< Supporting Information >

Label-free Target Identification Reveals Oxidative DNA Damage as the Mechanism of a Selective Cytotoxic Agent

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Experimental Procedures

Cell viability assay. 24 h after the cell seeding, cells were treated with compounds for 2 d. After washing, cell viability was assessed by MTT assay with EZ-Cytox [Dogen, South Korea].

TS-FITGE. Procedures for the TS-FITGE was done as previously described[1]. Briefly, protein concentration of the soluble fraction was quantified with Bio-Rad protein assay. 50 μg of the protein was precipitated with acetone followed by centrifugation for 7 min at 20,000g, 4 °C. The residual pellet was resuspended with 10 μl of conjugation buffer (30 mM Tris-HCl (pH 8.6), 2 M thiourea, 7 M urea, and 4% w/v CHAPS). 1 μl of 0.4 mM Cy3-NHS or Cy5-NHS was mixed to the proteins and incubated at 4 °C for 45 min. 320 μg of unheated sample for the internal standard was precipitated, resuspended with 64 μl of the conjugation buffer, and mixed with 6.4 μl of Cy2-NHS. The dye-conjugated protein was precipitated with cold acetone and resuspended with 50 μl of rehydration buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS, 40 mM DTT, and 1% IPG buffer). The compound-treated group (conjugated with Cy5) and DMSO-treated group (conjugated with Cy3) were mixed. The same amount of the unheated sample (conjugated with Cy2) was added for internal standard. Total 150 μg (50 μg for Cy2, Cy3, and Cy5 each) of proteins were loaded in a 24-cm Immobiline Drystrip gel [GE Healthcare], and isoelectric focusing was done by Ettan IPGphor 3 [GE Healthcare]. After the isoelectric focusing, proteins in the strip gel were separated by Ettan DALTsix electrophoresis system [GE Healthcare], and the gel was scanned with Typhoon Trio [GE Healthcare]. The Protein spot location and fluorescence signals were analyzed by DeCyder 2D software, ver. 7.2 [GE Healthcare]. Signal ratio of Cy5 to Cy3 were normalized, so that the modal peak of the logarithm value of the ratio was set to be zero. The data were presented in a box-and-whisker plot by GraphPad Prism 5, and outliers were chosen as thermally shifted spots.

In-gel digestion and mass spectrometry. The protein spots from silver-stained gel were excised, destained, and digested with trypsin. The mixture was evaporated in SpeedVac and then dissolved in 10% acetonitrile with 0.1% formic acid. The resulting peptides were desalted in a trap column (180 μm × 20 mm, Symmetry C18) and separated on a C18 reversed-phase analytical column (75 μm × 200 mm, 1.7 μm, BEH130 C18, Waters). Mass spectrometry was done by nanoAcquity UPLC-ESI-QTOF/MS (SYNAPT G2-Si HDMS, Waters) with an electrospray ionization PicoTip (±10 μm i.d., New Objective). The data were converted to .pkl files by Protein Lynx Global Server and searched by MASCOT engine with the SwissProt database.

Thermal proteome profiling. TPP procedures were conducted as described by Franken et al [2]. Soluble proteins from the cell extract were denatured by addition of urea buffer (6 M urea and 2 M thiourea in 50 mM triethylammonium bicarbonate (TEAB)) at 55 °C for 1 h, reduced with 10 mM of 1,4-dithiothreitol (DTT) at 55 °C for 1 h, and incubated with 45 mM of iodoacetamide for 1 h. 50 mM of TEAB buffer was added to dilute the urea concentration to lower than 1.2 M before trypsin digestion at 37 °C for 16 h. The tryptic peptides were desalted with Sep-Pak C18 cartridge [Waters] and resuspended with 50 mM of TEAB buffer containing 20% acetonitrile. Peptides from the 10 temperature points were reacted with each TMT-10plex isobaric mass tag [Thermo Scientific] at room temperature for 1 h, and quenched by the addition of hydroxylamine. The TMT-tagged peptides were pooled, and fractionated by reverse-phase liquid chromatography (Agilent HPLC 1200 series) with Zorbax extend C-18 rapid resolution column (4.6 × 100 mm, 3.5 μm, 80 Å, Agilent). Separation was performed at a flow rate of 0.33 ml/min for a 2-h gradient of 5–40% acetonitrile containing 20 mM ammonium formate and 20 mM ammonium hydroxide. The eluates were collected by 1-min interval and concatenated into final 6 fractions.

Each fraction was injected onto an EASY-nLC 1200 [Thermo Scientific] coupled to a Orbitrap Fusion Lumos mass spectrometer [Thermo Scientific]. Peptides were trapped on an Acclaim PepMap 100 column (75 μm × 2 cm, 3 μm, Thermo Scientific), separated on an EASY-Spray column (75 μm × 50 cm, 2 μm, Thermo Scientific) for a 2.5-h gradient of 3–35% acetonitrile containing 0.1% formic acid. MS scans were acquired in the Orbitrap at 120,000 resolution with automatic gain control (AGC) target of 2E5 and maximum injection time of 50 s. Precursor ions were selected with data-dependent mode, and MS2 spectra (AGC 1E4, maximum injection time 100 ms) were acquired with normalized collision energy (NCE) of 35 and analyzed in the linear ion trap. Using synchronous precursor selection (SPS) mode, the top 15 SPS precursors were selected for MS3 analysis (isolation window 2 Th, AGC target 5E4, maximum injection time 100 ms), fragmented by higher collision dissociation with NCE of 65, and analyzed at Orbitrap with 60,000 resolution.

Proteome Discoverer 2.1 [Thermo Scientific] was used for protein identification and quantification using SwissProt databases (released at April 12th, 2017) including human and human papilloma virus type 18. Missed cleavage was allowed up to 2; carbamidomethylation of cysteine residues and TMT modification of lysine residues were set as fixed modification; and methionine oxidation, and *N*-terminal acetylation and TMT modification of peptide *N*-termini were set as variable modifications. False discovery rate (FDR) of < 1% was applied. Data normalization, melting curve fitting, and T_m calculation were done with a TPP R package [3].

Western blotting. Cells were lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 1% IGEPAL CA-630, protease inhibitor cocktail) on ice for 15 min. Soluble fraction was collected after centrifugation at 20,000g for 15 min at 4 °C. The proteins were separated by SDS-PAGE and transferred to a PVDF membrane.

