not significantly increase the hydrolysis. This was further studied with Western blotting using higher protein concentrations, which showed significant hydrolysis after 10 min incubation. (Figure 3B). It is possible that biotin-mono-ADP-ribosylation may not be an ideal

substrate for MacroD1 and therefore a high enzyme concentration is required for the assay. In addition, SRPK2 may have several modification sites, some of which could be poor substrates for MacroD1 explaining high concentration of MacroD1 needed for the reaction. In order to ensure efficient 60% hydrolysis, we chose 800 nM MacroD1 and 80 min incubation time for the assay. Since DMSO is commonly used solvent for compound libraries, we tested DMSO tolerance of the assay (Figure 3C). The assay was found to be insensitive to DMSO up to 10 % concentration tested. No statistically significant deviation between 0 % DMSO control reaction and the DMSO reactions was found. Finally, we optimized the excitation and integration times of the plate reader and selected 600 ms excitation and 300 ms integration times for the further experiments (Figure 3D).

To validate the quality of the assay for screening, we tested the changes in plate-to-plate and day-to-day minimal and maximal signals with five different plates. The average Z' value for all the plates was 0.7, indicating a robust screening assay (Table 1).

To test the wider usability of the assay and especially SRPK2 as a general substrate for MAR hydrolysis, we measured the activity of five other macrodomains with the assay (Supplementary Figure 1). Human MacroD2 had higher activity than MacroD1 and showed better time-dependency in the assay (Supplementary Figure 1A). The trypanosomatid macrodomains *Trypanosoma brucei* MDO (TbMDO) and *T. cruzi* MDO (TcMDO) displayed much higher activity than the human macrodomains and even the lowest concentration used (200 nM) hydrolyzed almost all substrate (Supplementary Figure 1B,C). However, TARG1 and the third macrodomain of PARP14 did not show any activity in the assay, not even with the highest concentration (800 nM). This was surprising as TARG1 is classified as hydrolyzing enzyme of mono-ADP-ribosylation[17] whereas the third macrodomain of PARP14 is a reader of mono-ADP-ribosylation and was used as a control.[18] The

binding of these macrodomains to the SRPK2 was further studied with surface plasmon resonance. Based on the studies, TARG interacted unspesifically with the SRPK2 and this was not dependent on the modification (Supplementary Figure 2). The third macrodomain of PARP14 did not show binding to the SRPK2 at all.

Validatory Screening

To test the assay in real compound screening, we used a MicroSource Spectrum library of 2000 compounds which includes drugs, natural products and bioactive compounds. The screening was done with 100 µM compound concentration in singlets. The assay performed well in screening with an average Z' of 0.73 ± 0.07 over 6 plates. Altogether, five hits were identified (0.25 % hit rate) (Figure 4) that inhibited MacroD1 activity using a hit limit set to 50 % activity. Two additional compounds, namely protoporphyrin IX and haematoporphyrin were below the 50 % activity limit having activities of -190 and -51 %, respectively. These activity values were clear outliers and both of these compounds interfere with AlphaScreen signal by acting as singlet oxygen quenchers.[19]

Dose-response curves were measured for the hit compounds (Figure 5A-E) which revealed one of the initial hits (Azacitidine) as a false positive. The IC_{50} values for triclosan was 100 µM, ethacrynic acid 27 µM, thimerosal 5.2 µM and patulin 2.5 µM.

We also tested the hits using an orthogonal Western blot assay (Figure 5F). ADP-ribose was used as a control and it inhibited ADP-ribose hydrolysis, as expected. Azacitidine did not inhibit ADP-ribose hydrolysis and the result is consistent with dose-response measurements (Figure 5C). More potent hit compounds ethacrynic acid, triclosan, thimerosal and patulin inhibited the macrodomain catalyzed

reaction in the orthogonal assay: patulin had the highest potency, whereas ethacrynic acid, triclosan and thimerosal showed slightly weaker inhibition. Results were overall in agreement with the AlphaScreen assay.

DISCUSSION

ADP-ribosylation is used in cells for various functions, such as DNA-repair processes and cell signaling. A substantial amount of inhibitors have been developed for poly-ADP-ribosylating ARTDs via HTS methods, fragment-based screening and virtual screening. Despite functioning in the same pathways, proteins reversing the modification have not been targeted by small molecule inhibitors. Recently, a HTS method was developed against poly-ADP-ribose hydrolyzing PARG[16], but no screening assays for mono-ADP-ribosyl hydrolyzing macrodomains have been reported to date.

