Cell-permeable nicotinamide adenine dinucleotides for exploration of cellular protein ADP-ribosylation

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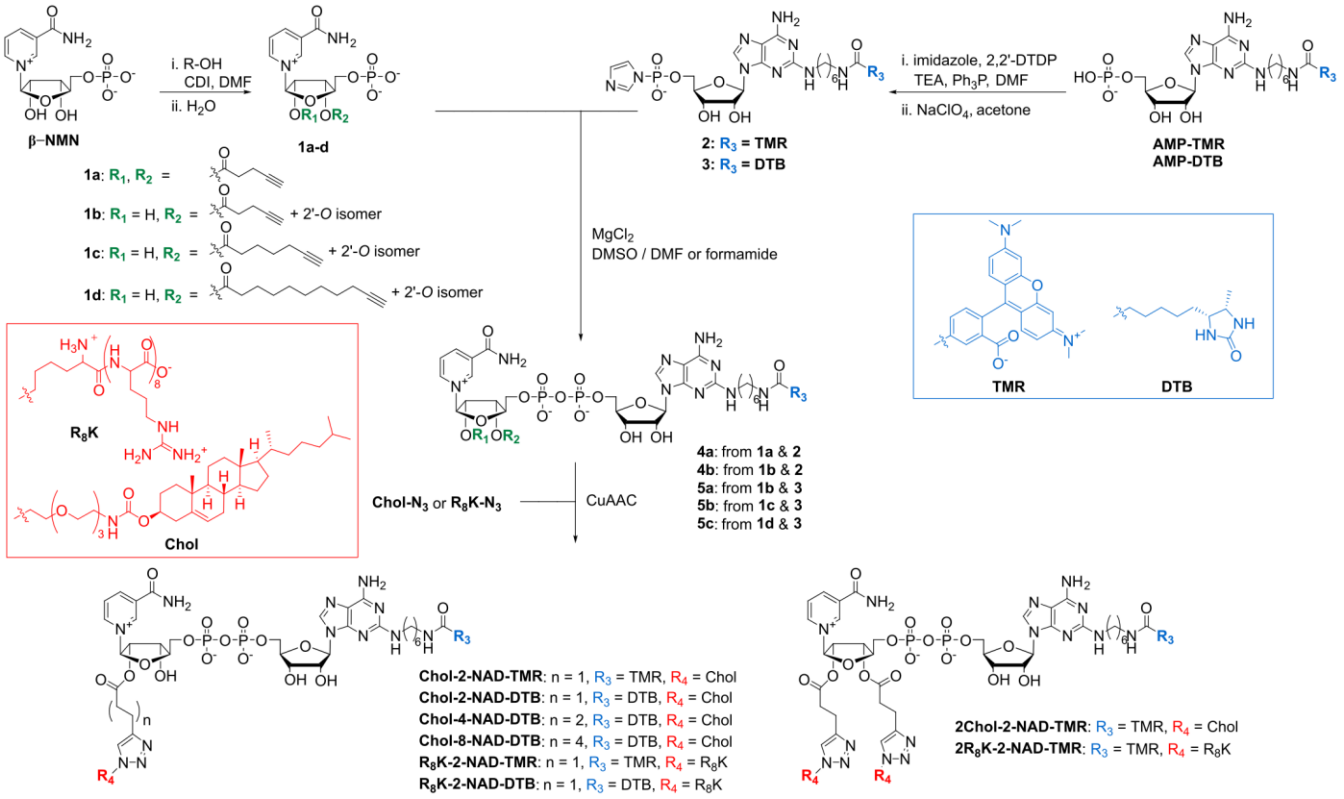
ABSTRACT: Posttranslational modifications (PTMs) greatly enhance the functional diversity of proteins, surpassing the number of gene-encoded variations. One intriguing PTM is ADP-ribosylation, which utilizes nicotinamide adenine dinucleotide (NAD^+) as a substrate and is essential in cell signaling pathways regulating cellular responses. Here, we report the first cell-permeable NAD^+ analogs and demonstrate their utility for investigating cellular ADP-ribosylation. Using a desthiobiotin-labelled analog for affinity enrichment of proteins that are ADP-ribosylated in living cells under oxidative stress, we identified protein targets associated with host-virus interactions, DNA damage and repair, protein biosynthesis, and ribosome biogenesis. Most of these targets have been noted in various literature sources, highlighting the potential of our probes for cellular ADP-ribosylome studies.