After blocked with 2% BSA in Tris-buffered saline (TBS) supplemented with 1% Tween-20 (TBST) for 1 h, the membrane was incubated with primary antibodies—anti-LTA4H (ab133512, abcam), anti-MTH1 (#43918, Cell Signaling), anti-MTRR (ab129159, abcam), anti-DIS3 (ab176802, abcam), anti-Erlin1 (ab171372, abcam), anti-ALDH2 (PA5-29517, Thermo Scientific), anti-ALDH1B1 (PA5-28554, Thermo Scientific), anti-CASP7 (#9492, Cell signaling), anti-PGAM(#12098, Cell signaling), anti-SDHA (#11998, Cell signaling), anti-β-actin (#4970, Cell signaling), anti-EXO1 (sc-56092, Santa cruz), anti-PCNA (sc-25280, Santa cruz), anti-PMS2 (sc-25315, Santa cruz), anti-MLH1 (sc-271978, Santa cruz), anti-MSH2 (sc-376384, Santa cruz), anti-MSH6 (sc-271080, Santa cruz), anti-ATM (pSer1981) (sc-47739, Santa cruz), anti-p53 (pSer15) (#9284, Cell Signaling), anti-caspase-3 (#9662, Cell Signaling), anti-PARP (#9532, Cell Signaling), and anti-GAPDH (#2118, Cell Signaling)—overnight at 4 °C. The membrane was washed with TBST and incubated with secondary antibodies—anti-rabbit HRP-linked (#7074, Cell Signaling), anti-mouse HRP-linked (#7076, Cell Signaling)—for 1 h at room temperature. The protein bands were developed with Amersham ECL prime western blotting detection reagent [GE Healthcare] and detected by Chemi-Doc [Bio-Rad].

Gene knockdown. The following Silencer Select siRNAs from Ambion [Thermo Scientific] were used: *ALDH1B1* (s1246), *ALDH2* (s1239), *CASP7* (s2424), *DIS3* (s229658), *ECH1* (s4436), *ERLIN1* (s22014), *ERLIN2* (s20839), *LTA4H* (s8307), *MTH1* (s9030), *MTRR* (s9060), *PGAM1* (s10839), *SDHA* (s12650), *EXO1* (s17502), *MLH1* (s298), *MSH2* (s8967), *MSH3*(s8970), *MSH6* (s6287), *PCNA* (s10134), and non-targeting negative control #2 (s4390846). 24 h after cell seeding, siRNA was transfected for 2 d with Lipofectamine RNAiMAX [Invitrogen] according to the manufacturer's instruction. For the experiments to assess the activity change of SB2001 after gene knockdown, cells were transfected with siRNA for 2 d and then treated with SB2001 for another 2 d, followed by an MTT assay.

***In vitro* LTA4H hydrolase assay.** Hexane solution of LTA_4 methyl ester [Cayman chemical] was evaporated under argon gas, and resulting LTA_4 methyl ester was hydrolyzed to LTA_4 with 50 mM NaOH in 80% acetone/20% H_2O for 40 min at room temperature. 5 μl of 100 ng/μl LTA4H protein [Sino Biological] was mixed with 0.45 μl of compound solution, and the volume was adjusted to 21 μl by addition of the assay buffer (50 mM Tris-HCl (pH 7.6) and 100 mM NaCl). After 15 min of incubation for binding, 1.5 μl of 750 μM LTA_4 was added to the reaction mixture. After 20 min of the reaction, 45 μl of methanol was added for quenching. Proteins were removed by centrifugation at 3,000 g for 10 min at 4 °C, and the supernatant was desalted using C18 tips [Thermo Scientific]. The eluate was analyzed by reverse-phase LC-MS [Shimadzu] on a 25-cm C18 column (250 × 4.6 mm, 4 μm, YMC) using 0.1% formic acid MeOH/H_2O as mobile phases. The LTB_4 produced by LTA4H activity was quantified by detection of absorption at 270 nm.

***In vitro* LTA4H aminopeptidase assay.** 220 ng of the recombinant LTA4H protein (ab174008, abcam) in 22 μl of assay buffer (50 mM Tris-HCl (pH 7.5) and 100 mM NaCl) was incubated with 2.5 μl of the compounds in a half-well area 96-well plate for 15 min at room temperature. 25 μl of 1 mM l-Ala-*p*-NA was added to each well, and the absorbance at 405 nm was observed for 30 min with a Synergy HT [Bio-Tek].

***In vitro* MTH1 activity assay.** Serially diluted compounds were dissolved in the assay buffer (100 mM Tris-acetate (pH 7.5), 40 mM NaCl, 10 mM $Mg(OAc)_2$, 0.005% Tween-20, and 2 mM DTT). The compounds were mixed with recombinant MTH1 protein (final 2 nM, Prospec) and incubated for 30 min at room temperature. After addition of PPiLight detection buffer and PPiLight converting buffer (Lonza), 8-oxo-dGTP (final 13.2 μM, Jena Bioscience) or 2-OH-dATP (final 8.3 μM, Jena Bioscience) was added as a substrate. After incubation for 30 min at room temperature, luminescence was measured with a Synergy HT [Bio-Tek].

Surface plasmon resonance. BIAcore T100 [GE Healthcare] was utilized to detect the direct binding. The buffer solution of the recombinant LTA4H protein (10276-H08B, Sino biological) or MTH1 protein (enz-010-b, Prospec) was changed to PBS with a 3K Amicon ultra centrifugal filter [Millipore]. Then, the protein was immobilized on a CM5 sensor chip [GE Healthcare] with the amine coupling kit [GE Healthcare] at pH 5.0 (for LTA4H) and pH 4.5 (for MTH1) acetate buffer. Final immobilization level of LTA4H and MTH1 reached 19,826 RU and 2,300 RU, respectively. For the binding kinetics study, compounds with various concentrations in the running buffer (for LTA4H: 4% DMSO, 10 mM Tris-acetate (pH 7.5), 100 mM NaCl, and 0.005% P20; for MTH1: 4% DMSO, 100 mM Tris-acetate (pH 7.5), 40 mM NaCl, 10 mM magnesium acetate, 1 mM DTT, and 0.005% P20) was injected for 60 s with a flow rate of 22 μl/min and dissociated with the running buffer for 300 s. The sensorgrams were fitted to the one-to-one binding model with BIAcore T100 evalutaion software [GE Healthcare].

Proteome expression profiling. Differential protein expression levels between HeLa and CaSki cells were analyzed using a quantitative mass spectrometry. The cells were harvested by scraping at each time point and lysed in the urea lysis buffer (50 mM TEAB (pH 8.2), 6 M urea, 2 M thiourea, 75 mM NaCl, 0.4% IGEPAL CA-630, and protease inhibitor cocktail). After centrifugation at 20,000g, 4 °C for 15 min, protein concentration in the supernatant was measured by Bio-Rad protein assay. 60 μg of the protein was reduced with 10 mM DTT at 55 °C for 1 h, and incubated with 45 mM iodoacetamide for 1 h. The mixture was diluted with 50 mM TEAB buffer and digested with trypsin at 37 °C for 16 h. The tryptic peptides were desalted with Sep-Pak C18 cartridge [Waters] and resuspended with 50 mM TEAB buffer containing 20% acetonitrile. Peptides from each condition was conjugated with TMT-10plex isobaric mass tag [Thermo Scientific] at room temperature for 1 h, and quenched by the addition of

hydroxylamine. The rest of the procedures for desalting, fractionation, mass spectrometry, and data analysis by Proteome discoverer were identical with that of the thermal proteome profiling. Upregulated or downregulated gene sets in HeLa cells compared to CaSki cells were analyzed with Gene Set Enrichment Analysis (GSEA) software [4] using the molecular signature database (MSigDB) including KEGG pathway database [5].

