Electronic Supplementary Information

A Poly-ADP-Ribose Polymer-Based Antibody-Drug Conjugate

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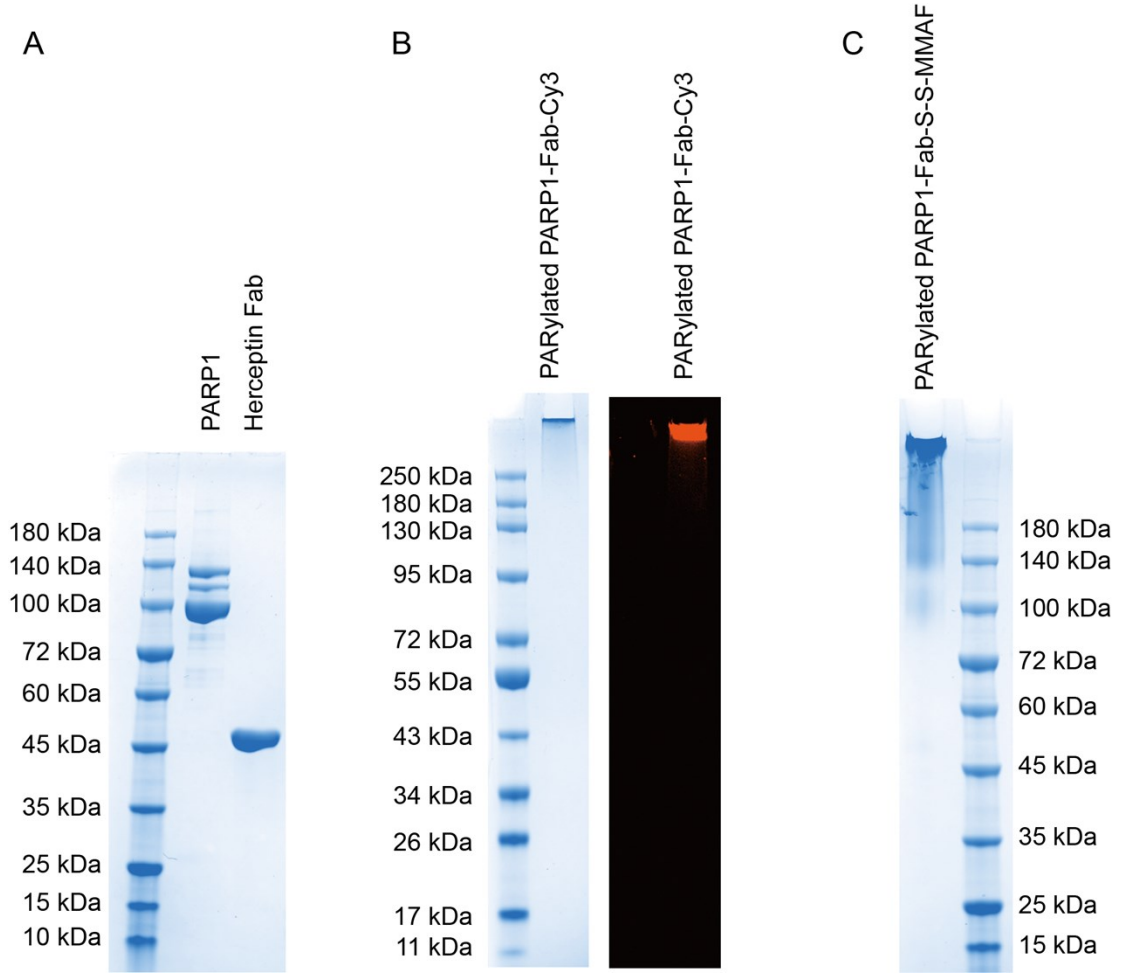

Figure S1. SDS-PAGE analysis of purified proteins and conjugates. (A) Coomassie stain of purified human PARP1 and Herceptin Fab. (B) Coomassie stain (left) and in-gel fluorescence (right) of PARylated PARP1-Fab-Cy3 conjugate. (C) Coomassie stain of PARylated PARP1-Fab-S-S-MMAF conjugate.