Posttranslational modifications (PTMs) contribute to the large variety of functional proteoforms.[1] One PTM is ADP-ribosylation, which is catalyzed by enzymes known as ADP-ribosyl transferases (ARTs), initially identified in the context of some bacterial toxins.[2] ARTs are also termed PARPs (poly(ADP-ribose)polymerases).[3] These enzymes employ NAD^+ as a donor of ADP-ribose, transferring it to specific amino acid side chains like Arg, Glu, Asp, Lys, Cys, and Ser, which leads to the formation of a mono-ADP-ribosylated (MARylated) protein.[4] This modification is often further extended by the consecutive attachment of ADP-ribose units through the 2′-*O* of adenosine leading to poly-ADP-ribosylation (PARylation). In mammals, there are 17 known members of the PARP family,[3, 5] each catalyzing either MARylation or PARylation. PARylation is recognized as a crucial mechanism for regulating various aspects of cellular physiology such as DNA damage response,[6] translation control,[7] and viral infectivity.[8] The role of MARylation is still not well understood, although it has been reported that it is involved in processes such as RNA metabolism, cellular transport, and stress respone.[9] Malfunction of the PTMs machinery can lead to various pathogenic processes, such as carcinogenesis.[10] Furthermore, inhibition of ADP-ribosylation can induce tumor cell death and suppress pro-inflammatory signaling by maintaining cellular bioenergetics and resulted in the development of approved drugs.[11] Hence, the development of new tools for further inside into ADP-ribosylation is of a great importance.

Several nucleotide-based tools have been designed for this purpose.[12, 13, 14, 15] However, due to the presence of a negatively charged phosphate chain, these nucleotides cannot penetrate the cell membrane, which hinders their utility for studies in living cells. Therefore, the identification of ADP-ribosylation targets has been primarily conducted in cell lysates. Besides invasive approaches for cellular delivery of NAD^+ analogs[16] that may result in cell damage and cell death,[17] transfection agents have only recently been used to be employed for cellular internalization of NAD^+ analogs.[14, 15, 18] Here, we report on the development of the first cell permeable NAD^+ analogs exploiting two mechanism of cellular uptake: modification by cholesterol (Chol, passive diffusion)[19] and by the cell penetrating peptide R_8K (direct translocation and endocytosis).[20] These NAD^+ modifications were attached through ester bonds at 2′-*O*/3′-*O* nicotinamide ribose that are expected to be hydrolyzed by cellular esterases, a strategy exploited in prodrug approaches.[21] To this end, we synthesized new double modified NAD^+ analogs, each containing either a TAMRA fluorescent tag (TMR) or a desthiobiotin affinity tag (DTB), in combination with a cell-permeability-promoting ligand (Chol or R_8K; **Scheme 1**). In brief, to modify the β-nicotinamide mononucleotide (**β-NMN**) with an ester that contains the cell-permeability-promoting ligand, it turned out that first esterification with an alkyne-containing acid and subsequent Cu-catalyzed azide-alkyne cycloaddition (CuAAC)[22] with the azide-containing ligand was the most proficient. Thus, **β-NMN** was reacted with 4-pentynoic acid to form 2′,3′-*O* diester (**1a**) or 2′-*O*/3′-*O* monoester mixture (**1b**). NMR analysis of the latter showed 1:2 ratio of 2′-*O* to 3′-*O* isomer (**SI**). In order to examine the impact of the linker length connecting the cell-permeable ligand with nicotinamide ribose, we also synthesized

Scheme 1. Synthesis of cell-permeable NAD⁺ analogs[a]



[a]For details on conditions, reagents and yields, see SI.