8-oxoG imaging. As avidin has been shown to bind to 8-oxoG specifically[6], avidin-alexa488 [Thermo Scientific] was used to measure cellular concentration of 8-oxo-dG. Cells were treated with the 1.6 mM of KBrO3 or 1 μM of SB2001 for 3 d, and fixed with methanol at –20 °C for 20 min. After incubation in TBS with 0.1% Triton X-100 for 15 min, blocking was performed with 2% BSA, 0.1% Triton X-100 in TBS for 2 h in room temperature. Cells were then incubated with 10 μg/ml avidin-alexa488 in the blocking solution for 1 h at 37 °C. After washing with 0.1% Triton X-100 in TBS for 5 min twice, DNA was stained with Hoechst 33342 [Thermo Scientific] for 10 min at room temperature. DeltaVision Elite [GE Healthcare] was used for imaging, and the intensity of 8-oxoG in the nucleus was analyzed with ImageJ.

Comet assay. Comet assay was performed according to the manufacturer's instruction of an hOGG1 FLARE assay kit [Trevigen]. HeLa and CaSki cells were treated with 300 nM SB2001 for 2 d or 100 μM hydrogen peroxide for 15 min at 37 °C and then trypsinized for harvest. Cells were washed, resuspended in PBS, and mixed with LMAa-garose. The cell suspension was spread on a FLARE slide glass and solidified on ice for 15 min. The slide was immersed in lysis buffer supplemented with 10% DMSO for 1 h and equilibrated with FLARE buffer. hOGG1 enzyme diluted in the Reaction buffer was added on the slide and incubated at humid 37 °C for 45 min. The slide was immersed in the alkali solution (200 mM NaOH (pH 13) and 1 mM EDTA) for denaturation, and electrophoresis was done at 25 V, 4 °C for 20 min. Then, the slide was washed and completely dried before immersing in the fixative solution (15% w/v trichloroacetic acid, 5% w/v zinc sulfate, and 5% glycerol) for 10 min. For silver staining[7], the slide was immersed in the mixture of 5 ml of Solution A (5% sodium carbonate) and 10 ml of Solution B (0.2% w/v ammonium nitrate, 0.2% w/v silver nitrate, 0.5% w/v tungstosilicic acid, 0.15% formaldehyde, and 5% w/v sodium carbonate) for 10 min three times, and the staining was stopped by immersing in 1% acetic acid for 5 min. Images were acquired wih IX71 microscope [Olympus], and tail moments was calculated as the percent comet tail multi-plied by the tail length by CometScore 2.0 software.

Data availability. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consor-tium via the PRIDE[8] partner repository with the dataset identifier PXD009903 (thermal proteome profiling,

Username: reviewer27088@ebi.ac.uk, Password: S8WdimS8) and PXD009904 (differential protein expression profiling, Username: reviewer80059@ebi.ac.uk, Password: whpR1qgT). The figures that will be published within a separate paper (ref. 19)—cell viability data of the other eleven cell lines, structure-activity relationship of SB2001, and *in vivo* data—are attached for the reviewing process.

Supplementary Figures

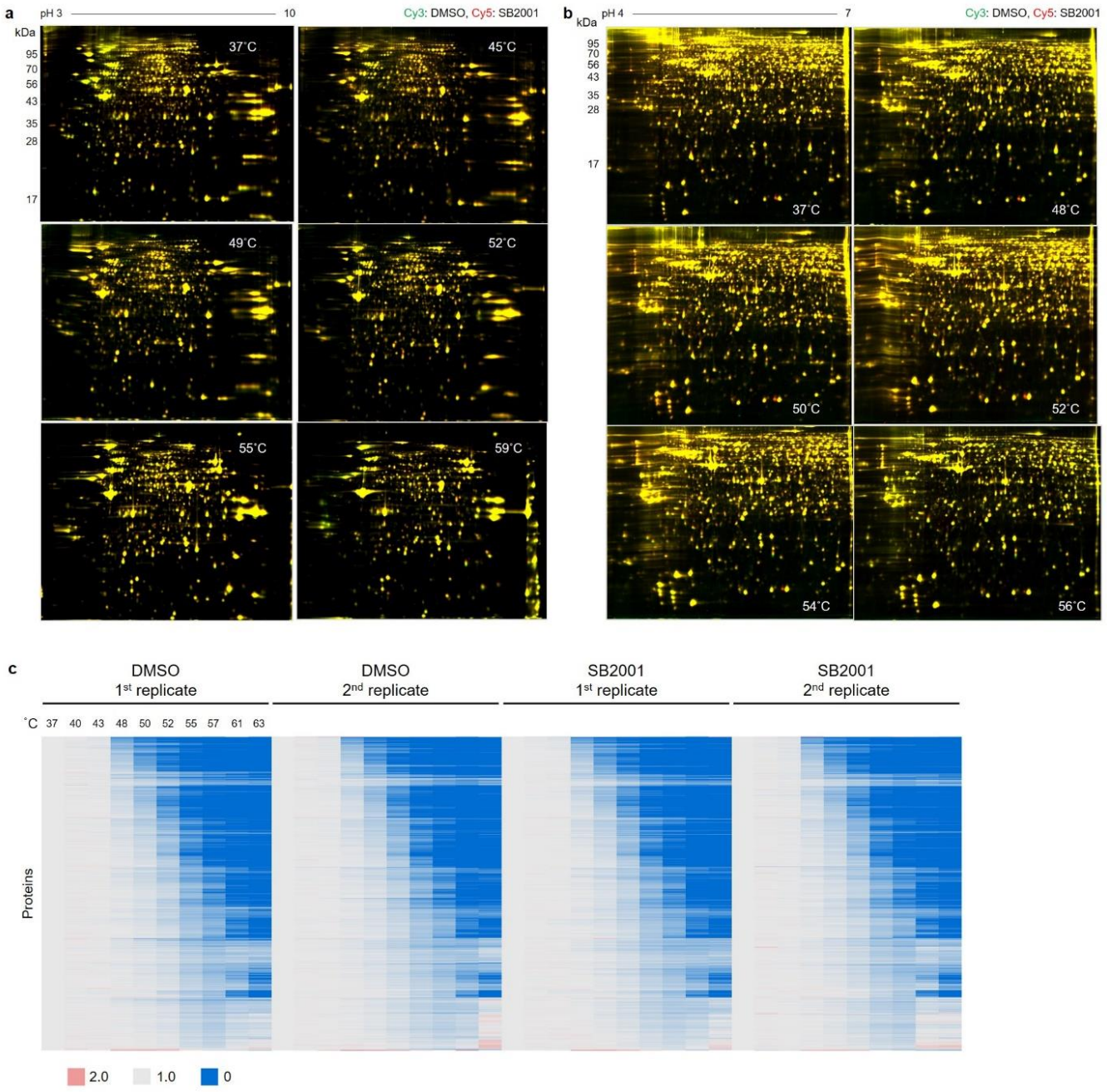

Fig. S1 Global melting proteomes (meltomes) upon treatment with DMSO and SB2001 (10 μM). (a) Whole gel images from TS-FITGE using strip gels with pH 3–10. Cy3 and Cy5 signals are overlaid. (b) Whole gel images from TS-FITGE using strip gels with pH 4–7. Cy3 and Cy5 signals are overlaid. (c) Heat maps representing the meltomes from TPP. For each protein, the concentration at each temperature was expressed as a fold-change relative to that of 37 °C. Hierarchical clustering was performed with Cluster 3.0[9] by complete linkage with Euclidean distance and visualized with TreeView.[10]