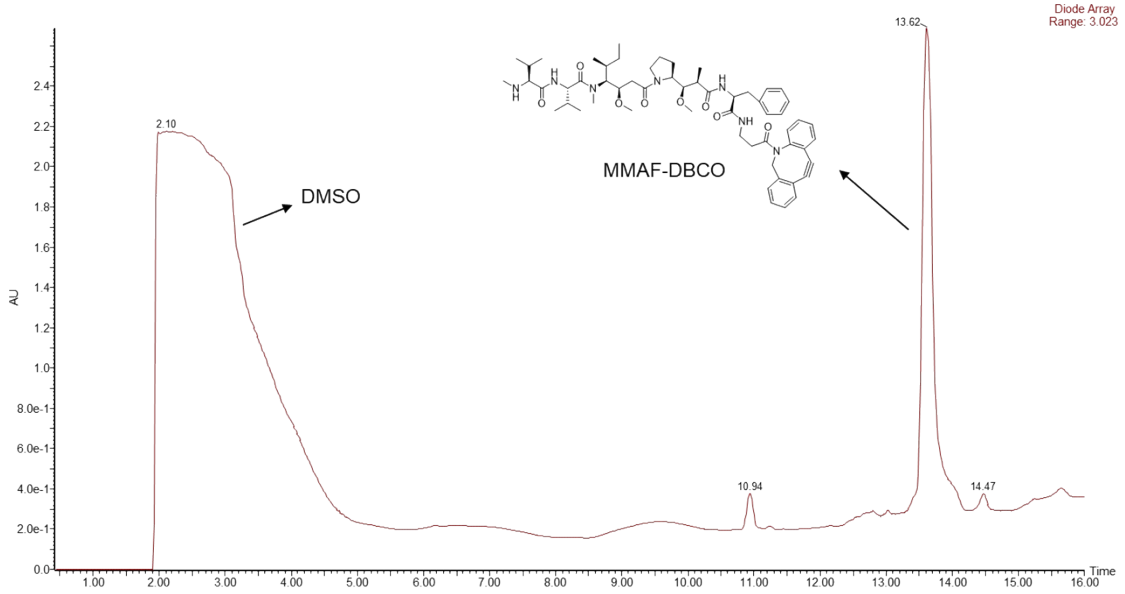

Figure S2. HPLC analysis of DBCO-MMAF molecule.

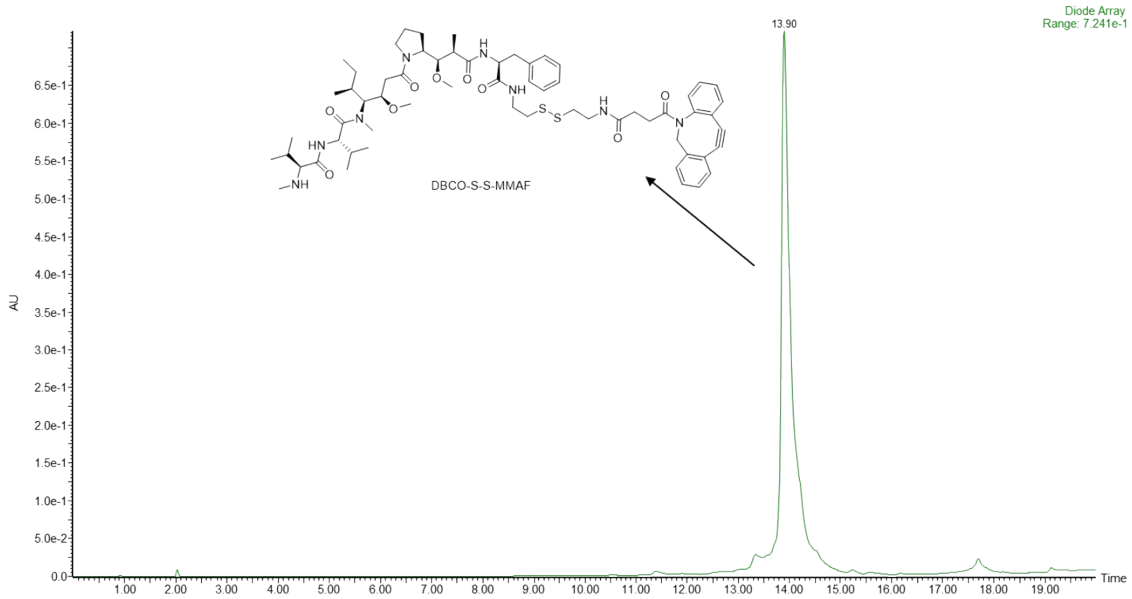

Figure S3. HPLC analysis of the DBCO-S-S-MMAF molecule.