6-heptynoic (**1c**) and 10-undecynoic (**1d**) **β-NMN** monoesters, each in a mixture of 2'-*O*/3'-*O* isomers. The synthesis of the adenosine-5'-monophosphate (AMP) residues started with the previously reported[18] analogs **AMP-TMR** and **AMP-DTB** that were converted into *P*-imidazolides **2** and **3**[23] and then coupled with **1** in DMF / DMSO or formamide, in the presence of Mg^{2+} to yield the NAD^+ analogs **4** and **5**. Ultimately, we obtained five NAD^+ analogs containing either a diester 4-pentynoic or monoester 4-pentynoic, 6-heptynoic or 10-undecynoic modification and either a TMR (**4a-b**) or DTB (**5a-c**) tag (**Scheme 1**). To further modify the NAD^+ probe with a cell permeability-promoting ligand, we employed CuAAC chemistry, utilizing either cholesteryl-TEG-azide (**Chol-N_3, Scheme 1**) or a short peptide consisting of eight arginines and *N*-terminal lysine modified with an azide group within the backbone (**R_8K-N_3**; **Scheme 1**). Interestingly, the synthesis of the latter required the presence of concentrated urea (**Figure S1**). The resulting products were purified using analytical or semi-preparative HPLC (**Table S1**). More information is detailed in the **SI**.

Next, we incubated HeLa cells with NAD-TMR probes functionalized with Chol- and R_8K-, and investigated them by fluorescence confocal microscopy. Images of fixed HeLa cells were captured after incubation with probes, and a control probe precursor, bearing TMR and two pentynoic substitutions (**4a**; **Figure S2**). The Chol-modified probe **Chol-2-NAD-TMR** exhibited a punctate cytoplasmic distribution, while the R_8K-modified analogs (**R_8K-2-NAD-TMR** and **2R_8K-2-NAD-TMR**) displayed an even distribution within the cytoplasm. Additionally, R_8K-labelled NADs were observed in the nucleoli, although this might have been caused by the fixation process.[24] Double cholesterol modified NAD-TMR (**2Chol-2-NAD-TMR**) precipitated significantly, making it challenging to control its concentration. Furthermore, the cells incubated with double peptide-modified NAD-TMR (**2R_8K-2-NAD-TMR**) at 20 μM exhibited signs of apoptosis, indicating increased cytotoxicity (**Figure S2**). Overall, mono-substitution with Chol or R_8K was sufficient for the delivery of NAD-TMR. Hence, for the further studies, we focused on mono-functionalized NADs. Additionally, we investigated cell-permeability of probes **Chol-2-NAD-TMR** and **R_8K-2-NAD-TMR** in living HeLa cells. The **Chol-2-NAD-TMR** probe was again localized in the cytoplasm, while R_8K was observed in both cytoplasm and nucleus, with a notably higher concentration in the latter (**Figure 1A**). We evaluated further the cell permeability of our probes using flow-cytometry analysis (**Figure 1B**). We proceeded to investigate the second set of probes functionalized with DTB. HeLa cells were incubated with Chol- and R_8K- labelled NAD-DTB compounds **Chol-2-NAD-DTB** and **R_8K-2-NAD-DTB**. Subsequently, the cells were fixed with paraformaldehyde and incubated with a Cy5-streptavidin conjugate to enable DTB visualization (**Figure 1C, S3**). As controls we used NAD-DTB analogs bearing 2'-*O*/3'-*O* alkyne linker (**5a-c**) and NAD-DTB.[18] Fluorescence confocal microscopy images of the cells indicated that both probes were localized in the cytoplasm. The peptide probe's (**R_8K-2-NAD-DTB**) presence within the nucleolus likely resulted from the cell fixation process once again.