a

pH 3–10, 55 °C	$\log_2$(Cy5/Cy3)
Median	0.01
95-percentile	0.19
5-percentile	– 0.19
LTA4H	1.17
ALDH1B1	0.49
ECH1	0.42
ALDH2	0.31
PGAM1	– 0.19

b

pH 3–10, 59 °C	$\log_2$(Cy5/Cy3)
Median	0.01
95-percentile	0.32
5-percentile	– 0.34
SDHA	0.32

c

pH 4–7, 52 °C	$\log_2$(Cy5/Cy3)
Median	0.00
95-percentile	0.23
5-percentile	– 0.34
MTH1	0.70

d

	Δ T_m (°C) (SB2001-DMSO)	Δ T_m (°C) (SB2001-SB2024)
MTRR	3.34	2.37
DIS3	3.17	3.61
ERLIN2	*n/a*	7.85
ERLIN1	1.50	1.88
CASP7	1.11	*n/a*
MTH1	0.99	2.93
ECH1	0.90	0.72

Fig. S2 The extents of thermal stability shift of target candidates from TS-FITGE and TPP. (a) The difference in fluorescence signals between Cy5 (SB2001, 10 μM) and Cy3 (DMSO) at 55 °C from TS-FITGE with pH 3–10 strip gel. (b) The difference in fluorescence signals between Cy5 (SB2001) and Cy3 (DMSO) at 59 °C from TS-FITGE with pH 3–10 strip gel. (c) The difference in fluorescence signals between Cy5 (SB2001) and Cy3 (DMSO) at 52 °C from TS-FITGE with pH 4–7 strip gel. (d) The average of shift in melting temperature (T_m) comparing DMSO versus SB2001 (10 μM), and SB2024 (10 μM) versus SB2001 from TPP. *n/a* denotes that the protein was not detected.

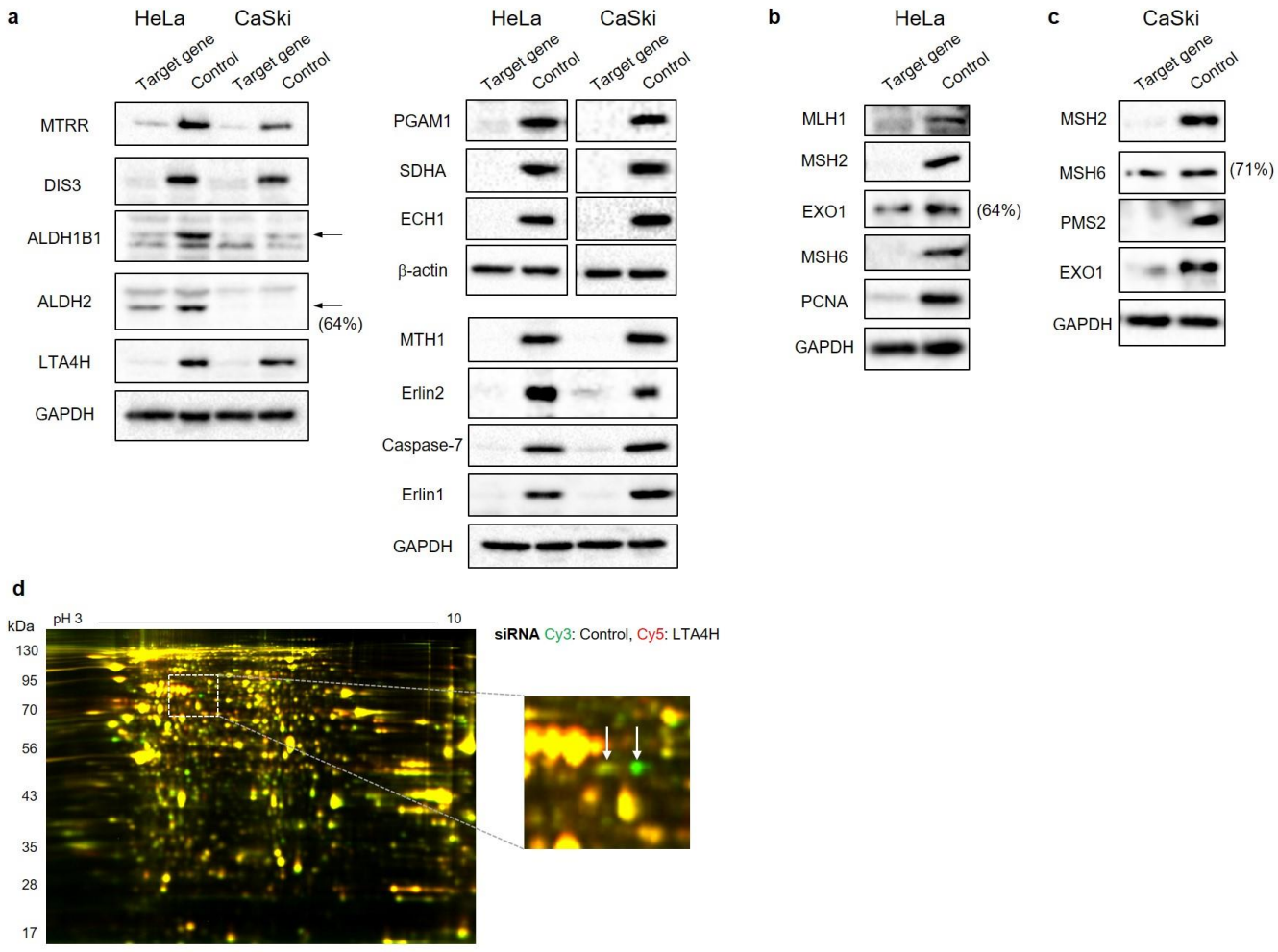

Fig. S3 Western blot to confirm the efficiency of gene knockdown by siRNA. (a) Knockdown of target candidates from TS-FITGE and TPP. (b) Knockdown of mismatch repair (MMR) genes in HeLa cells. (c) Knockdown of MMR genes in CaSki cells. (d) 2-D gel images after knockdown of LTA4H spots in HeLa cells. Images of Cy3 channel (control siRNA) and Cy5 channel (LTA4H siRNA) are overlaid. The area in the white box is magnified. LTA4H spots are pointed by arrows.