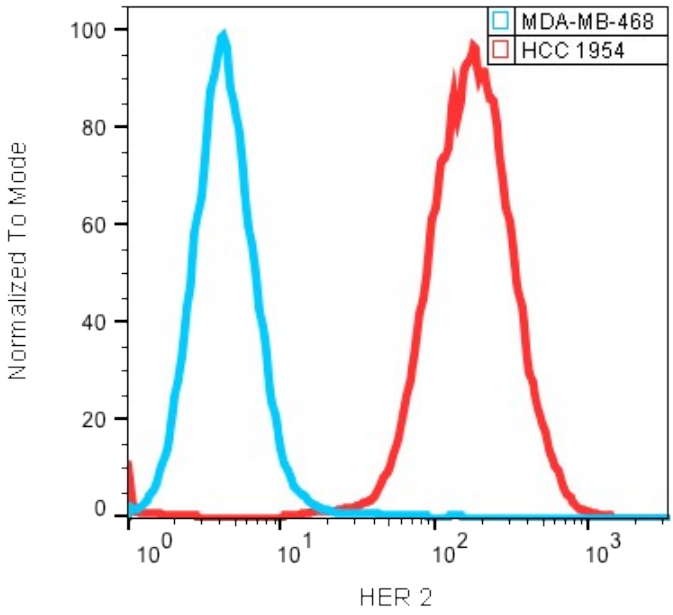

Figure S4. Flow cytometric analysis of HER2 expressions of HCC 1954 and MDA-MB-468 cell lines.

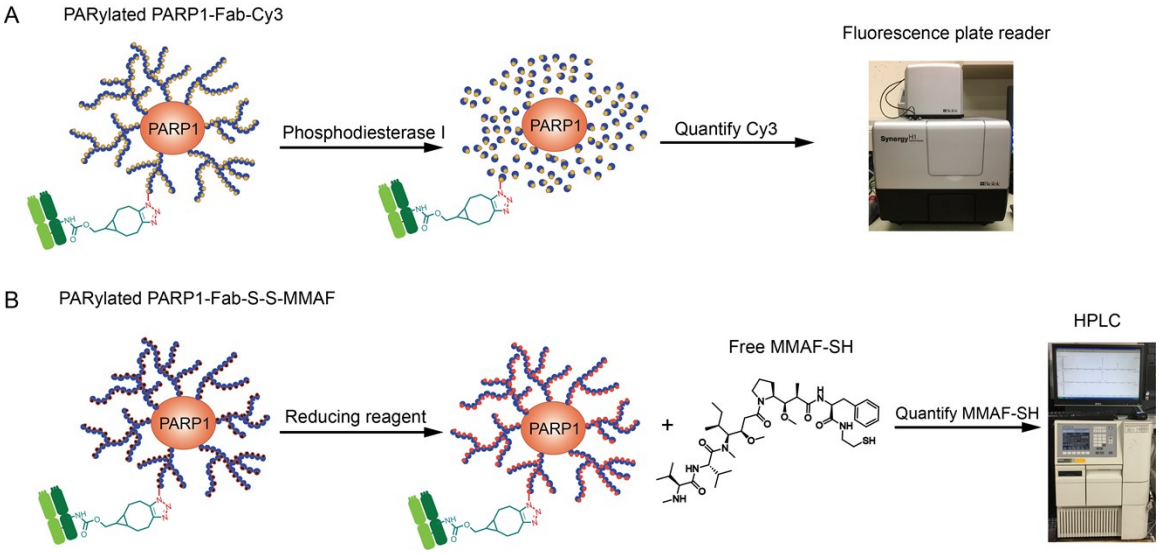

Figure S5. Schematic of quantification of the payloads of PARylated PARP1-Fab conjugates. (A) PARylated PARP1-Fab-Cy3 conjugate was treated with phosphodiesterase I to release Cy3 for quantification by a fluorescence plate reader. (B) PARylated PARP1-Fab-S-S-MMAF conjugate was reduced with dithiothreitol (DTT) to release free MMAF-SH for quantification by HPLC.