In this paper, we described an AlphaScreen based screening assay for inhibitors against mono-ADP-ribosyl hydrolyzing macrodomains. The assay was tested with four macrodomains, namely human MacroD1 and MacroD2, as well as two trypanosomatid macrodomains. Generally, the assay should be adaptable to other mono-ADP-ribosyl hydrolyzing macrodomains provided that they can utilize biotin-mono-ADP ribosylated SRPK2 or some other protein as a substrate. However this was not the case for PARP14 macrodomain and TARG, which both were inactive in the assay. Despite the potential limitations due to substrate protein and unnatural biotin-ADP-ribosylation, the assay is well-suited for inhibitor screening as it tolerates at least 10% DMSO allowing screening of compounds using high concentrations. The high DMSO tolerance also allows screening of fragment libraries, which usually require high compound (and DMSO) concentrations due to low binding affinity. The assay was develop using a 384-well plate format but should be further adaptable to a 1536-well plate format by utilizing an acoustic liquid dispenser.

The assay was validated for human MacroD1 and validatory screening was conducted with a library of 2000 compounds. The assay performance was good as indicated by Z′ (0.71) and (S/N 15.3) values. The validatory screening yielded five hits, and four were further validated as hits using dose-response measurements and an orthogonal assay. Currently, there are no inhibitors reported for mono-ADP-ribosyl hydrolyzing macrodomains. Therefore the μM inhibitors identified in the validatory screening, patulin and ethacrynic acid, belonging to different chemical classes could serve as starting points in future inhibitor development efforts. Both of these small compounds have a range of activities and are also used as medicines. Patulin has been recently identified as an antimicrobial agent against *Salmonella*[20] and ethacrynic acid has been shown to have potency to improve the antitumor effects of epidermal growth factor receptor tyrosine kinase inhibitors in breast cancer.[21] Taken into account the modest potency and reported activities of the compounds we would foresee that the hit molecules could be useful starting compounds for the development of macrodomain inhibitors when medicinal chemistry and structural biology methods are combined, especially as there are already several crystal structures of macrodomains such as MacroD1[4] and MacroD2[8] available in the Protein Data Bank.

The assay described for hydrolyzing macrodomains is simple and utilizes materials available commercially and therefore it will facilitate discovery of chemical probes for mono-ADP-ribosyl hydrolyzing macrodomains.

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Table 1. Assay performance

S/B	3.4 ± 0.2
S/N	15.3 ± 5.9
Z′	0.71 ± 0.09
Well-to-well CV (max/min, %)	4.59 ± 1.95 / 7.49 ± 1.2
Plate-to-plate CV (%)*	2.2
Day-to-day CV (%)*	13.8

* Calculated from Z′ values

FIGURE LEGENDS

Figure 1. AlphaScreen assay principle. Without an inhibitor macrodomain hydrolyzes biotinylated ADP-ribosyl from His-tagged SRPK2. Acceptor bead bound SRPK2 cannot interact with streptavidin coated Donor beads and there is no singlet oxygen transfer and no emission. With a macrodomain inhibitor, biotinylated ADP-ribosyl is not hydrolyzed from SRPK2. This leads to proximity generated singlet oxygen transfer and emission.

