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Activity-based assay for human mono-ADP-ribosyltransferases ARTD7/PARP15 and ARTD10/PARP10 aimed at screening and profiling inhibitors

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ABSTRACT

Poly(ADP-ribose) polymerases (PARPs) or diphtheria toxin like ADP-ribosyl transferases (ARTDs) are enzymes that catalyze the covalent modification of proteins by attachment of ADP-ribose units to the target amino acid residues or to the growing chain of ADP-ribose. A subclass of the ARTD superfamily consists of mono-ADP-ribosyl transferases that are thought to modify themselves and other substrate proteins by covalently adding only a single ADP-ribose moiety to the target. Many of the ARTD enzymes are either established or potential drug targets and a functional activity assay for them will be a valuable tool to identify selective inhibitors for each enzyme. Existing assays are not directly applicable for screening of inhibitors due to the different nature of the reaction and different target molecules. We modified and applied a fluorescence-based assay previously described for PARP1/ARTD1 and tankyrase/ARTD5 for screening of PARP10/ARTD10 and PARP15/ARTD7 inhibitors. The assay measures the amount of NAD^+ present after chemically converting it to a fluorescent analog. We demonstrate that by using an excess of a recombinant acceptor protein the performance of the activity-based assay is excellent for screening of compound libraries. The assay is homogenous and cost effective, making it possible to test relatively large compound libraries. This method can be used to screen inhibitors of mono-ARTDs and profile inhibitors of the enzyme class. The assay was optimized for ARTD10 and ARTD7, but it can be directly applied to other mono-ARTDs of the ARTD superfamily. Profiling of known ARTD inhibitors against ARTD10 and ARTD7 in a validatory screening identified the best inhibitors with submicromolar potencies. Only few of the tested ARTD inhibitors were potent, implicating that there is a need to screen new compound scaffolds. This is needed to create small molecules that could serve as biological probes and potential starting points for drug discovery projects against mono-ARTDs.

1. Introduction

ADP-ribosylation is a post-translational modification involved in several cellular activities like signaling, DNA repair, maintenance of genomic stability, apoptosis and cell proliferation (Hassa et al., 2006). Phylogenetic studies indicate that this is a fairly ancient mechanism and has been conserved from archaebacteria to multicellular eukaryotes during the course of evolution (Hassa and Hottiger, 2008). There are 18 members in the poly(ADP-ribose) polymerase (PARP) enzyme superfamily (Schreiber et al., 2006; Hottiger et al., 2010). Of these 18 enzymes some form ADP-ribose polymers (1–6) and some can only transfer one ADP-ribose unit (7–8, 10–12, 14–18), while ARTD9/PARP9 and ARTD13/PARP13 are thought to be inactive (Kleine et al., 2008). As most of the enzymes do not catalyze the formation of the ADP-ribose chain, but are mono-ADP-ribosyltransferases, it is more appropriate to refer to them as ARTDs (diphtheria toxin-like ADP-ribosyltransferases) (Hottiger et al., 2010).

The poly-ARTDs are implicated in a variety of cellular activities; the best understood and most exploited is the role of ARTD1 and ARTD2 in DNA damage repair. Cancer cells with compromised homologous recombination, for example due to loss of BRCA1 or BRCA2, display increased sensitivity to radiotherapy when complemented with ARTD inhibitors. This synthetic lethality is due to blocking the ARTD1 and ARTD2 promoted base excision repair (BER) (Jagtap and Szabó, 2005; Lord and Ashworth, 2008). Currently of special interest as drug targets are ARTD1, ARTD2, ARTD5 and ARTD6 due to their potential roles in the treatment of cancer (Kummar et al., 2012). The mono-ADP-ribosyltransferases are the least understood ARTD enzymes and recent breakthroughs suggest their involvement in various pathways ranging from immune signaling, cell proliferation, transcriptional regulation and cell death (Welsby et al., 2012). However, no specific inhibitors currently exist for these enzymes.

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Two mono-ARTDs, ARTD10/PARP10 and ARTD7/PARP15, are studied here. ARTD10/PARP10 ADP-ribosylates core histones and also interacts with the oncoprotein MYC (Yu et al., 2005; Kleine et al., 2012). It has been suggested to have a role in cell proliferation, which makes it a possible target for inhibitor development against cancer (Chou et al., 2006; Kleine et al., 2008; Herzog et al., 2013). ARTD7/PARP15 or BAL3 (B-aggressive lymphoma) is a macrodomain containing mono-ARTD. Macrodomains are protein domains known to bind to mono- or poly-ADP-ribose (Kleine and Lüscher, 2009; Han et al., 2011; Welsby et al., 2012; Forst et al., 2013). Other members of this macrodomain containing-subfamily are ARTD9 (BAL1) and ARTD8 (BAL2). ARTD7-9 have possible functions in the control of gene transcription and also have been found to be over-expressed in chemoresistant tumors of diffuse large B cell lymphoma (Aguiar et al., 2005).

While the role of different ARTD enzymes as potential drug targets for various cancers is undisputed, there is still very little known about these enzymes. Having selective inhibitors for each enzyme would provide chemical probes for analysis of their participation in cellular processes. As some enzymes are considered drug targets, it is important to establish methods for profiling the inhibitors early in the drug development. We present here an activity-based assay developed to screen and identify inhibitors for mono-ARTDs. The assay is modified from a previously described fluorescence method used for ARTD1 and ARTD5 (Putt and Hergenrother, 2004; Narwal et al., 2012a), but also for sirtuins (Feng et al., 2009) and for bacterial toxins (Maurer et al., 2011). The assay was not directly applicable for mono-ARTDs due to the non-processive nature of the reaction and high substrate consumption required for detecting a robust signal. We have circumvented this challenge by using an excess of an easily accessible recombinant protein as a target protein for modification in the reaction. The mono-ARTD assay we describe here is homogeneous, cost-effective and thus a feasible alternative to currently available commercial assays. We also profiled a panel of known ARTD/PARP inhibitors against ARTD7 and ARTD10 and identified the best inhibitors for these two enzymes. This screening method is directly applicable for other mono-ARTDs, provided that a good acceptor protein can be identified and used in the assay to increase the enzymatic activity.

