Epidithiodiketopiperazines inhibit protein degradation by targeting proteasome deubiquitinase Rpn11

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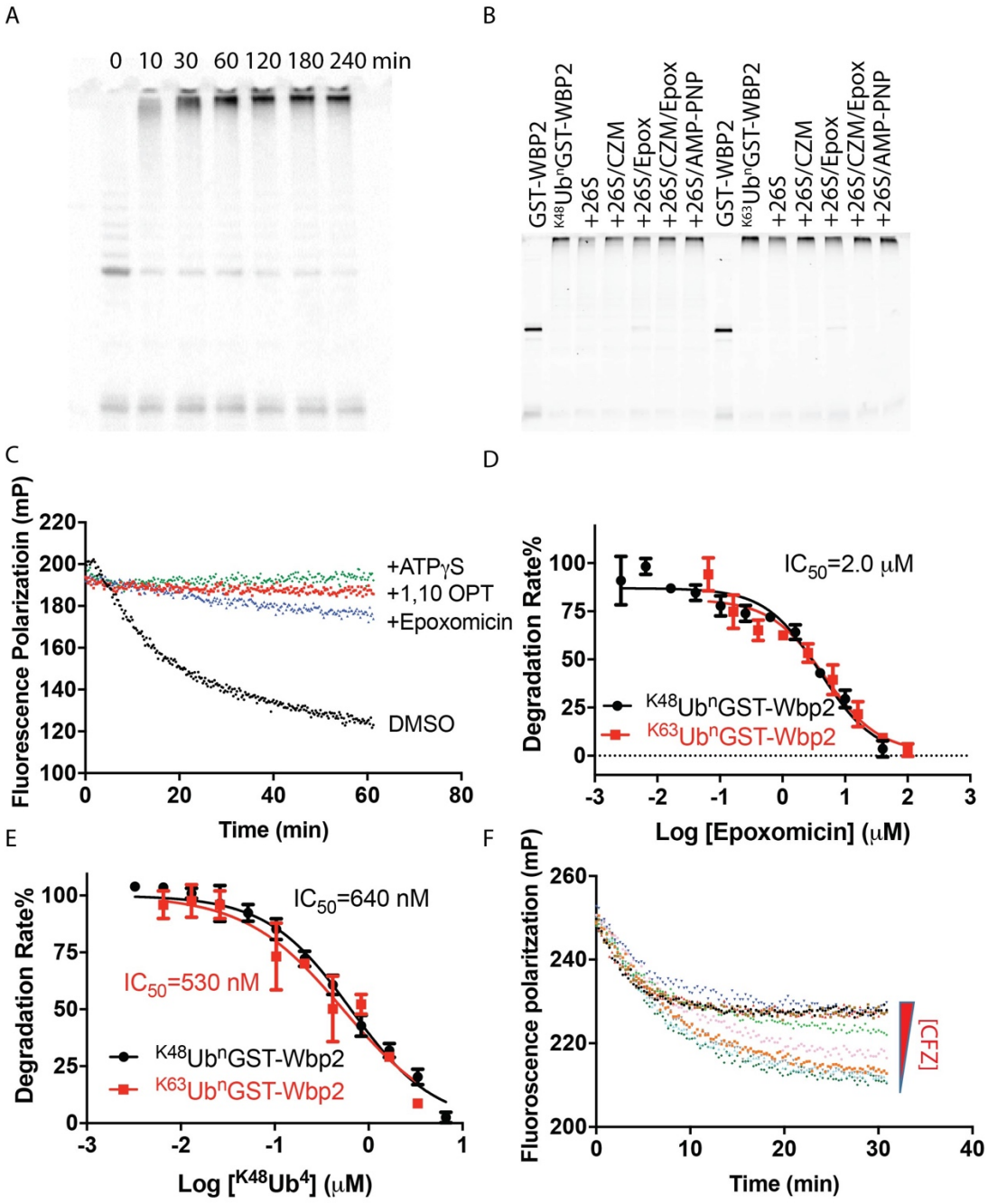


