Supplemental Material

A Selective Inhibitor of Ubiquitin-Specific Protease 4 Suppresses Colorectal Cancer Progression by Regulating β-Catenin Signaling

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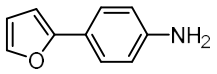
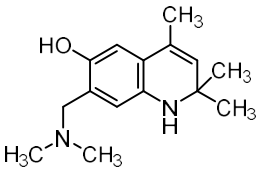
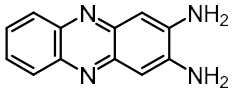
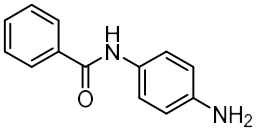
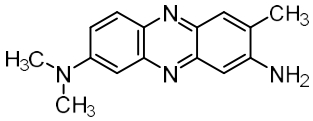
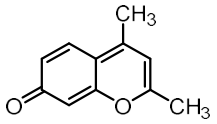
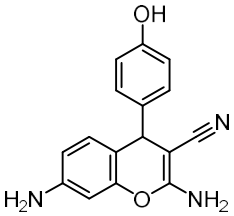
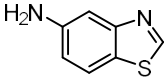
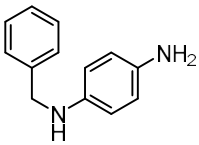
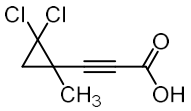
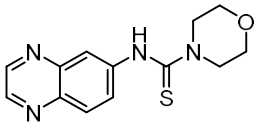
Supplementary Tables (S1–2)

Supplementary Table S1. Primers used in this study.

Primer name	Sequence (5'→3')[a]	Purpose
8839F	ATATATGGATCCCGTGAGCGACCGG	cloning of human *USP4* gene into plasmid pVFT1S
8839R	ATATATCTCGAGATCATCTCGACGTTGGTA AAATAG	
2330F	AAACTTGGATCCATGTTTGGCCCCGCTAAA	cloning of human *YOD1* gene into plasmid pVFT1S
2331R	TTCATGCTCGAGTCACACTTCTCCAAAGTT	
CCND1F	CCGTCCATGCGGAAGATC	qRT-PCR for cyclin D1 gene
CCND1R	ATGGCCAGCGGGAAGAC	
AXIN2F	CCTGCCACCAAGACCTACAT	qRT-PCR for *AXIN2* gene
AXIN2R	GTTTCCGTGGACCTCACACT	
USP4F	CCTGGGCTCTGTGGACTTG	qRT-PCR for *USP4* gene
USP4R	TGTTGATTTCGGCTTCATACTC	
CTNNB1F	ACCTTTCCCATCATCGTGAG	qRT-PCR for β-catenin gene
CTNNB1R	AATCCACTGGTGAACCAAGC	
GAPDHF	CTGGTAAAGTGG ATATTGTTGCCAT	qRT-PCR for *GAPDH* gene
GAPDHR	TGGAATCATATTGGAACATGTAAACC	

[a] The restriction sites are underlined.

Supplementary Table S2. USP4 inhibitors selected in the initial screening.

No.	ID	Chemical name	Structure	%Inhibition
1	2C6	[4-(2-furyl)phenyl]amine hydrochloride		65.4
2	2D7	7-[(dimethylamino)methyl]-2,2,4-trimethyl-1,2-dihydro-6-quinolinol		100
3	3G6	2,3-phenazinediamine hydrochloride		82.9
4	5F7	N-(4-aminophenyl)benzamide		76.5
5	5E9	N~8~,N~8~,3-trimethyl-2,8-phenazinediamine hydrochloride		85.9
6	6G10	2,4-dimethyl-7H-chromen-7-one		94.2
7	6D11	2,7-diamino-4-(4-hydroxyphenyl)-4H-chromene-3-carbonitrile		88.8
8	6A2	1,3-benzothiazol-5-amine		100
9	9A9	N-benzylbenzene-1,4-diamine		71.7
10	17C2	3-(2,2-dichloro-1-methylcyclopropyl)-2-propynoic acid		62.4
11	17B3	N-6-quinoxalinyl-4-morpholinecarbothioamide		100