2. Materials and methods

2.1. Protein expression

The expression constructs for the catalytic domain of ARTD10 (residues 809-1017) with a 6 × His tag at the C-terminus (pNIC-CH) and the catalytic domain of ARTD7 (residues 460-656) with a 6 × His tag at the N-terminus followed by a TEV cleavage site (pNIC28-Bsa4) were a gift from the Structural Genomics Consortium (Stockholm, Sweden). The construct for SRPK2 (residues 81-699) with a 6 × His tag at the N-terminus followed by a TEV cleavage site (pNIC-Bsa4) was also a gift from the Structural Genomics Consortium (Oxford, UK) (PDB: 2X7G). The original ARTD10 construct had two mutations (R922K, V979M; PDB: 3HKV). These mutations were corrected and inactive G888W mutant was made using a QuickChange™ Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA).

For expression, the plasmids were transformed into *Escherichia coli* Rosetta2 (DE3) cells. Glycerol stocks were used to inoculate 5 ml pre-cultures in Terrific Broth with 50 μg/ml kanamycin and 34 μg/ml chloramphenicol as selection markers. These cultures were grown overnight at 37 °C and were used to inoculate four cultures (750 ml each) of auto induction TB media with trace elements (Formedium, UK) supplemented with 8 g/l glycerol and the same selection markers. The cultures were incubated at 37 °C with shaking at 200 rpm until the OD_{600} reached 1. The temperature was then reduced to 18 °C and the cultures were allowed to grow overnight (14–16 h). The cells were collected the next day by centrifugation (5500*g*, 20 min, and 4 °C). The cell pellet was resuspended in lysis buffer (100 mM HEPES pH 7.5, 500 mM NaCl, 10% glycerol, 10 mM imidazole and 0.5 mM TCEP) and stored at −20 °C.

2.2. Protein purification

The cell suspension was thawed in warm water (5 min) and then 250 U benzonase (Sigma–Aldrich), 0.25 mg lysozyme and one EDTA-free protease inhibitor tablet (Roche, Switzerland) were added. The cells were lysed by sonication for 20 min with a 50% duty cycle (Branson 250 Sonifier). The sonicated solution was then centrifuged (35,000*g*, 20 min, 4 °C) to collect the supernatant. The supernatant was filtered using 0.45 μm filters before loading it onto a HisTrap HP column (GE Healthcare, UK) pre-charged with Ni^{2+} and pre-equilibrated with binding buffer (20 mM HEPES, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 0.5 mM TCEP, pH 7.5) at 4 °C. Further purification of the protein was performed using an Äkta purifier at room temperature (GE Healthcare, UK). The HisTrap column was attached to the purifier and then washed with a wash buffer (20 mM HEPES, 500 mM NaCl, 10% glycerol, 25 mM imidazole, 0.5 mM TCEP, pH 7.5). Protein was eluted from the column with a buffer supplemented with 500 mM (ARTD10 and ARTD7) or 300 mM imidazole (SRPK2). The eluted protein fractions were pooled together and concentrated to less than 800 μl using VIVASPIN 20 concentrators (Sartorius Stedim Biotech, France). For the purification of ARTD10 and ARTD7, the concentrated protein was loaded onto the size-exclusion column (Hiprep 16/60 Sephacryl S-100 HR, GE Healthcare) pre-equilibrated with gel filtration buffer (20 mM HEPES, 300 mM NaCl, 10% glycerol, 0.5 mM TCEP, pH 7.5). For SRPK2 the gel filtration buffer used contained 50 mM HEPES, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP at pH 7.5. Protein fractions were analyzed by separation on an SDS-PAGE gel and fractions containing the protein of interest were pooled and concentrated. Final protein concentrations were calculated using the extinction coefficients and absorbance at 280 nm. Finally the proteins were divided into aliquots (50 μL), which were flash frozen in liquid nitrogen and stored at −80 °C.

2.3. Western blot based activity assay

To test the activity of ARTD10 and ARTD7 and to confirm that SRPK2 is an acceptor of ADP-ribose western blot experiments were performed using biotinylated NAD^+ as a substrate (Trevigen, USA). The enzymatic reaction contained 1 μM biotinylated NAD^+ together with 500 nM ARTD10 and 2 μM SRPK2 in 50 mM Tris pH 7.0 or 350 nM ARTD7 and 2 μM SRPK2 in 50 mM Na2HPO4/NaH2-PO4 pH 7.0. The reactions were carried out at room temperature for 3 h, and they were stopped by adding 2 × Laemmli buffer (Bio-Rad, USA) and heating the mixture at 80 °C for 5 min. The samples were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane (Whatman, UK). The immobilized proteins on the membrane were blocked using 1% Casein in 1x TBS (Bio-Rad, USA) to avoid any unspecific binding of streptavidin conjugated horseradish peroxidase (PerkinElmer, USA), which was diluted to 1:15,000 to detect the ADP-ribosylated proteins.

2.4. Homogeneous activity assay

A previously described assay for ARTD1 (Putt and Hergenrother, 2004; Narwal et al., 2012a) was modified and adapted for measurement of mono-ARTD activity. The assay is based on the chemical conversion of the unused NAD^+ to a fluorophore. The decrease in