Turning a Scorpion Toxin into an Antitumor Miniprotein

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Synthesis of stoppin-1, stoppin-2 and stoppin-2 control. The amino acid sequences of stoppin-1, stoppin-2, and stoppin-2 control are as follows:

CYSFD CLWKC LAMGF SSGKC INSKC KC (stoppin-1)
CYSFD CLWKC LAMGF RRGKC RRRKC KC (stoppin-2)
CYSSD CRVKC VAMGF RRGKC RRRKC KC (stoppin-2 control)

Stoppin-1 was synthesized on Boc-Cys(4-MeBzl)-OCH_2-PAM resin using a custom-modified, machine-assisted chemistry tailored from the published in situ DIEA neutralization/HBTU activation protocol for Boc solid phase peptide synthesis. After chain assembly, the peptide was cleaved and deprotected by HF for 1 h in the presence of 5% *p*-cresol at 0 °C, followed by precipitation with cold ether. The crude product was purified by preparative RP-HPLC to homogeneity, and its molecular mass ascertained by ESI-MS. Oxidative folding of purified stoppin-1 was performed by dissolving the peptide at 1 mg/ml in 8 M GuHCl containing 12 mM reduced glutathione and 1.2 mM oxidized glutathione, followed by a rapid 4-fold dilution with 0.25 M $NaHCO_3$. The folding and disulfide formation proceeded productively at room temperature, and the final product was purified by HPLC and lyophilized.

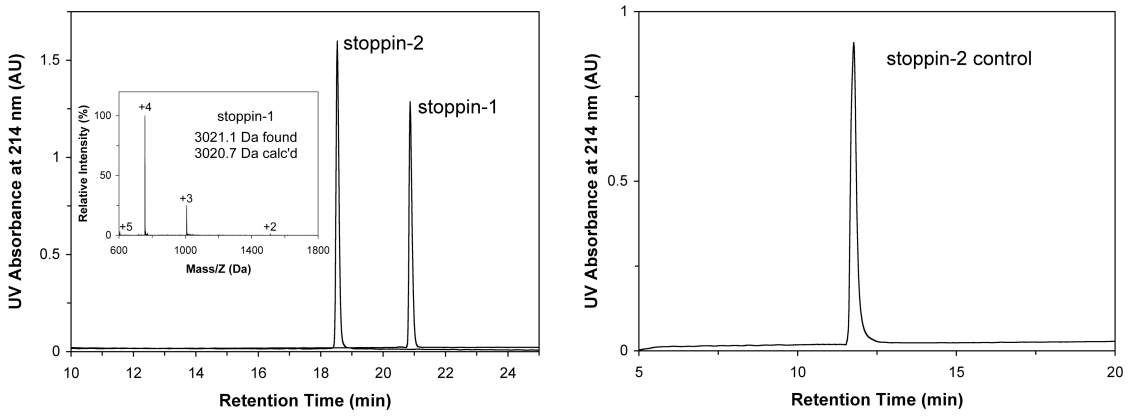

Figure S1. Stoppin-1, stoppin-2 and stoppin-2 control analyzed by HPLC and stoppin-1 by ESI-MS.

Shown in **Figure S1** (left) is stoppin-1 analyzed by HPLC and ESI-MS. The determined molecular mass of 3021.1 Da is in good agreement with the expected value of 3020.7 Da calculated on the basis of the average isotopic compositions of folded stoppin-1. To verify the correct folding of stoppin-1, we used a combination of trypsin and chymotrypsin to digest the miniprotein, and unambiguously identified all disulfide-containing peptide fragments by LC-MS analysis, establishing the native disulfide topology seen in BmBKTx1, i.e., Cys^1-Cys^4, Cys^2-Cys^5, and Cys^3-Cys^6.