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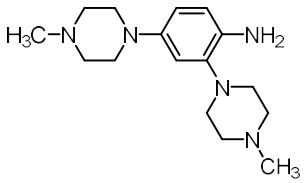
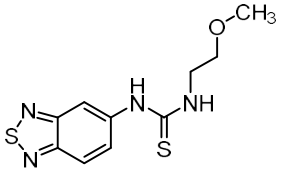
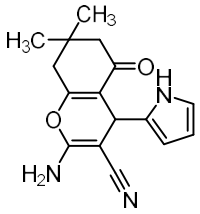
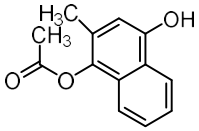
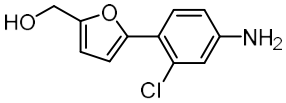
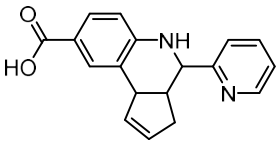
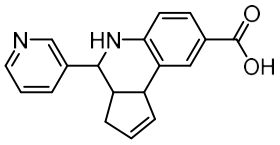
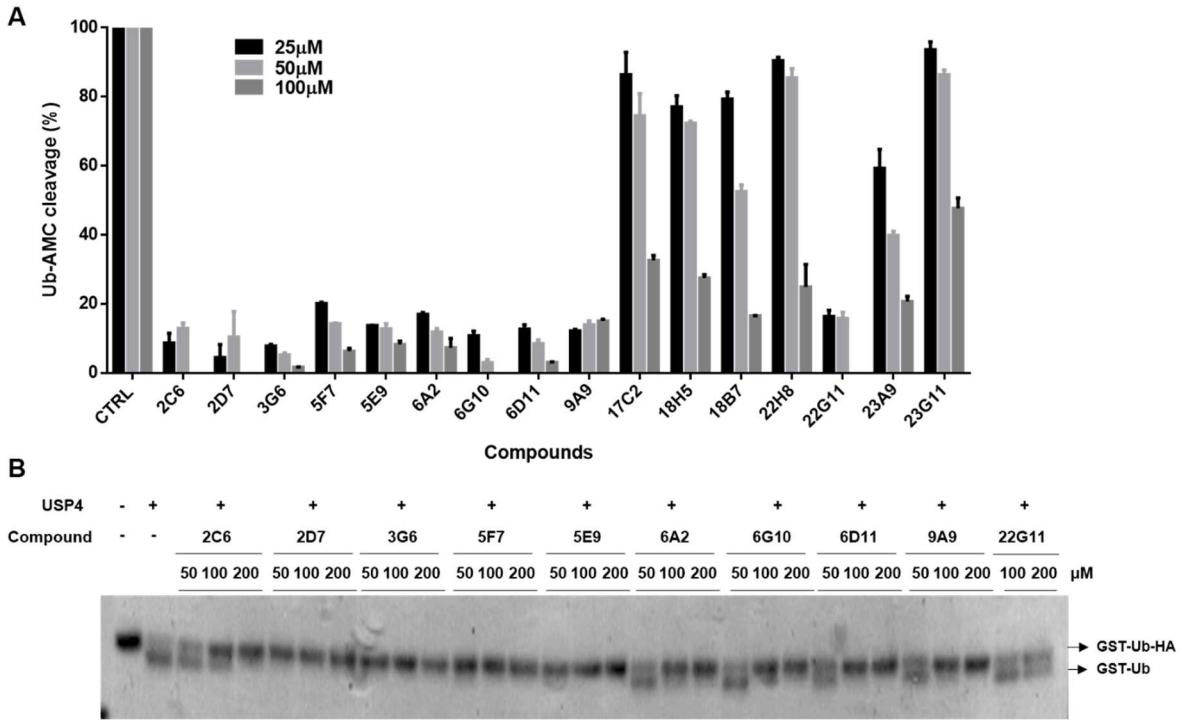
Supplementary Table S2. (*continued*) USP4 inhibitors selected in the initial screening.