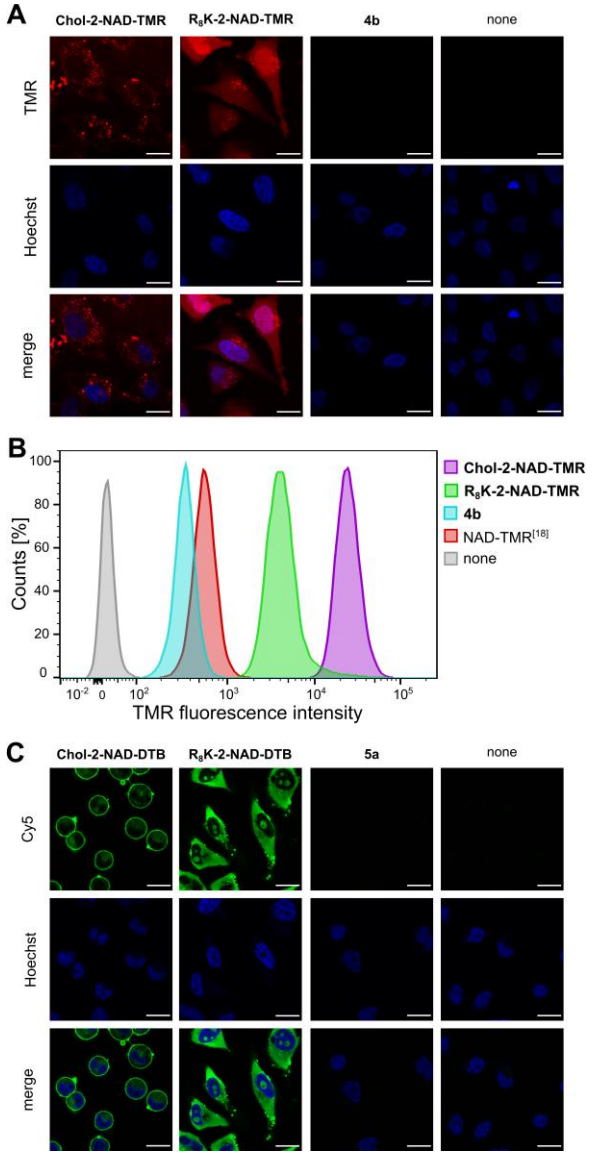


Figure 1. Cellular uptake of NAD-TMR conjugates featuring Chol- or R_8K- by living HeLa cells, investigated by **A.** confocal microscopy and **B.** flow-cytometry; **C.** confocal imaging of fixed HeLa cells previously incubated with NAD-DTB conjugates featuring Chol- or R_8K-. Scale bar: 20 μm.

We next assessed the substrate susceptibility of cholesterol-modified NAD-TMR (**Chol-2-NAD-TMR**), by conducting an *in vitro* auto-ADP-ribosylation with PARP1, using previously reported assay.[14] Briefly, **Chol-2-NAD-TMR** was incubated with PARP1 and short dsDNA, both alone and in a mixture with natural NAD^+ or NAD-TMR. Additionally, compounds were pre-incubated for 24 h or pig liver esterase (PLE) was added to induce hydrolysis of the ester bond connecting the cell-permeable ligand with nicotinamide ribose. Afterwards, the samples were analyzed using SDS-PAGE with TMR fluorescence detection or Coomassie staining (**Figure 2A**). The data shows that the NAD^+ analog was accepted to some extent as a substrate for PARP1, despite its modification. When subjected to a 24 h pre-incubation or used in a 1:1 mixture with natural NAD^+ or NAD-TMR, the formation of longer PAR chains was observed. Afterwards, we assessed substrate susceptibility of **Chol-2-NAD-DTB** to various PARP enzymes

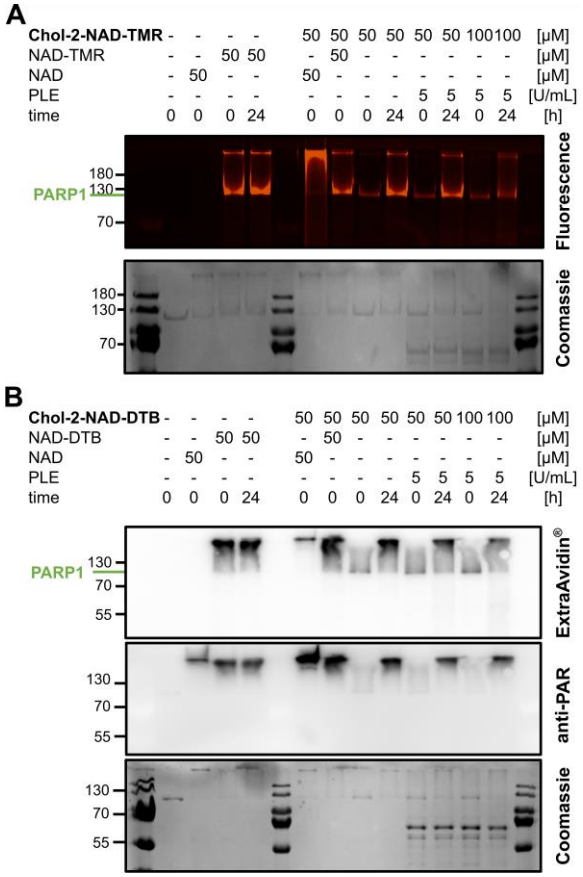


Figure 2. Acceptance of **A. Chol-2-NAD-TMR** or **B. Chol-2-NAD-DTB** by PARP1.