Synthesis of stoppin-2 and stoppin-2 control was essentially as described for stoppin-1. After oxidative folding and purification, stoppin-2 gave rise to a determined molecular mass of 3313.7 Da, in agreement with the calculated value of 3313.1 Da. For stoppin-2 control, a determined molecular mass of 3195.3 Da is within experimental error of the calculated value of 3194.9 Da. The folding efficiency of stoppin-2 was significantly lower than that of stoppin-1. We also prepared stoppin-2 by orthogonally protecting Cys^2 and Cys^5 with Acm first, followed by oxidative folding of the peptide with four free Cys residues, yielding three structurally distinct and chromatographically separable peptide intermediates. After disulfide decoding using proteolytic/CNBr fragmentation coupled with LC-MS, the desired intermediate with two native disulfides was treated with iodine at acidic pH to spontaneously deprotect and oxidize Acm-protected Cys^2 and Cys^5. Synthesis and functional and structural characterizations of ^{syn}MDM2 and ^{syn}MDMX were described elsewhere (Liu et al., manuscript in preparation).

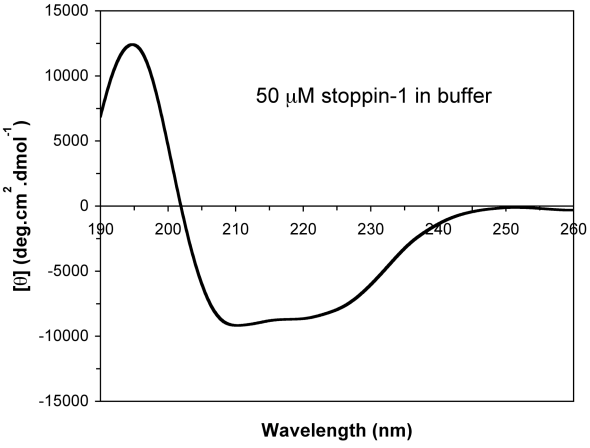

Figure S2. The CD spectrum of stoppin-1.

Circular dichroism spectroscopy. Far-UV CD spectra were obtained on a Jasco J-810 spectropolarimeter at room temperature using a 0.1-cm path length. Stoppin-1 was thoroughly dialyzed against 10 mM phosphate buffer containing, pH 7.2, and quantified by UV

absorbance measurements at 280 nm before data collection.

Trp fluorescence spectroscopy. Trp fluorescence spectra of 10 μM stoppin-1 and stoppin-2 in the presence and absence of 12 μM ^{syn}MDM2 or ^{syn}MDMX were recorded at 20°C in PBS on a Varian (Cary) Eclipse fluorimeter. The excitation wavelength was 295 nm and the width of both slits was set to 5 nm.

Surface plasmon resonance (SPR) spectroscopy. Competition binding kinetics were carried out on a Biacore T100 SPR instrument at 25°C in 10 mM HEPES, 150 mM NaCl, 0.005% surfactant P20, pH 7.4. $^{(15\text{-}29)}$p53 was immobilized in borate buffer, pH 8.5, to a CM5 sensor chip (17 RU's for ^{syn}MDM2 and 36 RU's for ^{syn}MDMX) using the EDC/NHS coupling chemistry and procedures recommended by the manufacturer. 50 nM ^{syn}MDM2 or 100 nM ^{syn}MDMX was incubated at room temperature for 30 min with varying concentrations of inhibitor, and injected at a flow rate of 20 μl/min for 2 min, followed by 4 min dissociation. The concentration of free ^{syn}MDM2 or ^{syn}MDMX in solution (not complexed with the inhibitor) was deduced, based on the binding RU's at equilibrium of p53 association, from a calibration curve established by RU measurements of different concentrations of ^{syn}MDM2 or ^{syn}MDMX injected alone. Non-linear regression analysis was performed using GraphPad Prism 4 to give rise to K_d values using the equation K_d = [peptide][MDM2/MDMX]/[complex].

Steady-state direct binding kinetics were carried out under the same condition as described for competition assays. Stoppin-1, stoppin-2 and stoppin-2 control were immobilized in an acetate buffer, pH 5.5, to CM 5 sensor chips, giving rise to 88, 93, and 89 RUs, respectively. Different concentrations of ^{syn}MDM2 were injected at a flow rate of 30 μl/min, followed by a 4-min dissociation. Non-linear regression analysis was performed using GraphPad Prism 4 to give rise to K_d values.