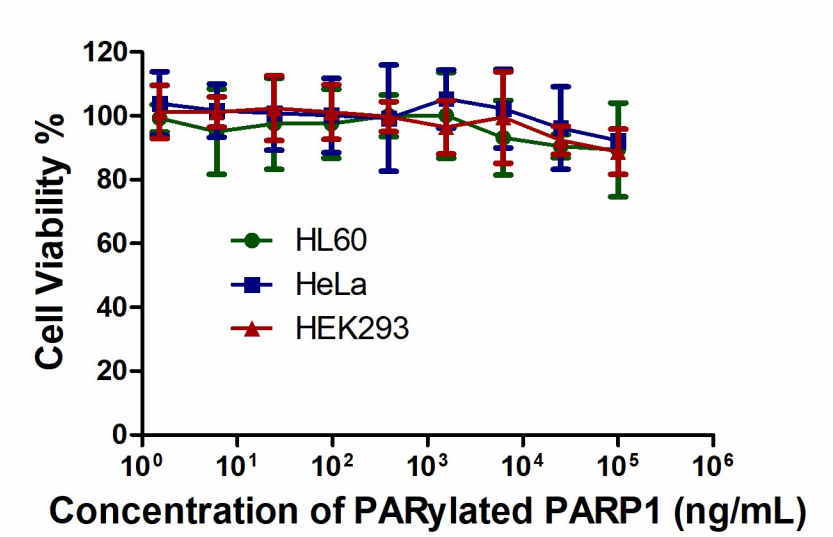

Figure S6. *In vitro* cytotoxicity of PARylated PARP1 for non-breast cancer cell lines. HL60, HeLa and HEK293 cells were incubated for 72 hours at 37°C with 5% CO_2 in the presence of various concentrations of PARylated PARP1. Cell viability was then measured by MTT assays. Data are shown as mean ± SD of triplicates.

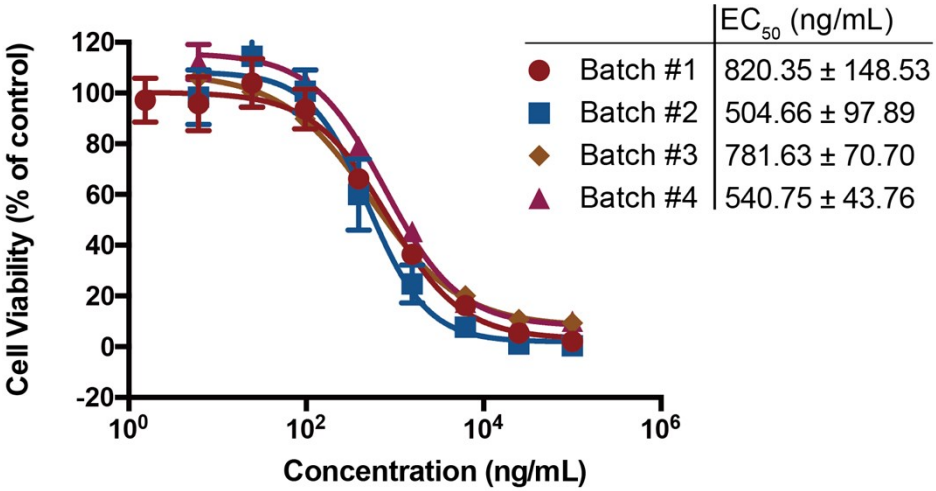

Figure S7. *In vitro* cytotoxicity of PARylated PARP1-Fab-MMAF conjugate for HCC 1954 ($HER2^+$) cells. Cells were incubated for 72 hours at 37°C with 5% CO_2 in the presence of various concentrations of PARylated PARP1-Fab-MMAF conjugates that were prepared with different batches of PARP1 and 3′-azido NAD^+. Cell viability was then measured by MTT assays. Data are shown as mean ± SD of duplicates.

Table S1. List of primers used for molecular cloning of full-length human PARP1.

PARP1-F	TGGTGCTCGAGCCACAGGGAGGTCTTAAAATTGAATTTCAGT
PARP1-R	CCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCGGAGTCTTCGGATAAGC

Experimental Methods

Materials and Cell Lines. All chemicals were purchased from common commercial sources and used as received unless otherwise specified. pBAD vector encoding trastuzumab Fab was a gift from Dr. Peter G. Schultz's laboratory at The Scripps Research Institute. Breast cancer cell lines (HCC1954 and MDA-MB-468) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% FBS at 37℃ in 5% CO_2.

Chemical Synthesis of 3′-azido NAD$^+$. The 3′-azido NAD^+ was prepared according to a previously published method[1].