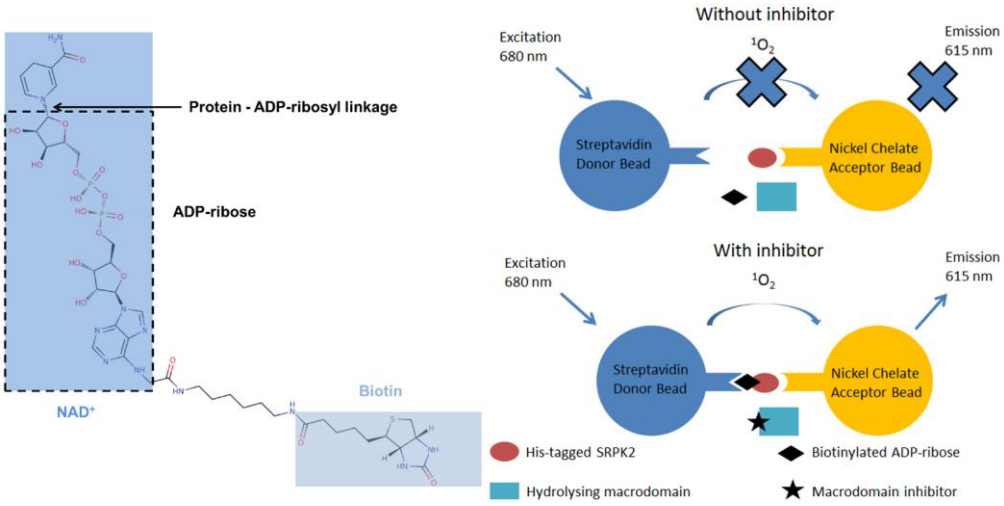
Figure 2. Assay sensitivity, ADP-ribosyl hydrolysis and AlphaScreen bead optimization. **(A)** The sensitivity of the assay was tested by a concentration series of modified SRPK2. Maximal signal (hook point) represents the maximal signal (cps) of the assay. Modified SRPK2 and acceptor beads were incubated for 30 min after which donor beads were added followed by an additional 60 min of incubation. **(B)** To test whether ADP-ribosylated SRPK2 can function as a substrate in MacroD1 catalyzed reaction, MacroD1 was titrated against SRPK2 (50 nM). MacroD1 and modified SRPK2 were incubated for 180 min after which acceptor beads were added followed by 30 min incubation and addition of streptavidin donor beads. The AlphaScreen signal was measured after 60 min incubation. **(C)** Order of addition of the AlphaScreen beads was determined with three sequences. After incubating 50 nM modified SRPK2 with 400 nM MacroD1 for 180 min, (a) Both beads were added together followed by 90 min incubation; (b) the acceptor beads were added and after 45 min incubation, donor beads were added followed by 45 min incubation; (c) the donor beads were added and after 45 min incubation, acceptor beads were added followed by 45 min incubation. **(D)** Optimal bead concentration was determined by incubating 50 nM modified SRPK2 with 400 nM MacroD1 for 180 min with various bead concentrations. AlphaScreen signal is shown for modified SRPK2, SRPK2 + MacroD1, and beads only. Also Z′ for each bead concentration is calculated. **(E)** The effect of bead incubation time to signal level was optimized using 5 µg / ml bead concentration.

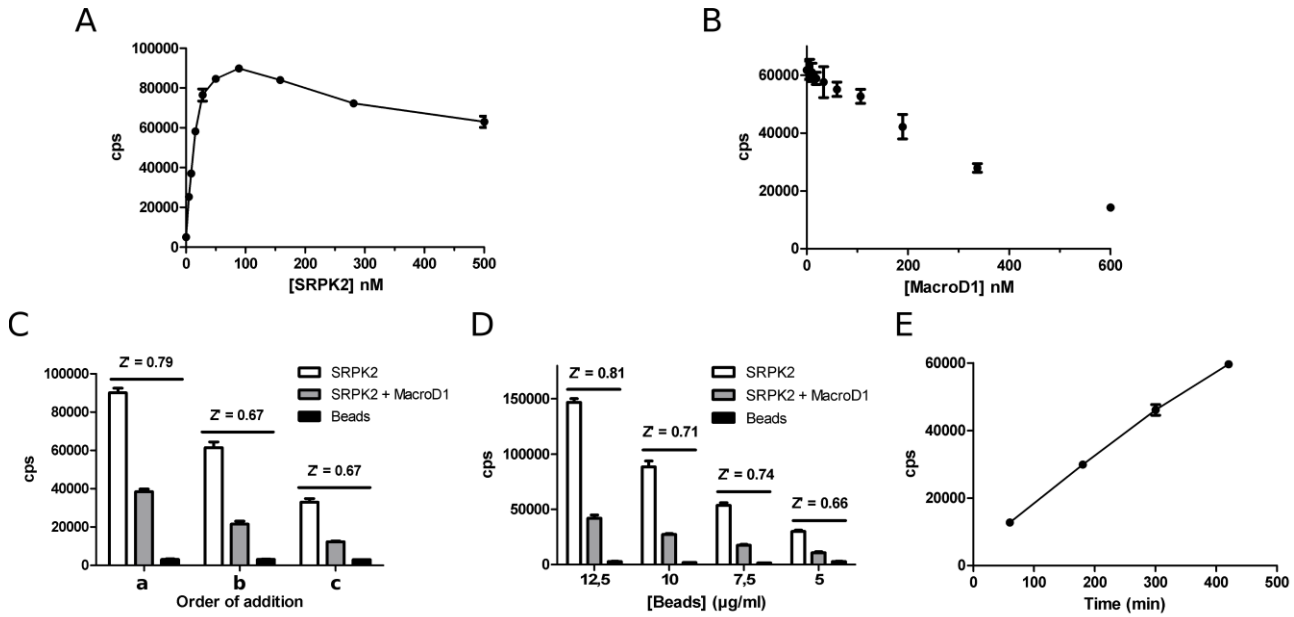
Figure 3. Time and concentration dependency of MacroD1, DMSO tolerance and plate reader parameters. (A) MacroD1 concentration and incubation time were optimized to shorten the assay duration. Various concentration and time points of MacroD1 were incubated with 50 nM SRPK2 to evaluate the enzymatic activity. **(B)** Western blot assay to verify substrate consumption. Modified SRPK2 (0.5 µM) was incubated with MacroD1 (4 µM) at room temperature for various times. Negative control (-) is modified SRPK2 only. Protein staining of the same membrane is shown below. **(C)** DMSO tolerance assayed with 50 nM SRPK2 incubated with 800 nM MacroD1 for 80 min in the presence of various DMSO concentrations. The results are presented as signal-% compared to the 0% DMSO control. DMSO concentration up to 10% did not show statistically significant difference compared to the control. **(D)** To further increase the signal window we optimized the plate reader parameters. SRPK2 (50 nM) was incubated with 5 µg/ml beads for 180 min and measured with various excitation times and signal integration times.