Figure S1. Development of an assay to monitor protein degradation. Related to **Figure 1** (A) *in vitro* ubiquitination of GST-Wbp2 with Ube1, UbcH5a and Rsp5-E6AP at room temperature. Reactions were fractionated by SDS-PAGE and analyzed using a Typhoon fluorescence scanner at different time points as indicated. (B) Proteasomal degradation of Ub^nGST–Wbp2. 20 nM $^{K48}Ub^n$GST–Wbp2 was incubated with 2.5 nM 26S proteasome at 37˚C for 2 hours in the absence and presence of different inhibitors (20 μM CZM or 5 μM epoxomicin/Epox or 1 mM AMP-PNP). Reactions were fractionated by SDS-PAGE and analyzed using a Typhoon fluorescence scanner. (C) Measurement of proteasome activity using fluorescence polarization assay. $^{K48}Ub^n$GST–Wbp2 (2.5 nM) was incubated with 1 nM 26S proteasome at 37 °C in the absence or presence of different inhibitors (10 μM Epox or 2 mM ATPγS or 2 mM 1,10-phenanthroline (1,10-PT)). (D) Epoxomicin inhibits the degradation of the Wbp2 substrate. Shown is the dose-response curve of proteasome activity measured at 37 °C using Ub^nGST–Wbp2 as substrate and proteasome with different concentrations of epoxomicin. (E) Shown are the dose-response curves of proteasome activity measured at 37°C using Ub^nGST–Wbp2 as substrate and proteasome in the presence of different concentrations of K48-linked Ub4. (F) Shown is the reaction kinetics measured at 37 °C using $^{K63}Ub^n$GST–Wbp2 as substrate and lysate from cells treated with different concentrations of carfilzomib.