Chemical Synthesis of MMAF-DBCO. To a stirred solution of MMAF (4.4 mg, 0.006 mmol, purchased from Ontario Chemicals, Inc. (Ontario, Canada)), HATU (3.0 mg, 0.0078 mmol, 1.3 eq) and DMAP (0.2 mg, 0.0018 mmol, 0.3 eq) in DMF (0.5 mL) was added with a solution of DBCO-amine (8.3 mg, 0.03 mmol, 5 eq, Click Chemistry Tools (Scottsdale, AZ)) and DIPEA (3 µL, 0.018 mmol, 3 eq) in DMF (0.5 mL) at 0^{o}C. Then, the reaction mixture was allowed to warm to room temperature and stirred at the same temperature until the reaction completed (monitored by HPLC). The reaction was concentrated *in vacuo* and purified via preparative HPLC (C18-A column, 150×10.0 mm, 5 µm) (mobile phase A: 0.1% formic acid (aq), mobile B: 0.1% formic acid in acetonitrile; flow rate = 2.0 mL/min; 0-2 min: 0-4% B, 2-4 min: 4-10% B, 4-6 min: 10-20% B, 6-12 min: 20-50% B, 12-14 min: 50-70% B, 14-16 min: 70-0% B) with detection of UV absorbance at 220 nm. Fractions containing the desired product were concentrated and lyophilized to yield the compound MMAF-DBCO (3.0 mg, 51%) as a colorless solid. MS (ESI) for $C_{57}H_{80}N_7O_8^+$ $(M+H)^+$ Cald:990.6068 Da, Obs: 990.6093 Da.

Chemical Synthesis of MMAF-S-S-DBCO. To a stirred solution of MMAF (4.4 mg, 0.006 mmol, Ontario Chemicals, Inc), HATU (3.0 mg, 0.0078 mmol, 1.3 eq) and DMAP (0.2 mg, 0.0018 mmol, 0.3 eq) in DMF (0.5 mL) was added with a solution of DBCO-S-S-amine (13.2 mg, 0.03 mmol, 5 eq, Conju-Probe, LLC (San Diego, CA)) and DIPEA (3 µL, 0.018 mmol, 3 eq) in DMF (0.5 mL) at 0^{o}C. Then, the reaction mixture was allowed to warm to room temperature and stirred at the same temperature until the reaction completed (monitored by HPLC). The reaction was concentrated *in vacuo* and purified via preparative HPLC (C18-A column, 150×4.60 mm, 5 µm) (mobile phase A: 0.1% formic acid (aq), mobile B: 0.1% formic acid in acetonitrile; flow rate = 1.0 mL/min; 0-2 min: 0-4% B, 2-4 min: 4-10% B, 4-6 min: 10-20% B, 6-12 min: 20-50% B, 12-17 min: 50-100% B, 17-20 min: 100-0% B) with detection of UV absorbance at 260 nm. Fractions containing the desired product were concentrated and lyophilized to yield the compound MMAF-S-S-DBCO (1.9 mg, 28%) as a colorless solid. MS (ESI) for $C_{62}H_{88}N_8O_9S_2Na^+$ $(M+Na)^+$ Cald: 1175.6013 Da, Obs: 1175.6029 Da.

Molecular Cloning. DNA sequence encoding full-length human PARP1 with a C-terminal His_6 tag was amplified through PCR using primers P1 and P2 (Table S1). The amplified DNA fragment was inserted into pET-28a (+) vector between XhoI and XbaI restriction enzyme sites.

Protein Expression and Purification. The bacterial expression and purification of PARP1 were carried out by following a previously published protocol with slight modifications[2]. The plasmid of pET-28a (+)-PARP1 was transformed into BL21 (DE3)