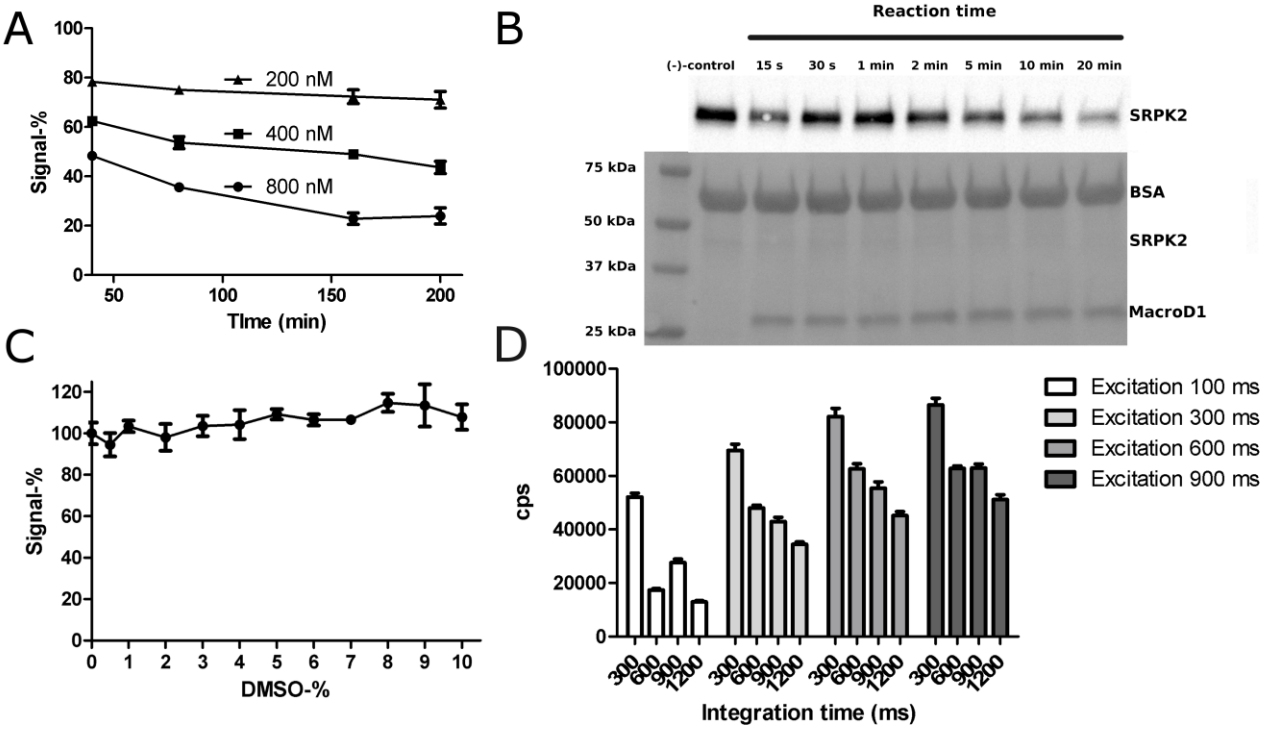
Figure 4. Screening of MacroD1 with MicroSource Spectrum library. (A) Relative activity of the compounds calculated from the positive controls (100 % activity) on the plate. Hit limit was set to 50 %. Two compounds with negative activity (protoporphyrin IX and haematoporphyrin) are not shown. **(B)** Structures of the hit compounds.