Tumor-killing activity of stoppin-1, stoppin-2, stoppin-2 control and Nutlin-3. HCT116 cells (10,000/well) were plated overnight in McCoy's 5A modified medium (1X) supplemented with 1% pen-strep and 10% heat inactivated FBS. SW480 cells (10,000/well) were plated overnight in DMEM 1X medium, 1% pen-strep and 10% heat inactivated FBS (all reagents were from GIBCO). Cells were treated the next day with the indicated compound and then OD and cell count assessed after 3 days (left column) or peptides were added every day for three days and then OD and cell count were assessed (right column). OD was measured with the MTS kit (Cell titer 96 aqueous non-radioactive cell proliferation assay, Promega). Trypan blue incorporation was used to determine the number of cells alive and dead. The concentration of DMSO is 0.2% (v/v).

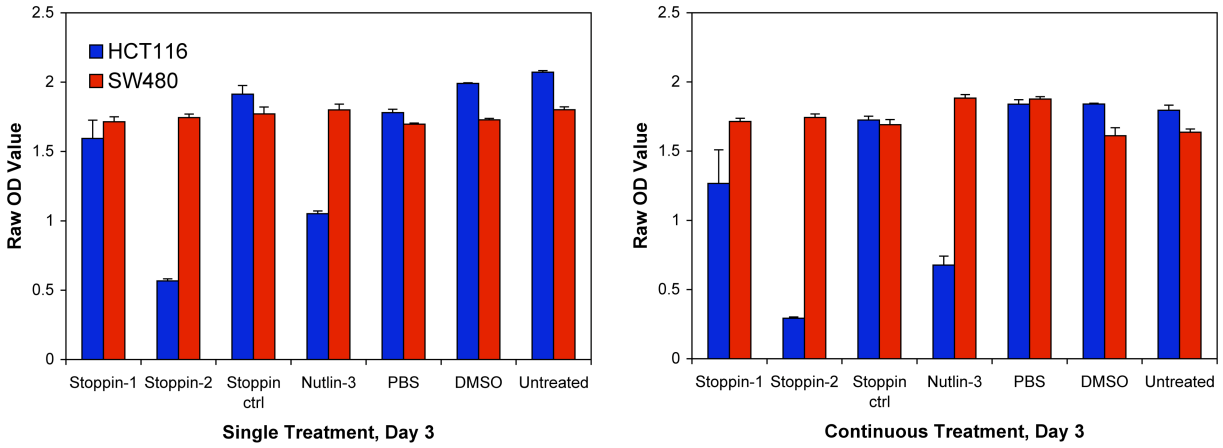

Figure S3. Tumor-killing activity of stoppin-1, stoppin-2, stoppin-2 control (100 μg/ml each) and Nutlin-3 (10 μg/ml) against HCT116 and SW480 cells as determined by MTS cell viability assay.

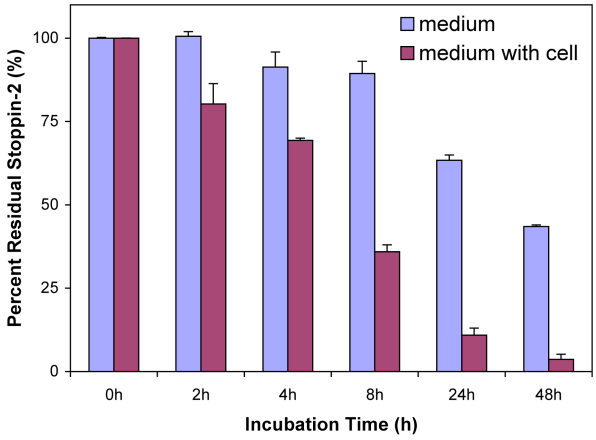

Figure S4. Proteolytic stability of stoppin-2 in assay media.

Stability of stoppin-2 in assay media. 100 μg/ml stoppin-2 was incubated in serum-containing assay media in the presence and absence of HCT116 cells. The supernatant was withdrawn at

different time intervals and analyzed by analytical HPLC for intact stoppin-2. Percent residual stoppin-2 was calculated by ratios of integrated peak areas of the intact peptide at a given time versus time zero (**Figure S4**).