Online Methods

Primary assay: multiplexed NRF2 reporter gene and CellTiter-Fluor cell viability assays in A549 cells. Five μL of A549 NRF2-ARE-Fluc cells at 4 x 10^5 cell/mL in OPTI-MEM medium containing 5% FBS were dispensed into white solid 1536-well plates (Greiner BIo # 789173-F), and cultured at 37 °C, 95% humidity, and 5% CO_2 for 2 hours. 23 nL of compounds dissolved in DMSO at different concentrations were transferred to the assay plates using a Kalypsys 1536-pin tool, the final concentration of DMSO concentration was maintained at 0.46%. Control compounds. Budesonide, a glucocorticoid with NRF2 inhibitory activity[1, 2], and staurosporine, an apoptosis inducer, were both added at a concentration of 2 mM and budesonide was also added as a 1:10 titration, starting at 200 μM, to achieve dose-response. After an 18-24 hour incubation at 37 °C, 95% humidity, and 5% CO_2, 1 μL of 5x CellTiter-Fluor non-lytic cell viability assay reagent (Promega) was added into the each well of the plates. The plates were then incubated for 30 minutes at room temperature before they were read on ViewLux (Perkin Elmer) using an excitation wavelength of 405 nm and an emission wavelength of 525 nm. This assay detects cytotoxic compounds. Finally, 2.5 μL of luciferin-based detection reagent containing DTT, CoA, ATP (Sigma #D0632, C-3019, A-7699), and Luciferin (Biosynth AG, #L-8240) were added into each well, the plates were incubated for 15 minutes, then were read on ViewLux using luminescent mode.

Counter assay 1: Biochemical firefly luciferase assay

This assay was used to remove compounds that inhibit luciferase reporter enzyme. 3 μL of substrate solution containing 50 mM Tris acetate, 13.3 mM Mg-acetate, 0.01 mM ATP, 0.01% Tween, 0.05% BSA and 0.01 mM *D*-Luciferin (Sigma, #L9504) was dispensed into 1536 well white solid bottom assay plate (Greiner Bio, #789173-F), followed by 23 nL of hit compounds dissolved in DMSO at different concentrations using a Kalypsys 1536-pin tool. Then 1 μL of firefly luciferase reagent containing 50mM Tris-acetate and 0.04 μM *P. pyralis* luciferase (Sigma, #L9506) was added. The final DMSO concentration was maintained at 0.56%. After incubation at room temperature for 10 minutes, the plates were read by Viewlux (PerkinElmer) using luminescent mode.

Counter assay 2: Multiplexed CMV driven luciferase reporter gene and CellTiter-Fluor cell viability assays

This assay was used to remove general transcriptional modulators and general cytotoxic compounds. The assay procedure was very similar to the primary assay, except cell line and control compound were changed. 5 μL of HEK293-CMV-Fluc cells at 4 x 10^5 cell/mL in OPTI-MEM medium containing 5% FBS were dispensed into white solid 1536-well plates (Greiner BIo # 789173-F), and cultured at 37 °C, 95% humidity, and 5% CO_2 for 2 hours. 23 nL of hit compounds dissolved in DMSO at different concentrations were transferred to the assay plates using a Kalypsys 1536-pin tool, the final concentration of DMSO concentration was 0.46%. Control compound PTC124 was added to achieve a final concentration of 75 μM. After an 18-24 h incubation at 37 °C, 95% humidity, and 5% CO_2, 1 μL of 5x CellTiter-Fluor non-lytic cell viability assay reagent (Promega) was added into the each well of the plates. The plates were then incubated for 30 minutes at room temperature before they were read on ViewLux (Perkin Elmer) using an excitation wavelength of 405 nm and an emission wavelength of 525 nm. Finally, 2.5 μL of luciferin-based detection reagent containing DTT, CoA, ATP (Sigma #D0632, C-3019, A-7699), and Luciferin (Biosynth AG, #L-8240) were added into each well, the plates were incubated for 15 minutes, then were read on ViewLux using luminescent mode.

Counter assay 3: GR-beta lactamase reporter gene assay

The GR-bla HeLa cells were dispensed at 1500 cells/5 μL/well in 1536-well black wall/clear bottom plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at a 37 °C /5% CO_2 incubator for 4 hours, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a pintool station (Kalypsys, San Diego, CA), followed by addition of 1 μL of dexamethasone (5 nM, final concentration in the well). The plates were incubated at 37 °C for 18 hours. After 1 μL of LiveBLAzerTM B/G FRET substrate was added using a Flying Reagent Dispenser (Aurora Discovery, San Diego, CA), the plates were incubated at room temperature for 2 hours, and fluorescence intensity was measured by an Envision plate reader (PerkinElmer, Shelton, CT).

