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Olaparib based photo-affinity probes for PARP-1 detection in living cells

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Abstract The poly-ADP-ribose polymerase (PARP) is a protein from the family of ADP-ribosyltransferases that catalyzes poly adenosine diphosphate ribose (ADPR) formation in order to attract the DNA repair machinery to DNA damage sites. Inhibition of PARP activity by olaparib can cause cell death which is of clinical relevance in some tumor types. This demonstrates that quantification of PARP activity in the context of living cells is of great importance. In this work we present the design, synthesis and biological evaluation of photo-activatable affinity probes inspired by the olaparib molecule which are equipped with a diazirine for covalent attachment upon activation by UV light and a ligation handle for the addition of a reporter group of choice. SDS-PAGE, western blotting and label-free LC-MS/MS quantification analysis show that the probes target the PARP-1 protein and are selectively outcompeted by olaparib suggesting binding in the same enzymatic pocket.

One of the crucial steps in the repair of DNA double-strand breaks is ADP-ribosylation of chromatin,[1–6] a process driven by several members of the PARP family[7–9] otherwise known as ARTDs (poly-ADP-ribosyltransferases).[10] This family of transferases represents clinically relevant target proteins for drugs directed to tumors that are deficient in their DNA repair.[11,12] The ability to monitor ARTDs *in vivo* is necessary if one wants to study the processes of the drug-target engagement and pharmacokinetics. Small molecule probes that are capable to specifically and covalently bind to their target enzymes in living cells and in tissues have been developed for various classes of enzymes[13–17] and used in successful studies involving *in vivo* protein detection and quantification. One way to attain such a probe is to convert a known reversible enzyme inhibitor into a covalent probe suitable for affinity-based protein profiling (AfBPP).[17–20] AfBPP probes consist of a recognition element that reversibly interacts with the target of interest on which a photoreactive group and ligation handle are installed, without affecting the affinity of the parent structure. Irradiation by UV light activates the photoreactive group, forming a covalent bond between the probe and the target protein in the process called photo-affinity labeling (PAL).[19–22] The bioorthogonal ligation handle is used to couple a reporter group, e.g. a fluorophore for in-gel analysis or a biotin for affinity enrichment with subsequent tandem mass spectrometry in chemical proteomics. In this work we present such a covalent probe for the detection of PARP-1 in live cells using AfBPP (Figure 1). Prior work on the detection of PARP was focused solely on non-covalently binding molecules with the aim of either off-target finding[23,24] or in vivo imaging[25,26] We set out to design, synthesize and biologically validate a PARP-1-selective photoaffinity probe for use in the standard proteomics protocols. Such well-established workflows make use of UV-induced PAL through a diazirine tag and copper-catalyzed alkyne-azide cycloaddition that enable the pull-down of the protein for subsequent analysis by standard mass-spectrometry methods.
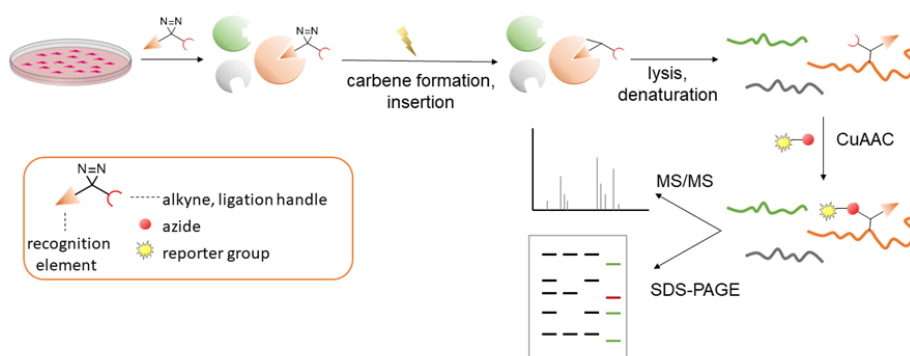

Figure 1. Live Raji cells were treated with probes 3 or 6. Irradiation at 350 nm leads to activation of the diazirine group, forming a highly reactive carbene that can rapidly insert into nearby N-H, O-H or C-H bonds. Protein extraction was done by cell lysis under denaturing conditions. Reporter or affinity handles were introduced by CuAAC click chemistry. Proteins were analyzed by SDS-PAGE and/or western blot analysis or by affinity purification, digested to peptides and LFQ proteomics analysis.