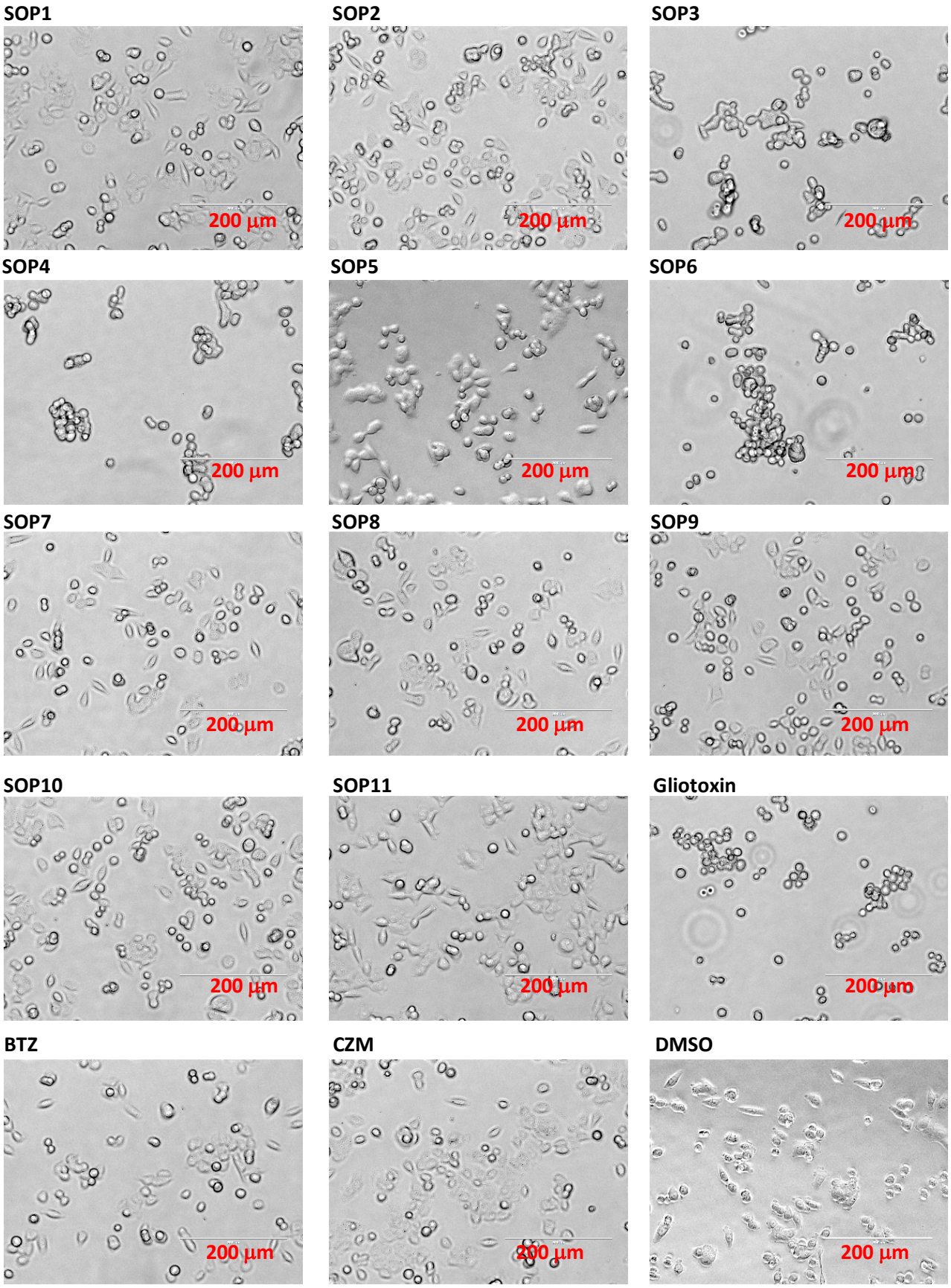


Figure S2. Effects on the cell morphology. Related to **Figure 4.** Shown are microscopic images of HCT116 cells, taken 3 hours after treatment with 10 µM of the indicated compounds except for BTZ, which was used at 1 µM.

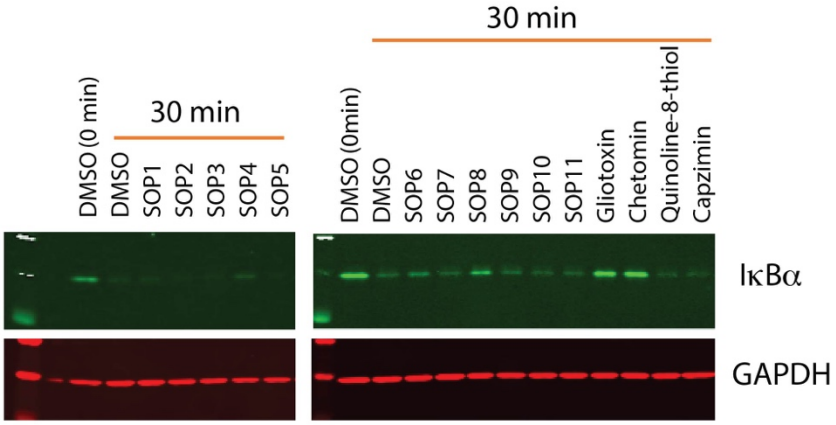


Figure S3. Screening the effects of ETPs on the degradation of IκBα in TNF-α-stimulated Hela cells. Related to **Figure 4.** Hela cells were stimulated with TNF-α for 30 mins in the presence of 1 μM compounds as indicated. Degradation of IκBα was evaluated by western blot.

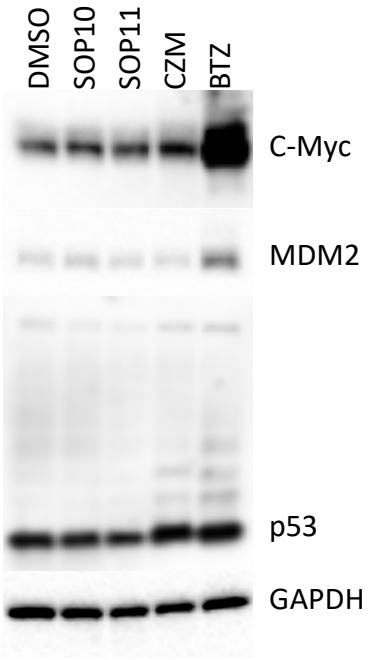


Figure S4. ETP showed little effect on stabilization of multiple proteasome substrates. Related to **Figure 4.** HCT116 cells were incubated with either ETP compounds (10 μM), CZM (10 μM), or BTZ (1 μM) for 6 hours, and cell lysates were fractionated by SDS–PAGE and immunoblotted with antibodies against c-Myc, MDM2, p53 or GAPDH.