Figure 5. Dose-response measurements and validation of the hit compounds. Dose response measurements **(A-E)** were done with hit compounds from validatory screening. The compounds were assayed in quadruplicates using half-logarithmic dilutions. An orthogonal Western blot assay **(F)** to validate the hits obtained from the screening. Modified SRPK2 (1 µM), MacroD1 (4 µM) and the hit compounds (100 µM) were incubated at room temperature for 2 h 30 min. Negative control (-) is the

modified SRPK2 only. Positive control (+) contains the modified SRPK2 and MacroD1 without compound. Protein staining of the same membrane is shown below.


Protein - ADP-ribosyl linkage
ADP-ribose
NAD+
Biotin
Without inhibitor
Excitation
680 nm
$^1O_2$
Emission
615 nm
Streptavidin
Donor Bead
Nickel Chelate
Acceptor Bead
With inhibitor
Excitation
680 nm
$^1O_2$
Emission
615 nm
Streptavidin
Donor Bead
Nickel Chelate
Acceptor Bead
His-tagged SRPK2
Biotinylated ADP-ribose
Hydrolysing macrodomain
Macrodomain inhibitor


A
cps
[SRPK2] nM
B
cps
[MacroD1] nM
C
Z' = 0.79
Z' = 0.67
Z' = 0.67
SRPK2
SRPK2 + MacroD1
Beads
cps
a
b
c
Order of addition
D
Z' = 0.81
Z' = 0.71
Z' = 0.74
Z' = 0.66
SRPK2
SRPK2 + MacroD1
Beads
cps
12,5
10
7,5
5
[Beads] (µg/ml)
E
cps
Time (min)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Signal-%
200 nM
400 nM
800 nM
TIme (min)
B
Reaction time
(-)-control
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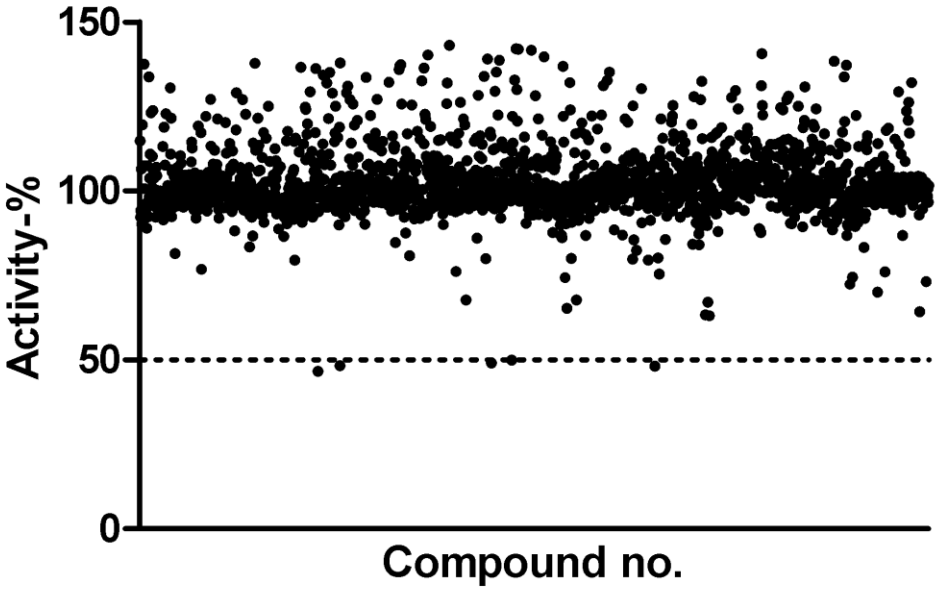
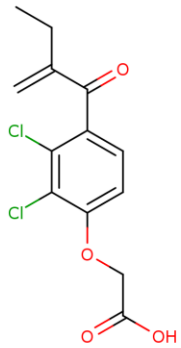
30 s
1 min
2 min
5 min
10 min
20 min
SRPK2
75 kDa
50 kDa
37 kDa
25 kDa
BSA
SRPK2
MacroD1
C
Signal-%
DMSO-%
D
cps
Excitation 100 ms
Excitation 300 ms
Excitation 600 ms
Excitation 900 ms
Integration time (ms)