No.	ID	Chemical name	Structure	%Inhibition
12	17D11	2,4-bis(4-methyl-1-piperazinyl)aniline		94.3
13	18H5	N-2,1,3-benzothiadiazol-5-yl-N'-(2-methoxyethyl)thiourea		100
14	18B7	2-amino-7,7-dimethyl-5-oxo-4-(1H-pyrrol-2-yl)-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile		59.8
15	22H8	4-hydroxy-2-methyl-1-naphthyl acetate		100
16	22G11	[5-(4-amino-2-chlorophenyl)-2-furyl]methanol		94.7
17	23A9	4-(2-pyridinyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-carboxylic acid		74.2
18	23G11	4-(3-pyridinyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-carboxylic acid		68.9

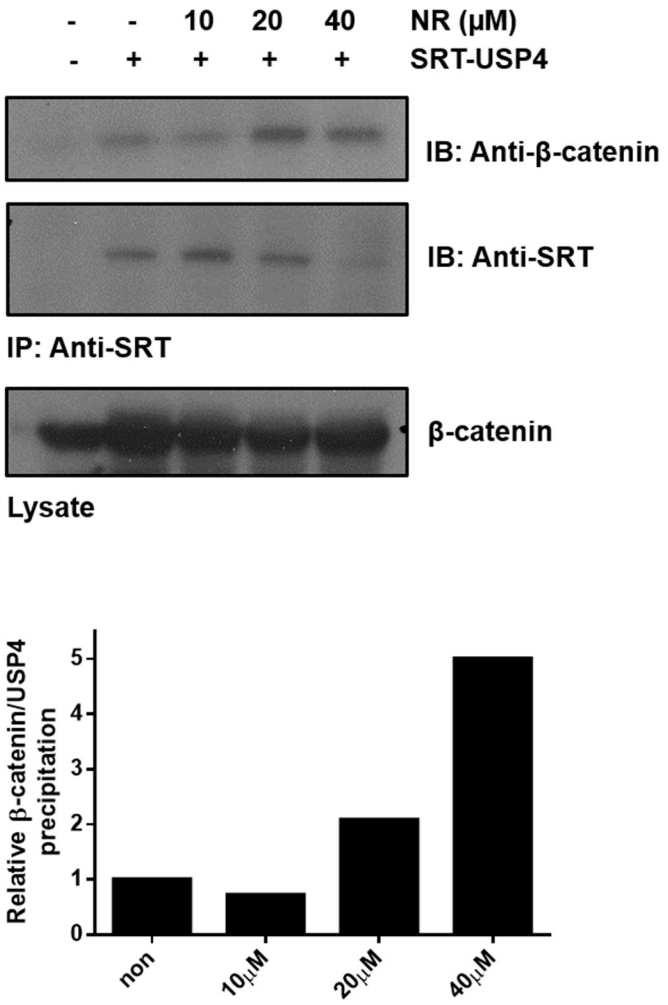
Supplementary Figures (S1-3)



Supplementary Fig. S1. Inhibitory activity of USP4 inhibitors selected in the initial screening.