Confirmation assay 1: multiplexed NRF2 reporter gene and CellTiter-Fluor cell viability assays in H838 cells

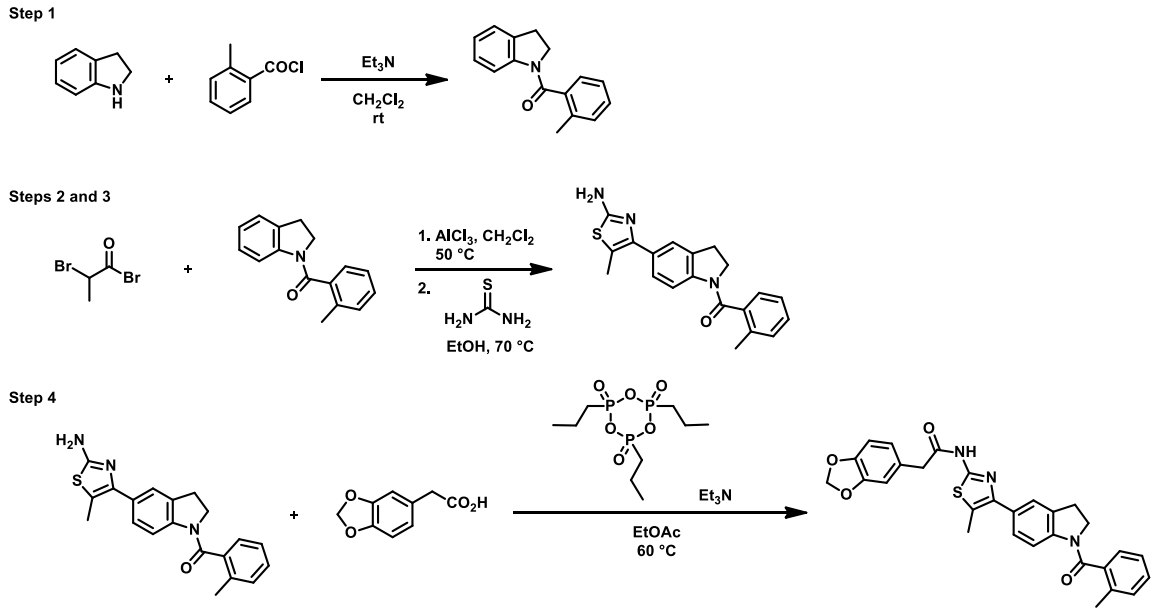
The assay is very similar to primary assay except that the cell line was changed. 5 μL of H838 NRF2-ARE-Fluc cells at 4 X 10^5 cell/mL in OPTI-MEM medium containing 5% FBS were dispensed into white solid 1536-well plates (Greiner BIo # 789173-F), and cultured at 37 °C, 95% humidity, and 5% CO_2 for 2 hours. 23 nL of compounds dissolved in DMSO at different concentrations were transferred to the assay plates using a Kalypsys 1536-pin tool, the final concentration of DMSO concentration was maintained at 0.46%. Control compounds budesonide and staurosporine were both added at a concentration of 2 mM and Budesonide was also added as a 1:10 titration, starting at 200 μM, to achieve dose-response. After an 18-24 hour incubation at 37 °C, 95% humidity, and 5% CO_2, 1 μL of 5x CellTiter-Fluor non-lytic cell viability assay reagent (Promega) was added into the each well of the plates. The plates were then incubated for 30 minutes at room temperature before they were read on ViewLux (Perkin Elmer) using an excitation wavelength of 405 nm and an emission wavelength of 525 nm. Finally, 2.5 μL of luciferin-based detection reagent containing DTT, CoA, ATP (Sigma #D0632, C-3019, A-7699), and Luciferin (Biosynth AG, #L-8240) were added into each well, the plates were incubated for 15 minutes, then were read on ViewLux using luminescent mode.

Confirmation assay 2: multiplexed NRF2 reporter gene and CellTiter-Fluor cell viability assays in H1437 cells

The assay is very similar to primary assay except that the cell line was changed. 5 μL of H1437 NRF2-ARE-Fluc cells at 4 X 10^5 cell/mL in OPTI-MEM medium containing 5% FBS were dispensed into white solid 1536-well plates (Greiner BIo # 789173-F), and cultured at 37 °C, 95% humidity, and 5% CO_2 for 2 hours. 23 nL of compounds dissolved in DMSO at different concentrations were transferred to the assay plates using a Kalypsys 1536-pin tool, the final concentration of DMSO concentration was maintained at 0.46%. Control compounds budesonide and staurosporine were both added at a concentration of 2 mM and budesonide was also added as a 1:10 titration, starting at 200 μM, to achieve dose-response. After an 18-24 hour incubation at 37 °C, 95% humidity, and 5% CO_2, 1 μL of 5x CellTiter-Fluor non-lytic cell viability assay reagent (Promega) was added into the each well of the plates. The plates were then incubated for 30 minutes at room temperature before they were read on ViewLux (Perkin Elmer) using an excitation wavelength of 405 nm and an emission wavelength of 525 nm. Finally, 2.5 μL of luciferin-based detection reagent containing DTT, CoA, ATP (Sigma

#D0632, C-3019, A-7699), and Luciferin (Biosynth AG, #L-8240) were added into each well, the plates were incubated for 15 minutes, then were read on ViewLux using luminescent mode.

Synthesis of ML385



Scheme 1. Synthetic route to **ML385**.