(PARP1-3, 6, 10, 14, and TNKS1-2), using previously described *in vitro* auto-ADP-ribosylation assays.[18] The same experimental conditions and control samples were examined as for **Chol-2-NAD-TMR**. Reaction mixtures were analyzed through immunoblotting using ExtraAvidin®-Peroxidase for DTB visualization or poly(ADP-ribose) monoclonal antibody (10H) to visualize PAR chains (**Figure 2B**, **S4**). We concluded, that probe **Chol-2-NAD-DTB** serves as a substrate for all of the tested PARPs. We observed weak auto-ADP-ribosylation by PARP1 and 2. TNKS1-2, (PARylating enzymes) most likely utilized the probe primarily for MARylation or the short PAR chains formation. The PARPs, classified as MARylating proteins, also demonstrated the ability to utilize **Chol-2-NAD-DTB** as a substrate. Preincubation of **Chol-2-NAD-DTB** for 24 h resulted in more efficient ADP-ribosylation, attributed to the hydrolysis of the ester bond. In the presence of natural NAD^+ or NAD-DTB, longer PAR chains were formed, indicating that probe **Chol-2-NAD-DTB** is not the preferred substrate. Application of the **R_8K-2-NAD-DTB** for *in vitro* protein ADP-ribosylation showed similar outcome (**Figure S5**).

Afterwards we moved to the application of NAD-DTBs for protein ADP-ribosylation in HeLa cells. Initially, cells were treated with NAD^+ probes and ADP-ribosylation was induced by H_2O_2-mediated oxidative stress. Subsequently, cell lysates were subjected to analysis using immunoblotting with ExtraAvidin®-Peroxidase (DTB visualization), an anti-PAN-binding reagent that interacts with ADP-ribose, and SDS-PAGE with Coomassie staining as a loading control

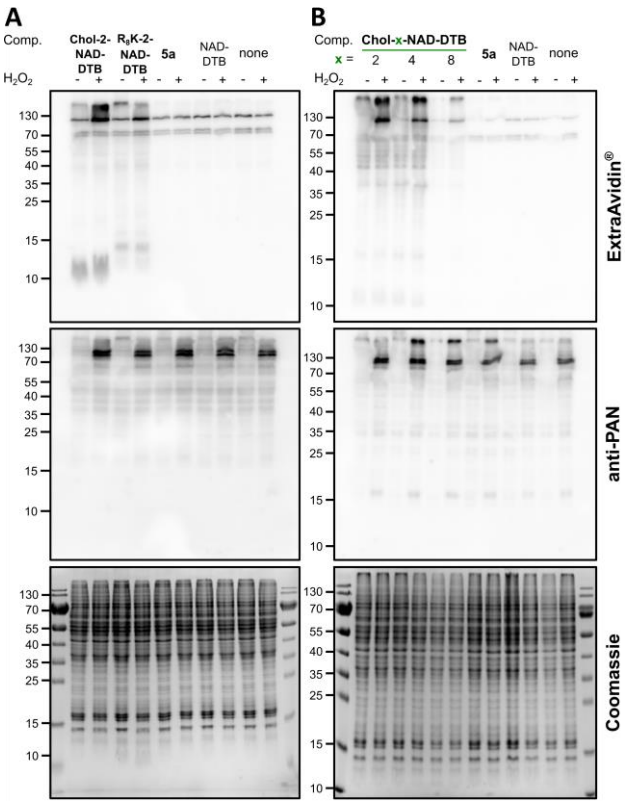


Figure 3. Analysis of lysates from HeLa cells, previously incubated with NAD-DTB probes containing different **A.** cell-permeable ligand and **B.** length of the linker connecting ligand with nicotinamide ribose.