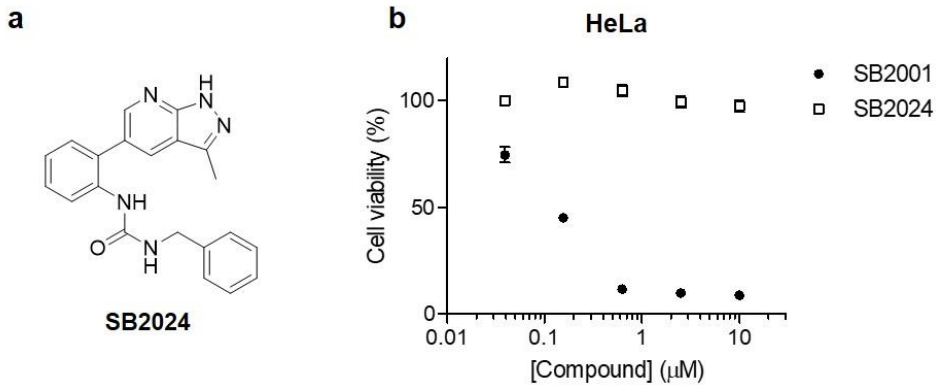

Fig. S4 SB2024, an inactive analogue of SB2001. (a) The chemical structure of SB2024. (b) Cell viability upon treatment with SB2001 or SB2004 for 2 d in HeLa cells. Data are presented as the mean ± SEM (*n* = 2).

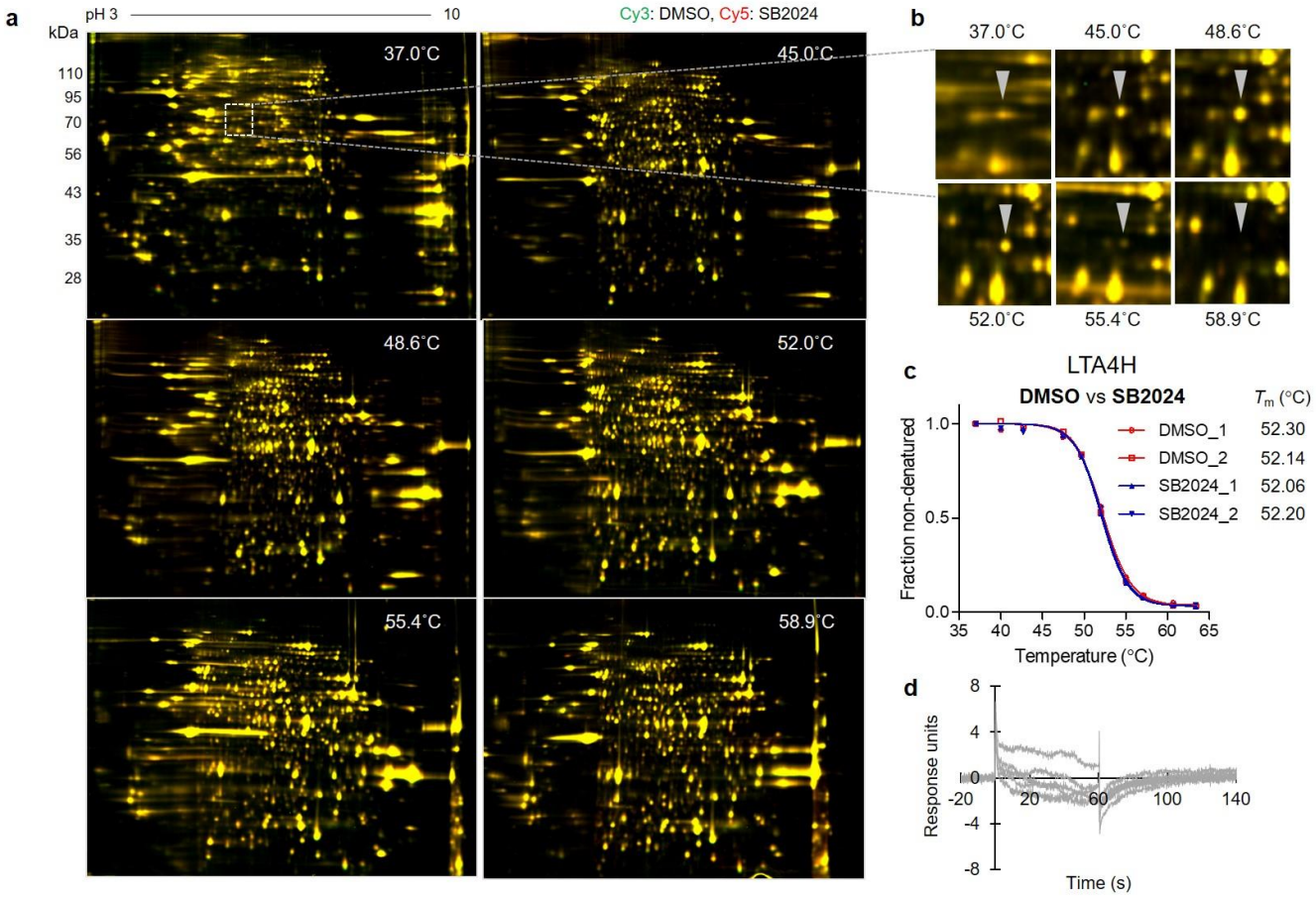

Fig. S5 Validation of the target candidate LTA4H of SB2001 using SB2024 (10 μM) as a negative control compound. (a) Whole gel images from TS-FITGE upon treatment with SB2024. (b) Magnified images of the white box area in (a) to visualize the LTA4H spot. (c) TPP melting curves of LTA4H comparing DMSO and SB2024. (d) Sensorgrams of surface plasmon resonance analysis showing the kinetics of SB2024 (0.625 to 10 μM) to immobilized LTA4H. Representative data from two independent experiments.