cells. A single colony was picked and inoculated into 60 mL of LB Broth media with kanamycin (50 μg mL^{-1}) for overnight growth at 37 ℃ in an incubator shaker at a speed of 250 rpm (Series 25, New Brunswick Scientific, NJ). The overnight bacterial culture was then diluted into six 1-liter LB Broth media with kanamycin (50 μg mL^{-1}) for continued growth at 37 ℃ in an incubator shaker (250 rpm). When the OD_{600} reached 0.6–0.8, 100 mM $ZnSO_4$ was added to the bacterial culture for a final concentration of 0.1 mM. Once the OD_{600} reached 0.8-1.0, the bacterial culture was chilled on ice for 1 hour. Protein expression was then induced with 0.5 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG) for overnight at 16 ℃. Bacterial cells were harvested by centrifugation at 4,550 × g for 50 minutes at 4 ℃, resuspended in equilibrium buffer (25 mM HEPES pH 8.0, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF)), and lysed using a French Press (GlenMills, NJ) at 25,000 psi for three cycles. Cell debris was removed by centrifugation at 27,000 × g for 100 minutes at 4 ℃ and supernatants were filtered through 0.45 μm membranes. The filtrate was loaded on a gravity flow column packed with 5 mL of Ni-NTA agarose resin (Thermo Fisher Scientific, Waltham, MA), followed by washing with 50 mL of low-salt wash buffer (25 mM HEPES pH 8.0, 500 mM NaCl, 20 mM imidazole), 50 mL of high-salt wash buffer (25 mM HEPES pH 8.0, 1 M NaCl, 20 mM imidazole) and 50 mL of low-salt wash buffer. Proteins were then eluted with 25 mL of elution buffer (25 mM HEPES pH 8.0, 500 mM NaCl, 400 mM imidazole), followed by addition of 25 mL of no-salt buffer (50 mM Tris pH 7.0, 1 mM EDTA, 0.1 mM dithiothreitol (DTT)). The eluted protein was then loaded onto a 5-mL HiTrap Heparin HP Column (GE Healthcare, Princeton, NJ).Upon completion of protein loading by a peristaltic pump, the heparin column was placed on an ÄKTA Pure chromatography system to elute PARP1 using a gradient of 0-100% buffer B (50 mM Tris pH 7.0, 1 mM EDTA, 0.1 mM DTT, 1 M NaCl) in buffer A (50 mM Tris pH 7.0, 1 mM EDTA, 0.1 mM DTT, and 250 mM NaCl) at a flow rate of 1 mL min^{-1}. PARP1 was eluted starting at 40% buffer B and the collected fractions were combined and concentrated to below 500 μL using Amicon centrifugal filters with 30 kDa cutoff (EMD Millipore, Temecula, CA). The concentrated proteins were injected on to a size-exclusion chromatography column Superdex 200 Increase 10/300 GL (GE Healthcare, Princeton, NJ) and eluted using gel filtration buffer (25 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1 mM DTT). The fractions containing full-length PARP1 were combined, concentrated using Amicon centrifugal filters with 30 kDa cutoff, analyzed by SDS-PAGE, flash frozen using liquid nitrogen, and stored at -80°C.

To express trastuzumab Fab, DH10B cells transformed with the trastuzumab Fab plasmid were inoculated in LB Broth with ampicillin (100 μg mL^{-1}). The overnight bacterial culture (5 mL) was then diluted into 1-liter LB Broth with ampicillin (100 μg mL^{-1}) for growth at 37 ℃ in an incubator shaker at speed of 250 rpm (Series 25, New Brunswick Scientific, NJ). When the OD_{600} reached 0.6-0.8, protein expression was induced with 0.2% L-arabinose for overnight at 22 ℃. Cells were then harvested by centrifugation at 4,550 × g for 50 minutes at 4 ℃, resuspended in lysis buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM PMSF, 20 mg mL^{-1} lysozyme and 20% (w/v) sucrose) for periplasmic protein extraction. Cells were stirred for 20 minutes and vigorously shaken for another 20 minutes. Cell debris was removed by centrifugation at 27,000 × g for 1 hour and supernatants were filtered through a 0.45 μm membrane. The Fabs were purified from the filtrates by Protein G chromatography. Specifically, the

filtrates were loaded on a gravity flow column packed with 1 mL of Protein G resin (GenScript, Piscataway, NJ), followed by washing with PBS. Proteins were then eluted with elution buffer (100 mM glycine, pH 2.7), neutralized with 1 M Tris buffer (pH=8.0) at 10:1 ratio, dialyzed in PBS buffer at 4 ℃ for overnight and another 6 hours in fresh PBS buffer, and concentrated using Amicon centrifugal concentrators (EMD Millipore, Temecula, CA) with a 10 kDa cutoff. Purified Fab was analyzed by SDS-PAGE and stored at -80°C.

Trastuzumab Fab NHS-BCN Linker Conjugation. The endo-BCN-PEG_4-NHS ester linker (purchased from BroadPharm, San Diego, CA) was dissolved in 100% DMSO as 100 mM stock. A 20-fold molar excess of endo-BCN-PEG4-NHS ester linker was added into trastuzumab Fab in PBS. The solution was mixed gently and allowed to react at room temperature for 2 hours. To remove unreacted linkers, the reaction mixture was buffer exchanged to PBS with a dilution factor over 1,000,000 using Amicon centrifugal concentrators (EMD Millipore, Temecula, CA) with a 10 kDa cutoff.