(**Figure 3A**). The DTB signals were detected in all samples containing cell-permeable probes and corresponded to the ADP-ribose ones, which confirmed the incorporation of DTB-tagged ADP-ribose units into the proteins. The difference in the DTB signal intensity between lysates from non-stressed and H_2O_2-stressed HeLa cells was the most significant for compound **Chol-2-NAD-DTB**, hence, we focused on cholesterol-tagged NADs for further structure activity studies. We investigated different linker lengths connecting cholesterol-TEG-triazole with the 2'-*O*/3'-*O* position of nicotinamide riboside (three – **Chol-2-NAD-DTB**, five – **Chol-4-NAD-DTB**, or nine –**Chol-8-NAD-DTB** carbon atoms). The most significant increase in DTB signal after H_2O_2 treatment was observed for the probe with the shortest linker, **Chol-2-NAD-DTB** (**Figure 3B**). In the end the sensitivity of this probe was higher comparing to our previous approach[18] allowing for a tenfold reduction of its concentration. We also assessed the cytotoxicity of NAD-DTBs (**Figure S6**). Cholesterol-modified probes exhibited greater cytotoxicity compared to peptide-modified ones, albeit still minor at 10 µM.

Next, we employed the optimized probe **Chol-2-NAD-DTB** for affinity enrichment of proteins that are ADP-ribosylated upon H_2O_2-induced oxidative stress in HeLa cells. Using a modified workflow established for NAD-DTB,[18] proteins were enriched, trypsin-digested (**Figure 4A**, **S7**), and analyzed via LC-MS/MS (four biological replicates, each measured in technical duplicate). Using a label-free quantification approach[25] we found 1465 proteins and identified 121 potential protein targets (**Figure S4B**, **SI**) after statistical validation using ANOVA (FDR = 0.05, s0 = 0.1) and post-hoc Tukey HSD (FDR = 0.05). To gain a deeper insight into the localization and potential function of the identified proteins, we used the DAVID tool[26] to analyze their respective genes (**Figure 4C; SI**). Our analysis revealed a comparable number of proteins localized in the nucleus (56) and cytoplasm (67), associated with processes that have been linked to ADP-ribosylation, such as host-virus interactions, DNA damage, mRNA processing or ribosome biogenesis.[27] Further evaluation by comparison with the ADPriboDB 2.0 database[28] revealed that genes of only 8 out of our 121 significantly enriched proteins were not present in this database (**Table S3**). However, these 8 hits have close relationships with proteins included in the database, encoded by genes like FMNL2, STAT5A, PREP, CD151, OSBPL8, ACTR3, and AP3D1. Limited data exists on the ADP-ribosylation of IFIT-5 (gene: IFIT5), although recent findings indicate that PARP9 can increase IFIT1 expression in B cells.[29] Additionally, we investigated protein-protein interaction networks using the STRING database,[30] revealing two primary networks: one involved in DNA repair and metabolic processes, the other in RNA-related processes and ribosome biogenesis, which have already been discussed in the context of ADP-ribosylation (**Figure S8**).[7, 31] Numerous proteins were also found to play roles in stress responses. We compared these proteins with previously identified ADP-ribosylation targets.[12, 18, 32] Overlap between various enrichment strategies ranged from 1 to 27% (**Figure S9**), comprising 3 to 22% for methods not involving nucleotides and 1 to 27% for nucleotide-derived probes. Overall, we found 56% of our protein hits within the selected literature data (**SI**). The lack of the remaining proteins may result from a different detection system (non-nucleotide approaches, nucleosides with low specificity), utilization of living cells instead of cell extracts (cellular compartmentalization), different cell lines or additional cell-stress by the transfection reagent.

In conclusion, we report the first cell-permeable NAD^+ analogs and demonstrate their suitability for investigating protein ADP-ribosylation in living cells. Utilizing DTB-tagged probes, we characterized ADP-ribosylome changes during oxidative stress in HeLa cells. Enhanced sensitivity of these probes allowed for a tenfold reduction of its concentration compared to our previous method (NAD-DTB with DOTAP transfection).[18] Using our approach we identified proteins previously described as ADP-ribosylation targets or closely associated with them in addition to potential novel targets. We believe that the herein reported cell-permeable NAD^+ probes offer reliable tools for a comprehensive investigation of ADP-ribosylation in living cells and to increase the understanding of cellular responses to stress.