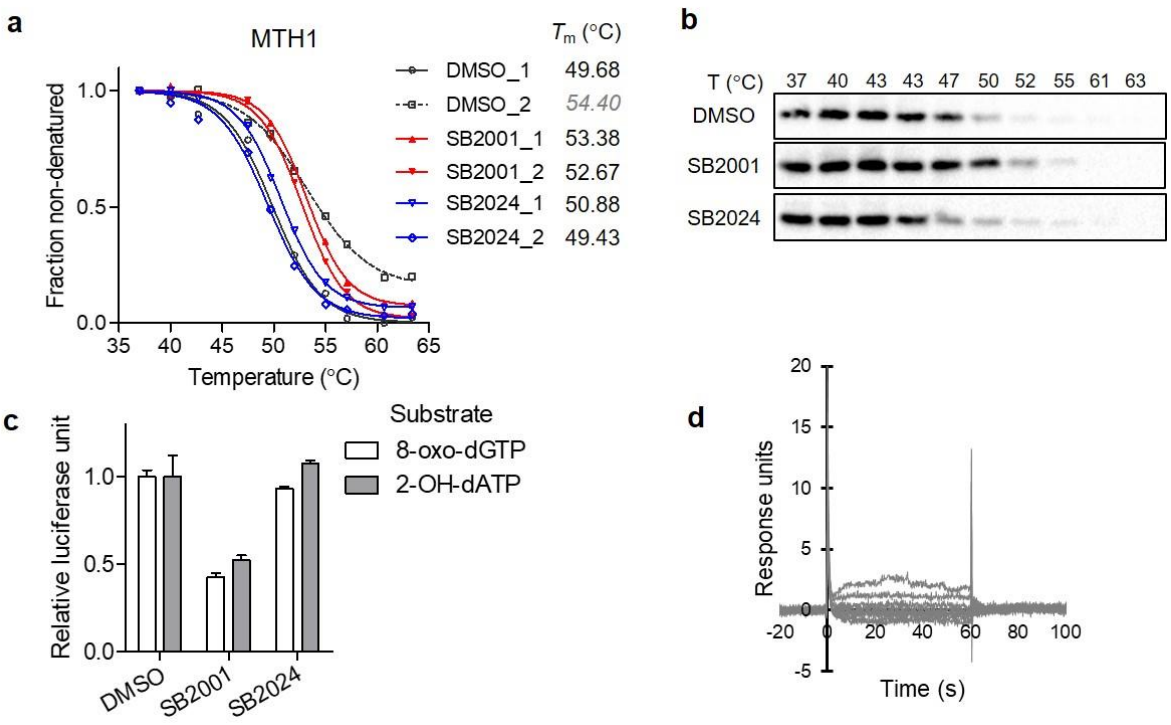

Fig. S6 Validation of the target candidate MTH1 of SB2001 using SB2024 as a negative control compound. (a) TPP melting curves of MTH1 comparing DMSO, SB2001, and SB2024. (b) CETSA with MTH1 antibody. (c) *In vitro* MTH1 enzymatic assay with 8-oxo-dGTP or 2-OH-dATP as a substrate. The concentrations of SB2001 and SB2024 were 10 μM. Data are presented as the mean ± SEM (n = 2). (d) Surface plasmon resonance analysis by injecting SB2024 (0.16 to 40 μM) to the immobilized MTH1. Represnetative data from two independent experiments.

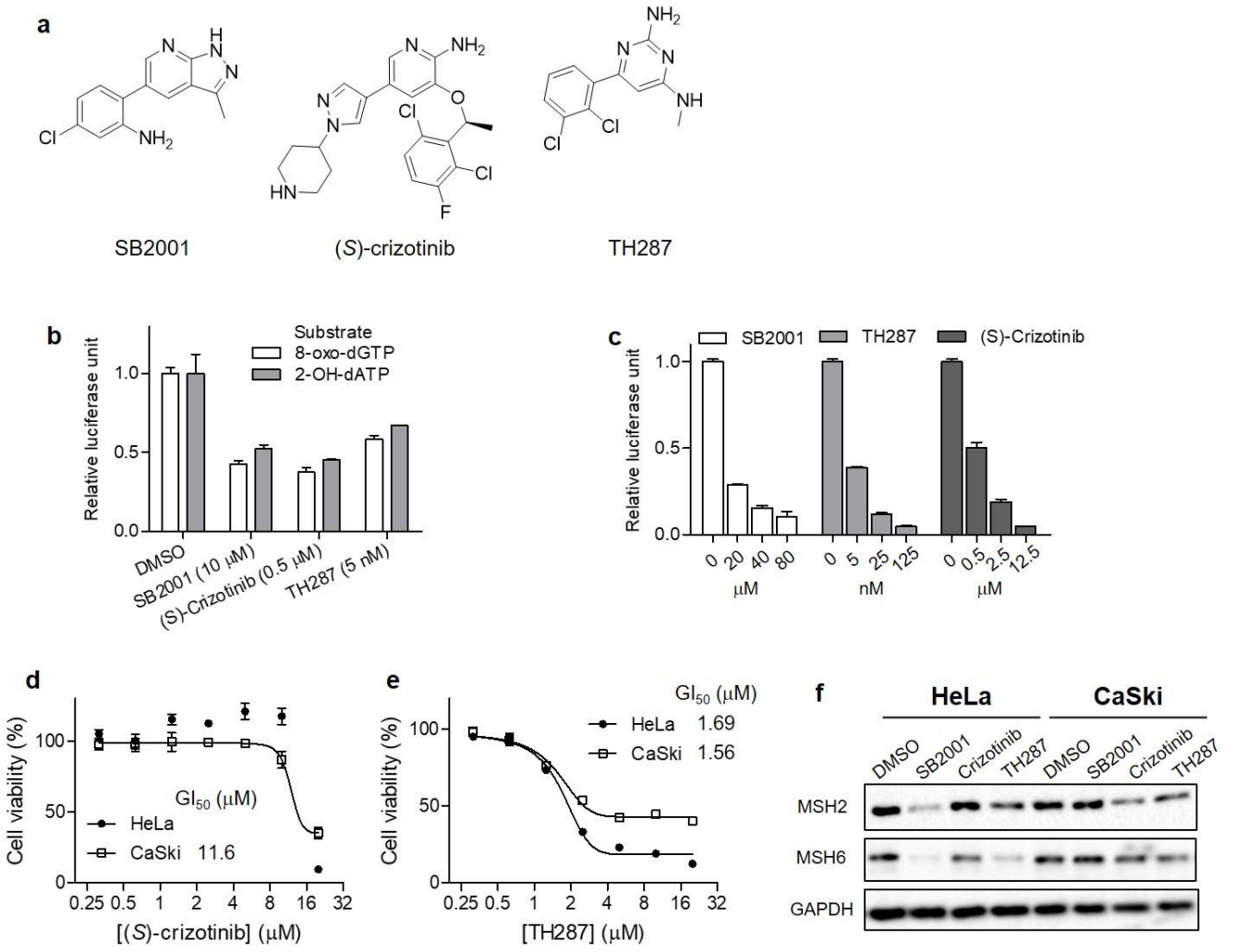

Fig. S7 Comparison of SB2001 to the typical MTH1 inhibitors. (a) The chemical structures of SB2001, (*S*)-crizotinib, and TH287. (b) *In vitro* MTH1 enzymatic assay with SB2001 (10 μM), (*S*)-crizotinib (0.5 μM), and TH287 (5 nM). Data are presented as the mean ± SEM (*n* = 2). (c) *In vitro* MTH1 enzymatic assay with 8-oxo-dGTP upon treatment of high concentration of the inhibitors. Data are presented as the mean ± SEM (*n* = 2). (d) Cell viability after treatment of (*S*)-crizotinib for 2 days in HeLa and CaSki cells. Data are presented as the mean ± SEM (*n* = 2). (e) Cell viability upon treatment with TH287 for 2 d in HeLa and CaSki cells. Data are presented as the mean ± SEM (*n* = 2). (f) Changes in expression levels of MSH2 and MSH6 upon treatment with the compounds for 2 d in HeLa and CaSki cells. The concentration of SB2001, (*S*)-crizotinib, and TH287 was 750 nM, 15 μM, and 3 μM, respectively.