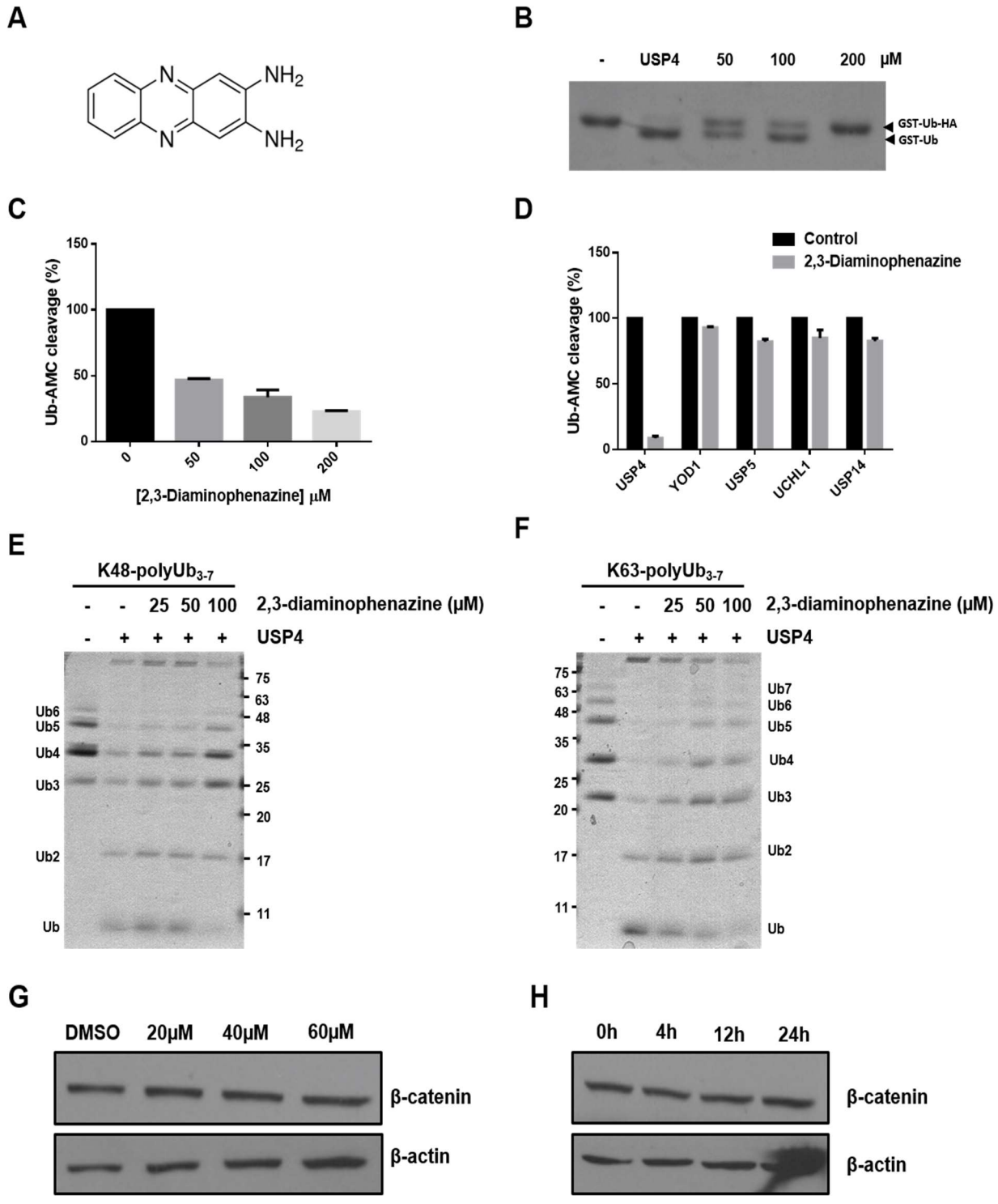
(A) Indicated concentrations of selected compounds (Supplementary Table S2) were incubated with USP4 and Ub-AMC for 20 min at room temperature. Percentage of Ub-AMC cleavage was calculated based on the fluorescent AMC measured by a fluorescence reader. The 5E9 compound was identified as neutral red.

(B) His-tagged USP4 was pre-incubated with different concentrations of selected compounds and then reacted with GST-Ub-HA for 4 h at 37 °C. The mixtures were separated by 12% SDS-PAGE and stained with Coomassie Blue dye.



Supplementary Fig. S2. NR enhances the binding of USP4 and β-catenin.

SRT-USP4 was transfected into HCT116 cells, and the indicated concentration of NR was added to the cell medium for 24 h. Co-precipitated β-catenin protein was detected by Western blotting after IP with anti-SRT antibody (top). The band intensity of β-catenin was measured by densitometry and normalized to the precipitated USP4 (bottom).



Supplementary Fig. S3. 2,3-diaminophenazine inhibits USP4 activity *in vitro* but not in HCT116 cells.

(A) Chemical structure of 2,3-diaminophenazine.

(B, C) 2,3-diaminophenazine inhibited USP4 deubiquitinating activity *in vitro*. Recombinant USP4 protein was incubated with various concentrations of 2,3-diaminophenazine, and then the hydrolysis of certain substrates was measured using a GST-Ub-HA deubiquitination assay (B) and Ub-AMC deubiquitination assay (C).

(D) 2,3-diaminophenazine did not affect the activity of other DUBs. USP4, YOD1, USP5, UCHL1, and USP14 were incubated with and without 200 μM of 2,3-diaminophenazine, and the release of AMC was measured using a Ub-AMC deubiquitination assay.

(E, F) 2,3-diaminophenazine prevented USP4 deubiquitinating activity on both K48 and K63 linkages. The monomerization of the K48-polyubiquitin chain (E) and K63-polyubiquitin chain (F) by USP4 was tested with increasing concentrations of 2,3-diaminophenazine and observed using Coomassie gel staining.

(G, H) 2,3-diaminophenazine did not affect β-catenin expression in HCT116 cells. (G) HCT116 cells were treated with various concentrations of 2,3-diaminophenazine for 24 h. (H) Cells were treated with 40 μM of 2,3-diaminophenazine and harvested at the indicated time points. The expression of β-catenin was obtained using Western blot analysis. β-actin was used as a loading control.