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The clinical PARP inhibitor olaparib was chosen as the starting point for the design of the recognition element of photoaffinity probes **3** and **6**, because it inhibits PARP-1 and PARP-2 with a nanomolar potency and shows exceptional selectivity over other cellular targets [24, 27, 28] The synthesis of **3** and **6** is depicted in Scheme 1. For PAL, a plethora of photo reactive groups is reported.[29] In order to limit the interference of the tag with the activity and selectivity of the probes, we chose the compact diazirine-alkyne linkers **2** and **5** developed by Li et al.[30] The nitrogen atom of the piperazine moiety distal from the phtalazinone substructure of olaparib is known to tolerate a wide range of modifications.[28,31] With this in mind, we attached the photoaffinity tag to this nitrogen to minimize its influence on binding efficiency to PARP-1, giving the design of probe **3**. A second, simplified probe **6**, in which we removed the piperazine bridge and attached the diazirine-alkyne tag directly to benzylphtalazinone core of the olaparib structure was also synthesized. The preparation of the probes was done via the HCTU-mediated condensation of either commercially available **1** or known compound **4**[25,28] with the linkers **2** and **5** respectively.
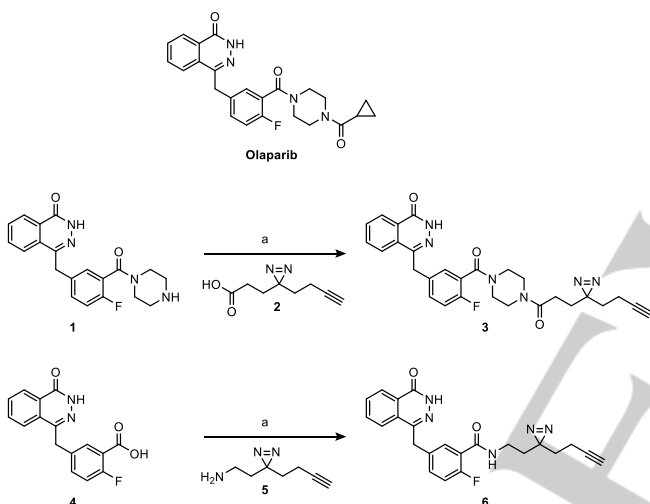
Scheme 1. Structure of olaparib and the synthesis of the probes **3** and **6** derived from olaparib. Reagents and condition: a) HCTU, DIPEA, DMF, 33% for **3**, 75% for **6**.

With the AfBPs **3** and **6** in hand, their ability to bind PARP covalently *via* PAL in live cells was tested on human B-cell-derived Raji cell line. Raji cells were chosen as the model because they are easy to grow in suspension and have an advantageous nucleus to cytoplasm ratio that maximizes the PARP abundance. The cells were incubated with probes **3** or **6** (10 µM) and irradiated with UV light for 30 minutes to ensure complete activation of the diazirine and covalent labeling of PARP. During irradiation, the cells were cooled to 4°C to counteract the heat generated from the irradiation. The cells were then harvested and lysed. The lysate was treated with the azide-containing fluorophore AlexaFluor 647 under click-reaction conditions and further processed for in-gel fluorescence analysis. The gel revealed a fluorescent band at the expected height of around 116 kDa (Figure 2, lanes 3) which was probe-dependent as cells treated with only the DMSO vehicle (lanes 2) revealed no labeling. The same cell lysates were also coupled to Diazo Biotin-Azide (DBA, SI Figure 4) and an anti-biotin Western-Blot (WB) revealed the same band at 116 kDa (SI Figure 1) verifying that the detected bands are probe-specific. To further show probe-specific labeling,

the UV irradiation step was omitted yielding no signal, showing that the observed labeling is UV-dependent rather than circumstantial activation. As an additional control, the gel used for in-gel fluorescence readout was analyzed by western blot with an anti-PARP-1 antibody showing bands at the same height confirming it to be PARP-1 (SI Figure 1). To further verify our target, we performed a click reaction with *N*-(3-azidopropyl)biotinamide (SI Figure 5) on the lysates and enriched the probe-bound proteins on streptavidin beads. SDS-PAGE followed by WB analysis with the anti-PARP-1 antibody proved the probe-bound protein to be PARP-1 (SI Figure 2).
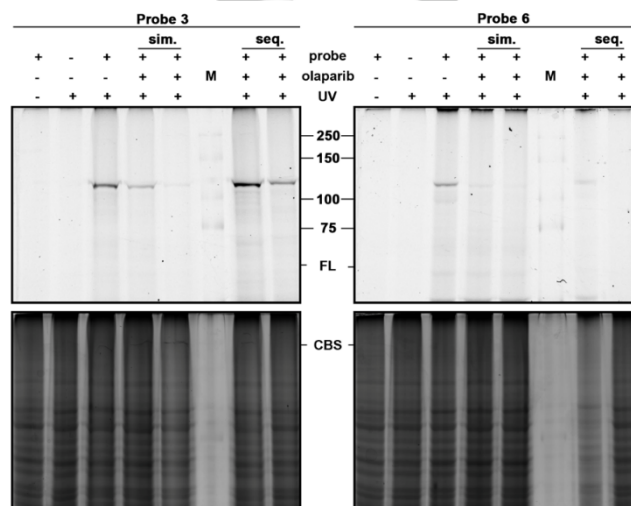

Figure 2. SDS-PAGE analysis and fluorescent imaging of Raji cells exposed to probes **3** and **6** (10 µM) and no UV control. sim: simultaneous incubation with 1:1 (10 µM olaparib, 10 µM probe) or 5:1 (50 µM olaparib, 10 µM probe) ratio. seq: sequential incubation with olaparib, wash and probe at 1:1 ratio or 2:1 olaparib:probe ratio. FL: fluorescence channel. CBS: coomassie blue staining for loading control of the gel in the upper panel. M: protein ladder