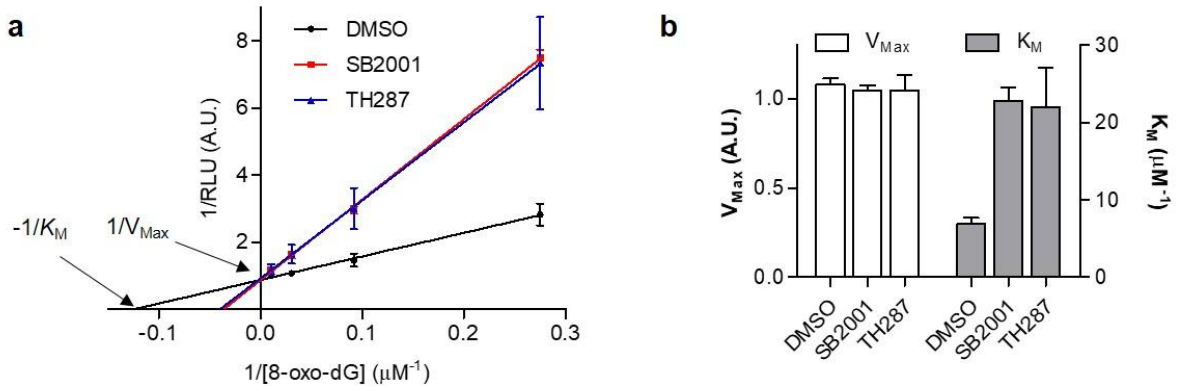

Fig. S8 Binding mode of SB2001 to MTH1 by an *in vitro* hydrolysis assay. (a) Lineweaver-Burk plot with various concentrations of 8-oxo-dGTP (0.135 to 98.2 μM) upon treatment of SB2001 (8.23 μM) or TH287 (3.29 nM). (b) Comparison of V_{Max} and K_M upon treatment of SB2001 and TH287. Data are presented as the mean ± SEM (n = 2).

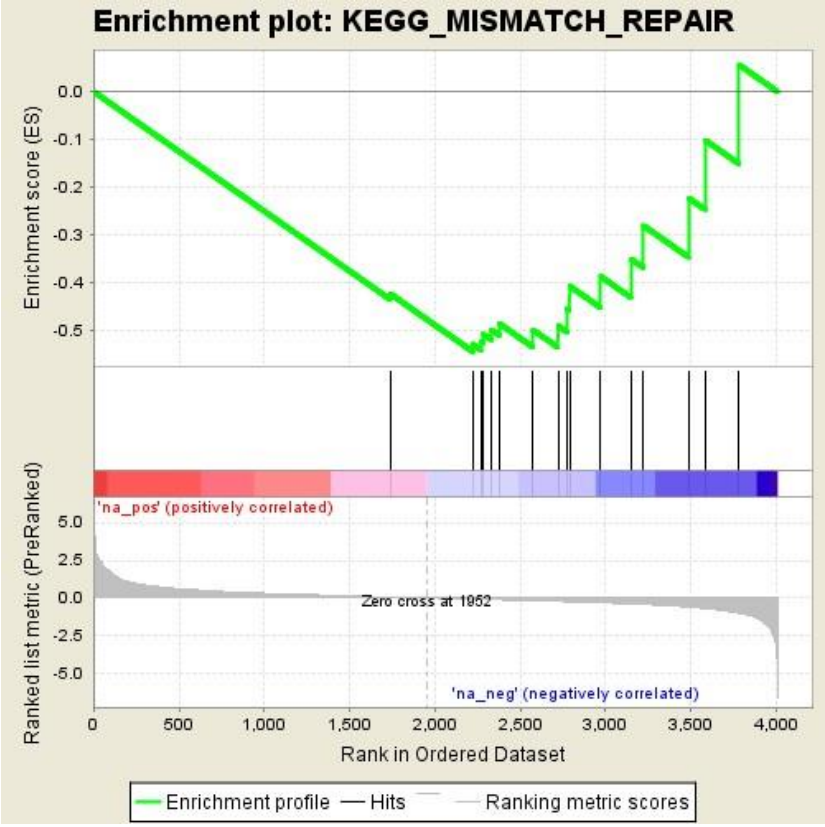

Fig. S9 Enrichment plot of DNA mismatch repair pathway from gene set enrichment analysis comparing the expression levels in HeLa and CaSki cells. Proteins are ordered as the protein with lower expression levels in HeLa than CaSki cells are in the right-hand side.

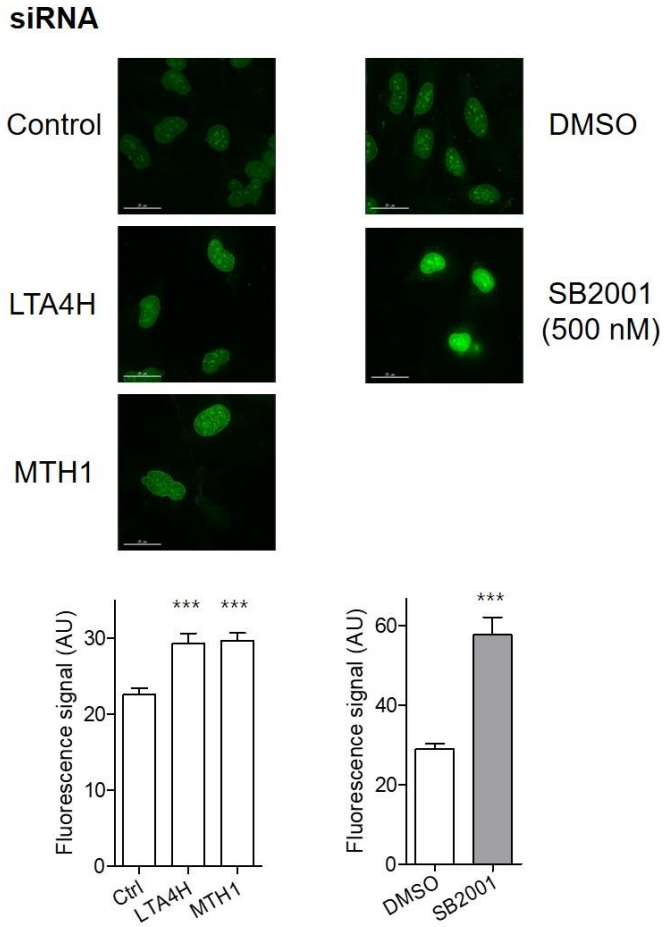

Fig. S10 Knockdown of either LTA4H or MTH1 increases oxidative DNA damage in HeLa cells. Fluorescence imaging of 8-oxoG was performed in HeLa cells. Fluorescence intensities in the nuclear region were quantified (n > 10). Representative data from two biological replicates. Data are presented as the mean ± SEM. P value was derived from a two-tailed Student's t-test. *** P < 0.001.