PARP1 Automodification. Large-scale auto-PARylation of purified PARP1 was performed at 30 ℃ for 12 hours. Purified PARP1 (5 μM) was incubated with 250 μM of 3′-azido NAD^+ or NAD^+ in a reaction buffer containing 30 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM DTT and 100 ng μL^{-1} activated DNA (purchased from Sigma-Aldrich, St. Louis, MO). The removals of reaction buffer and unreacted 3′-azido NAD^+ or NAD^+ were performed through buffer exchange using Amicon centrifugal concentrators (EMD Millipore, Temecula, CA) with a 30 kDa cutoff in PBS buffer.

Conjugation of PARylated PARP1. Trastuzumab Fab-BCN was added into PARylated PARP1 solution with a 1.5-fold molar excess. The conjugation was allowed to react at room temperature for 72 hours. DBCO-MMAF or DBCO-Cy3 were then added onto the reaction mixtures at a molar ratio of 1:20 (PARylated PARP1:payloads). The solutions were gently mixed and allowed to react at room temperature for another 72 hours.

PARylated PARP1 conjugates were purified using size-exclusion chromatography. Prior to injections, precipitates were removed by passing the solutions through a 0.22 μm filter. The filtrates were injected on to a size-exclusion chromatography column Superdex 200 Increase 10/300 GL (GE Healthcare, Princeton, NJ) and eluted with PBS. The first peak eluted was collected and concentrated using Amicon centrifugal concentrators (EMD Millipore, Temecula, CA) with a 30 kDa cutoff. Purified PARylated PARP1 conjugates were examined by SDS-PAGE. PARylated PARP1-Fab-Cy3 conjugate was imaged by an iBright FL1000 gel imager (Thermo Fisher Scientific, Waltham, MA).

Immunoblot analysis. Purified proteins and conjugates (3 μg) were boiled with 100 mM DTT in NuPAGE LDS sample buffer (Thermo Fisher Scientific, MA) at 98°C for 10 minutes, separated in 4-20% ExpressPlus-PAGE gels (GenScript, Piscataway, NJ), and then transferred to Immun-Blot PVDF membranes (Bio-Rad Laboratories, Inc., CA) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Inc., CA). The membranes were subsequently blocked with 5% non-fat milk in PBST (PBS with 0.1% Tween-20) for 1 hour at room temperature, followed by incubation with appropriate primary antibodies for 1 h at room temperature, which included an anti-pADPr (10H, Santa Cruz Biotechnology, TX) and anti-His_6 (HIS.H8, Thermo Fisher Scientific, MA)

monoclonal antibodies. After 1-hour incubation with the secondary anti-mouse IgG-HRP (Thermo Fisher Scientific, MA), the membranes were developed by additions of SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, MA) and imaged using a ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Inc., CA).

Flow Cytometric Analysis. HER2 expression levels of HCC 1954 and MDA-MB-468 cells were evaluated by flow cytometry. Equal numbers of cells were incubated with the trastuzumab followed by the anti-human IgG Fc-FITC in PBS with 2% FBS at 4°C for 1 hour. The binding of PARylated PARP1-Fab-Cy3 and PARylated PARP1-Cy3 conjugates to HER2-positive cell line HCC 1954 and HER2-negative cell line MDA-MB-468 were evaluated by flow cytometry. Cells were incubated with PARylated PARP1-Cy3 conjugate at 20 μg mL^{-1} and PARylated PARP1-Fab-Cy3 conjugate at 20 μg mL^{-1} and 100 μg mL^{-1} for 30 minutes at 4°C and washed three times with PBS containing 2% FBS. Samples were analyzed using a Fortessa X20 flow cytometer (BD Biosciences, San Jose, CA). Data were processed by FlowJo software (Tree Star Inc., Ashland, OR).

Confocal Microscopy of Cellular Uptake of PARylated PARP1-Fab-Cy3 Conjugate. HCC 1954 cells (3×10^4) were seeded onto glass cover slips in 24-well cell culture plates and incubated at 37°C with 5% CO_2 overnight. Cells were then treated with 16 μg mL^{-1} of PARylated PARP1-Fab-Cy3 conjugate in the absence or presence of trastuzumab Fab (800 nM) at 37 ℃ for 3 h. The cells were gently washed three times with PBS, fixed with 4% paraformaldehyde for 20 minutes, and stained with DAPI for 20 minutes. After three more PBS washes, cells were mounted on slides and imaged with a Leica SP8 confocal laser scanning microscope (Leica Microsystems Inc., Buffalo Grove, IL) equipped with HC PL APO 63x/1.40 Oil CS2 oil immersion objective lenses using DAPI and rhodamine (for Cy3) filters. Images were processed using the LAS X software (Leica Microsystems Inc., Buffalo Grove, IL) and ImageJ.