Having verified that probes **3** and **6** were able to label PARP in a UV-dependent covalent manner, we reasoned they should also be outcompeted by olaparib, as they should bind to the same protein active site. The probes and olaparib were incubated simultaneously in a 1:1 ratio after which a decrease of band intensity was observed (lanes 4). After increasing the amount of olaparib to a 5:1 drug/probe ratio, labeling by the probe was fully diminished (lanes 5). Interestingly, in a sequential competition experiment where first olaparib was administered, removed and then incubated with the probes in a 1:1 ratio, no apparent competition was observed (lanes 7) and when a 2:1 drug/probe ratio was applied, the bands appeared to decrease slightly (lanes 8) but proved not as effective as simultaneous competition. This effect might be due to rapid dissociation of olaparib from the PARP binding pocket during the washing step.

Next, we wanted to show the probes ability to bind PARP in conditions frequently encountered in the field of ADP-ribosylation research. H_2O_2 is often used to induce double strand breaks, facilitating PARP activation. Therefore, Raji cells were treated with the probes as before with addition of H_2O_2 during incubation. The addition of this oxidizing agent neither changed the ability of the probes to label PARP nor the efficiency of the labeling (SI Figure 3).The latter result suggests that olaparib is able to bind to PARP in the absence of extensive DNA-damage and appears consistent with previously reported findings.[23]
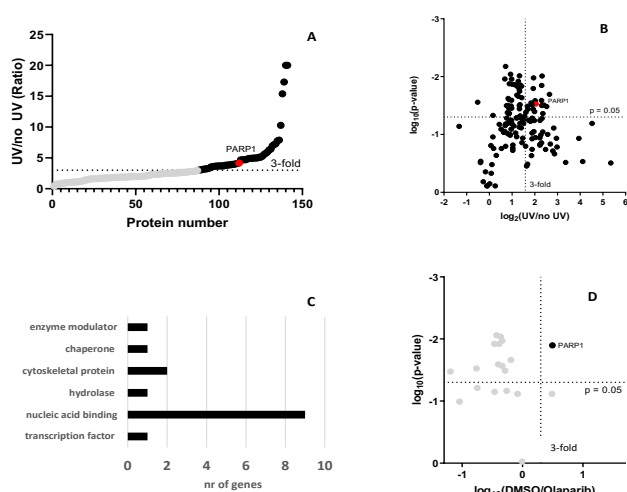
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Figure 3. LFQ proteomics analysis of probe **3** in living Raji cells of proteins with at least 2 unique peptides. A: Waterfall plot of UV-dependent enrichment by probe **3** of proteins identified in the proteomics analysis, proteins showing >3-fold UV-dependent enrichment are in black. B: Volcano plot of UV-dependent enrichment of proteins by probe **3**, proteins showing >3-fold UV-dependent enrichment and with a p-value < 0.05 using a Student's t-test are in black. PARP-1 is marked in red. C: Significantly UV-enriched proteins sorted with the gene function analysis module of the PANTHER classification system (v.14.0) software.[32] D: Competition analysis of significantly UV-enriched probe targets, proteins outcompeted >3-fold by olaparib pre-addition with a p-value < 0.05 using a Student's t-test are in black.

To identify and quantify the bands seen by SDS-PAGE and western blot analysis we used a LC-MS/MS based label-free quantification (LFQ) method.[16] The results show that PARP-1 is significantly enriched in the UV-dependent manner by probe **3** indicating that the observed band at 116 kDa can be attributed to PARP-1 (Fig 3A, 3B). The analysis of proteins significantly enriched in the photo-affinity experiment (UV-irradiated) and in presence of olaparib (Fig 3D) shows that PARP-1 is outcompeted. This competition strongly indicates that the probe binds in the same protein pocket as olaparib. Several other proteins were selectively enriched by the probe upon UV irradiation having a nucleic acid binding motif as common feature, indicating that the probe targets these proteins possibly by their nucleic acid binding or perhaps by the presence of the diazirine group (Fig 3C).[33] PARP-1 was not significantly enriched by probe **6** in a photo-affinity experiment (SI Figure 6), which agrees with the in-gel fluorescence data for this probe that showed fainter bands for PARP-1 compared with the background. Apparently, removal of the piperazine ring diminishes the binding affinity of probe **6** to PARP-1.

In conclusion, we present for the first time design and synthesis, of two synthetically accessible olaparib-based photoaffinity probes (**3** and **6**) for PARP-1 detection in living cells. We show evidence of efficacy and provide a detailed protocol for application of these probes. Both **3** and **6** proved to be stable under oxidative conditions often employed for PARP activation while probe **3** was successfully used to label PARP-1 selectively in a live cell model and to analyze its presence by in-gel fluorescence and LFQ LC-MS/MS methods.

Note added in proof. When this paper was under review a similar approach for PARP-targeting has been published by Tate and coworkers. [34]

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