A



B



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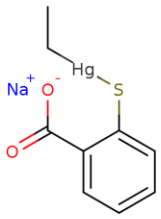
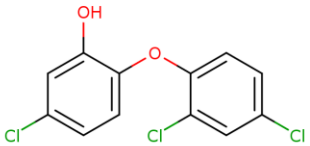
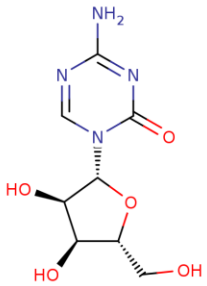


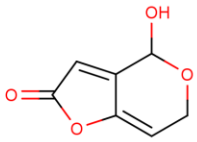
thimerosal



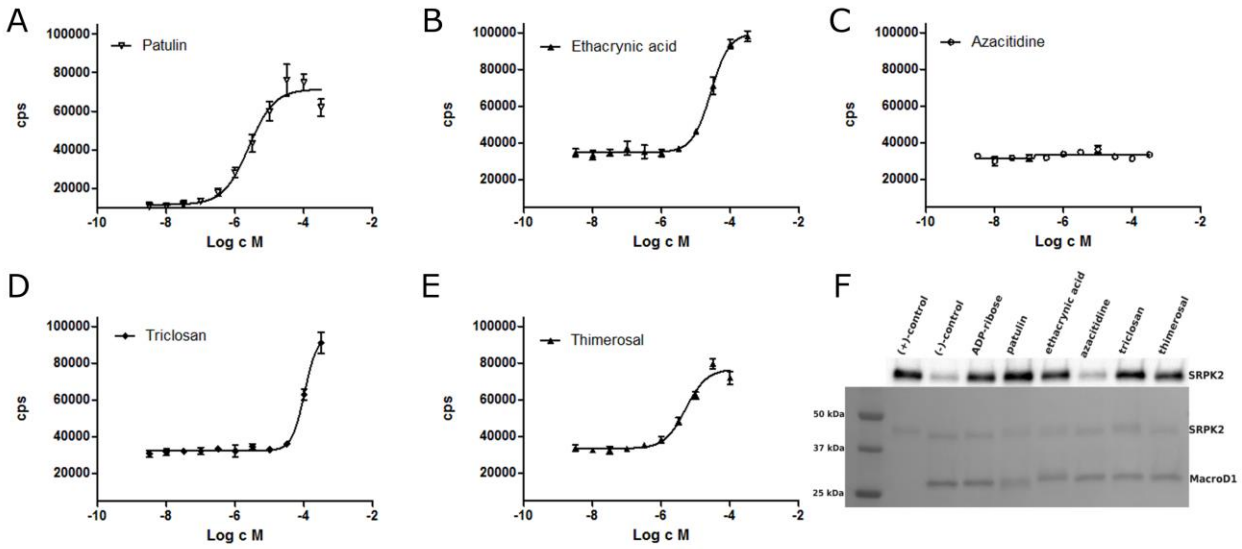
triclosan



azacitidine



patulin


A
Patulin
cps
Log c M
B
Ethacrynic acid
cps
Log c M
C
Azacitidine
cps
Log c M
D
Triclosan
cps
Log c M
E
Thimerosal
cps
Log c M
F
(+)-control
(-)-control
ADP-ribose
patulin
ethacrynic acid
azacitidine
triclosan
thimerosal
SRPK2
50 kDa
37 kDa
25 kDa
SRPK2
MacroD1