Stability Test of PARylated PARP1-Fab-Cy3 Conjugate. PARylated PARP1-Fab-Cy3 conjugate was diluted with RPMI 1640 medium with 10% FBS to a final concentration of 0.25 mg mL^{-1} and incubated at 37 ℃ for 0 h, 24 h, 48 h, and 72 h. Collected samples were separated in 4-20% ExpressPlus-PAGE gels (GeneScript, Piscataway, NJ), and Cy3-derived fluorescence signals were detected using an iBright FL1000 gel imager (Thermo Fisher Scientific, Waltham, MA). Fluorescence intensities were quantified by the iBright FL1000 gel imager (Thermo Fisher Scientific, Waltham, MA) and normalized to those of samples incubated for 0 h. The experiment was repeated once and three fluorescence measurements were performed for each experiment. Data are shown as mean ± SD (n=6).

Quantification of Cy3 on PARylated PARP1-Fab-Cy3 Conjugate. PARylated PARP1-Fab-Cy3 conjugate was treated with 0.4 mg mL^{-1} of phosphodiesterase I (PDE I) (purchased from Worthington Biochemical Corporation, Lakewood, NJ) at 37 ℃ overnight. PDE-treated PARylated PARP1-Fab-Cy3 conjugate and DBCO-Cy3 standards were added into black 96-well plates for fluorescence measurements (excitation at 550 nm; emission at 600 nm) using a BioTek Synergy H1 Hybrid Multi-Mode Microplate reader (BioTek, VT). Cy3 concentrations of PARylated PARP1-Fab-Cy3 conjugate were determined on the basis of standard curves.

Quantification of MMAF on PARylated PARP1-Fab-S-S-MMAF Conjugate. PARylated PARP1-Fab-S-S-MMAF conjugate was treated with 50 mM DTT overnight at room temperature to reduce disulfide bonds and to release MMAF-SH from the conjugate. The supernatants of reaction mixtures and MMAF standards were analyzed by reverse-phase HPLC (C18-A column, 150×10.0 mm, 5 μm) (mobile phase A: 0.1% formic acid (aq), mobile B: 0.1% formic acid in acetonitrile; flow rate = 1.0 mL/min; 0-2 min: 0-4% B, 2-4 min: 4-10% B, 4-6 min: 10-20% B, 6-12 min: 20-50% B, 12-17 min: 50-100% B, 17-20 min: 100-0% B) with detection of UV absorbance at 220 nm. Peak areas of MMAF were integrated and plotted against the concentrations of MMAF as the standard curves. MMAF concentrations on PARylated PARP1-Fab-S-S-MMAF were determined on the basis of standard curves.

***In vitro* Cytotoxicity Assay**s. HCC 1954, MDA-MB-468, HL60, HeLa, and HEK293 cells were seeded in 96-well cell culture plates the day prior to the experiments (5,000 cells per well for HCC 1954, HL60, HeLa, and HEK293; 6,000 cells per well for MDA-MB-468). The cells were treated with various concentrations of PARP1-Fab-MMAF conjugate, DBCO-MMAF, PARylated PARP1, or PARylated PARP1-Fab for 72 hours. Ten μL of 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was then added to the wells and incubated for 2 hours at 37℃. Subsequently, 100 μL of lysis buffer (20% SDS in 50% dimethylformamide, 0.5% (v:v) 80% acetic acid, 0.4% (v:v) 1 N HCl, pH 4.7) was added and incubated for 2 hours at 37℃. The absorbance was measured at 570 nm using a BioTek Synergy H1 Hybrid Multi-Mode Microplate reader (BioTek, VT). Cell viability was calculated as:

$$\%\text{Cell Viability} = [(\text{absorbance}_{\text{experimental}} - \text{absorbance}_{\text{spontaneous average}})/(\text{absorbance}_{\text{maximum viability average}} - \text{absorbance}_{\text{spontaneous average}})] \times 100$$

References

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2. Langelier, M. F.; Planck, J. L.; Servent, K. M.; Pascal, J. M., Purification of human PARP-1 and PARP-1 domains from Escherichia coli for structural and biochemical analysis. *Methods Mol Biol* **2011,** *780*, 209-26.