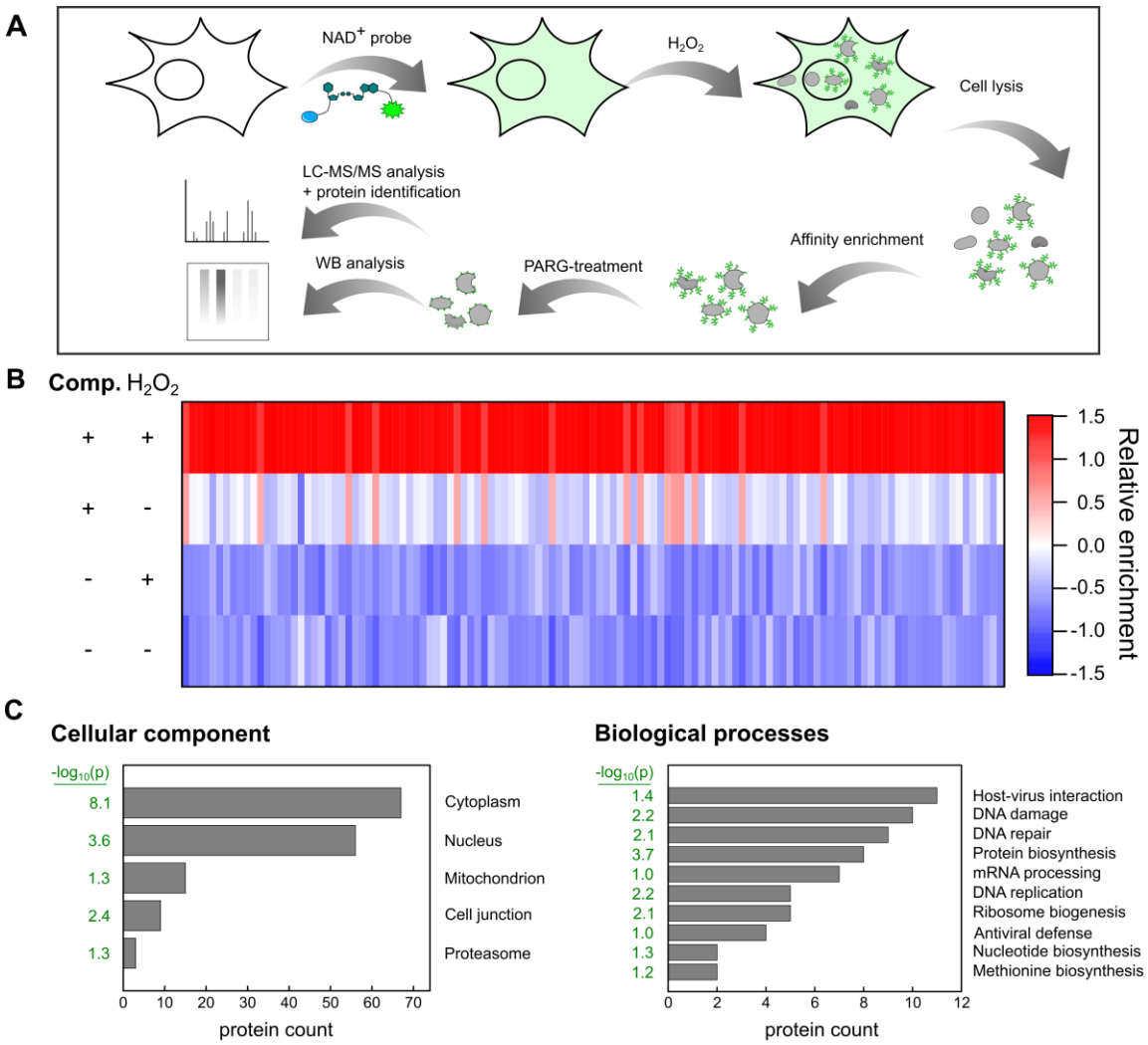


Figure 4. Affinity enrichment of ADP-ribosylated proteins in response to H_2O_2-induced stress using probe **Chol-2-NAD-DTB A.** Experimental workflow overview; **B.** Heat-map representing 121 identified protein targets (**Comp. = Chol-2-NAD-DTB**); **C.** DAVID analysis of the enriched proteins, categorized based on cellular component and biological processes, with $-\log_{10}(p)$ values depicted in green.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, synthesis of compounds with NMR and HR MS characterization, additional figures, a list of identified proteins together with DAVID analysis and comparison to the literature data are included in the supporting information files.

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Notes

The authors declare no competing financial interests.

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