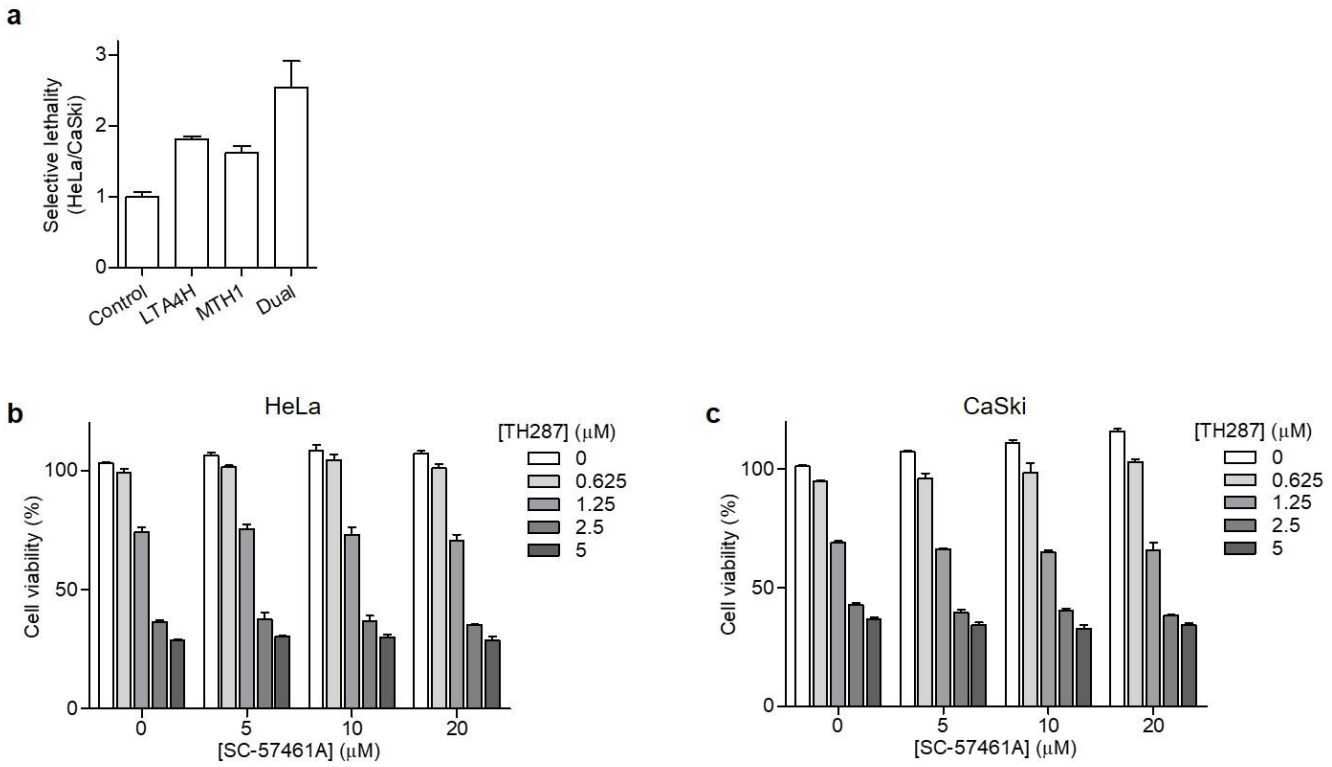

Fig. S11 Dual inhibition of LTA4H and MTH1. (a) Selective lethality between HeLa and CaSki cells after single or dual knockdown of LTA4H and MTH1. (b) Combination treatment of SC-57461A and TH287 in HeLa and CaSki cells for 2 d. Data are presented as the mean ± SEM (*n* = 2).

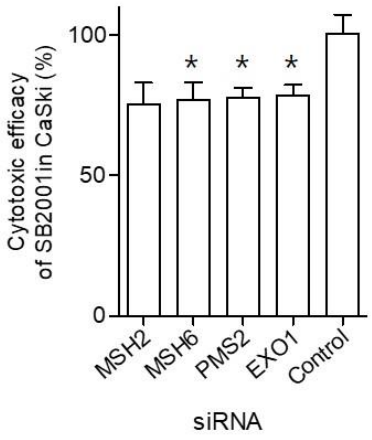

Fig. S12 Cytotoxic efficacy of SB2001 (40 μM) in CaSki cells after knockdown of mismatch repair gene by siRNA. Data are presented as the mean ± SEM (*n* = 2). *P* value was derived from a two-tailed Student's t-test. * *P* < 0.05.

REFERENCES

1. H. Park, J. Ha, J. Y. Koo, J. Park and S. B. Park, *Chem. Sci.*, 2017, **8**, 1127–1133.
2. H. Franken, T. Mathieson, D. Childs, G. M. A. Sweetman, T. Werner, I. Togel, C. Doce, S. Gade, M. Bantscheff, G. Drewes, F. B. M. Reinhard, W. Huber and M. M. Savitski, *Nat. Protocols*, 2015, **10**, 1567–1593.
3. D. Childs, N. Kurzawa, H. Franken, C. Doce, M. Savitski and W. Huber, *R package version 3.0.3.*, 2016.
4. A. Subramanian, P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, A. Paulovich, S. L. Pomeroy, T. R. Golub, E. S. Lander and J. P. Mesirov, *Proc. Natl. Acad. Sci. USA*, 2005, **102**, 15545–15550.
5. M. Kanehisa, M. Furumichi, M. Tanabe, Y. Sato and K. Morishima, *Nucleic Acids Res.*, 2017, **45**, D353–D361.
6. L. Struthers, R. Patel, J. Clark and S. Thomas, *Anal. Biochem.*, 1998, **255**, 20–31.
7. S. Nandhakumar, S. Parasuraman, M. Shanmugam, K. Rao, P. Chand and B. Bhat, *J. Pharmacol. Pharmacother.*, 2011, **2**, 107–111.
8. J. A. Vizcaíno, A. Csordas, N. del-Toro, J. A. Dianes, J. Griss, I. Lavidas, G. Mayer, Y. Perez-Riverol, F. Reisinger, T. Ternent, Q.-W. Xu, R. Wang and H. Hermjakob, *Nucleic Acids Res.*, 2016, **44**, D447–D456.
9. M. J. L. de Hoon, S. Imoto, J. Nolan and S. Miyano, *Bioinformatics*, 2004, **20**, 1453–1454.
10. R. D. M. Page, *Bioinformatics*, 1996, **12**, 357–358.