	K63 (%)	K48 (%)	K33 (%)	K29 (%)	K27 (%)	K11 (%)	K6 (%)
$^{K63}Ub^{n}$GST-Wbp2	94.13±0.28	1.69±0.06	0.34±0.03	2.13±0.03	0.04±0.01	1.49±0.16	0.19±0.02
$^{K48}Ub^{n}$GST-Wbp2	5.29±0.06	87.18±1.60	0.22±0.03	Not detected	0.03±0.00	3.78±0.27	3.52±0.67

Table S1. Mass spectrometry characterization of the polyubiquitinated GST-Wbp2. Related to **Figure 1.**

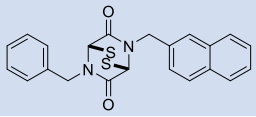
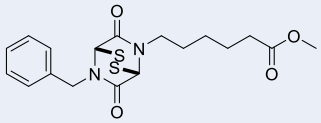
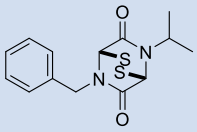
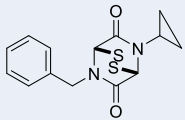
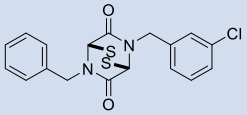
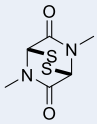
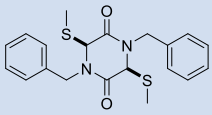
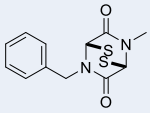
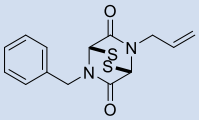
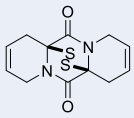
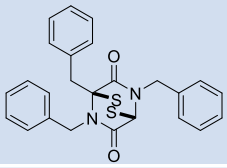
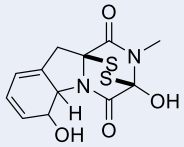
Reference	Structure	Rpn11 IC_{50} (μM)	Csn5 IC_{50} (μM)	AMSH IC_{50} (μM)	$Ub^{G76V}GFP$ IC_{50} (μM)	GI_{50} (μM)
SOP1		2.6±0.6	0.2±0.06	0.6±0.04	5.7±0.6	8.6±2.0
SOP2		1.1±0.3	1.2±0.3	3.2±0.2	10.7±1.6	>20
SOP3		1.3±0.4	0.4±0.1	0.5±0.04	5.0±0.8	5.7±1
SOP4		0.2±0.05	0.5±0.1	0.3±0.04	14.6±3.1	5.8±1
SOP5		3.1±1.0	0.7±0.3	1.0±0.1	8.0±1.1	>20
SOP6		3.8±1.2	2.9±0.5	2.1±0.2	4.6±1.2	1.4±0.1
SOP7		>100	>100	>100	>50	>100
SOP8		3.7±1	1.2±0.2	0.9±0.04	>20	>20
SOP9		1.9±0.4	0.9±0.3	0.4±0.03	>20	>20
SOP10		0.7±0.2	0.6±0.2	1.0±0.04	4.6±1.1	8.2±1.0
SOP11		1.3±0.3	0.6±0.2	0.9±0.1	2.7±0.7	4.7±0.5
Gliotoxin		6.9±3.4	14±3	3.6±0.6	0.4	0.3±0.04

Table S2. Summary table of the ETPs. Related to **Figure 3** and **Figure 5.**