Preparation of 2-(benzo[d][1,3]dioxol-5-yl)-*N*-(5-methyl-4-(1-(2-methylbenzoyl)indolin-5-yl)thiazol-2-yl)acetamide (ML385):

Scheme 1, step 1: To a solution of indoline (4.91 mL, 43.8 mmol) and triethylamine (12.21 mL, 88 mmol) in dichloromethane (30 mL) was added 2-methylbenzoyl chloride (6 mL, 46.0 mmol). The reaction became very warm. The reaction mixture stirred at rt overnight. The reaction mixture was diluted with 0.5N NaOH and dichloromethane. The layers were separated and the aqueous layer was re-extracted with dichloromethane. The combined organic layers were dried with $MgSO_4$ and concentrated *in vacuo* to afford an oil. The residue was taken up in hexanes with a small amount of ethyl acetate. A precipitate formed. The precipitate was filtered and washed with hexanes. Indolin-1-yl(*o*-tolyl)methanone (9.17 g, 88%) was isolated as a tan solid and used without further purification; LCMS : *m/z* $(M+H)^+$ = 238.1.

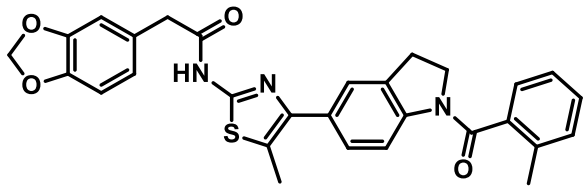
Scheme 1, step 2: To a solution of indolin-1-yl(*o*-tolyl)methanone (1.5 g, 6.32 mmol) in dichloromethane (20 mL) was added aluminum trichloride (2.53 g, 18.96 mmol) followed by 2-bromopropanoyl bromide (1.986 mL, 18.96 mmol). The resulting reaction mixture

was heated at 50 °C for 5 hr. The reaction mixture was cooled to rt and poured onto ice water. The resulting mixture was treated with a saturated aqueous solution of potassium sodium tartrate (Rochelle's salts) and stirred rapidly for 20 min. The mixture was neutralized with 0.5 N NaOH. The layers were separated and the aqueous layer was re-extracted with dichloromethane. The combined organic layers were dried with $MgSO_4$ and concentrated *in vacuo* to afford 2-bromo-1-(1-(2-methylbenzoyl)indolin-5-yl)propan-1-one as a black oil; LCMS : *m/z* $(M+H)^+$ = 372.0, 374.0. This material was used in the following step without purification.

Scheme 1, step 3: Thiourea (1.203 g, 15.80 mmol) was added to a solution of 2-bromo-1-(1-(2-methylbenzoyl)indolin-5-yl)propan-1-one (6.32 mmol) in ethanol (20 mL). The reaction mixture was heated at 70 °C for 16.5 hr. The reaction mixture was cooled to rt, diluted with water, and basified with ammonium hydroxide. The mixture was diluted with dichloromethane and extracted (2x). The combined organic layers were dried with $MgSO_4$ and concentrated *in vacuo* to afford (5-(2-amino-5-methylthiazol-4-yl)indolin-1-yl)(*o*-tolyl)methanone (2.2 g, quant) as an orange-brown foam; LCMS : *m/z* $(M+H)^+$ = 350.1. This was used in the following step without additional purification.

Scheme 1, step 4: To a solution of 2-(benzo[*d*][1,3]dioxol-5-yl)acetic acid (452 mg, 2.507 mmol), (5-(2-amino-5-methylthiazol-4-yl)indolin-1-yl)(*o*-tolyl)methanone (730 mg, 2.089 mmol), and triethylamine (1.456 mL, 10.45 mmol) in ethyl acetate (10 mL) was added 50 wt. % propylphosphonic anhydride solution in ethyl acetate (2.5 mL, 4.2 mmol). The mixture was stirred at rt for 5 min and then heated at 60 °C for 5 hr. The reaction mixture was cooled to rt and diluted with water, 0.5 N NaOH, and ethyl acetate. The layers were separated and the aqueous layer was reextracted with ethyl acetate. The combined organic layers were dried with $MgSO_4$, and concentrated *in vacuo* to afford a residue. The residue was taken up in dimethyl sulfoxide and subsequently purified by reverse phase chromatography utilizing a gradient of 10 to 100% acetonitrile/water to give **ML385** (496 mg, 46-60% over 3 steps).

Characterization of ML385



*Purity >95% as determined by LC/MS and ^{1}H NMR analyses.

2-(benzo[d][1,3]dioxol-5-yl)-*N*-(5-methyl-4-(1-(2-methylbenzoyl)indolin-5-yl)thiazol-2-yl)acetamide: LC-MS Retention Time: t_1 = 6.186 min; ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C) δ 11.95 (s, 1H), 8.14 (s, 1H), 7.51 (dq, *J* = 1.8, 0.9 Hz, 1H), 7.43 – 7.14 (m, 5H), 6.94 – 6.65 (m, 3H), 5.95 (s, 2H), 3.65 (s, 2H), 3.13 (t, *J* = 8.4 Hz, 2H), 3.07 (d, *J* = 0.9 Hz, 2H), 2.42 (s, 3H), 2.26 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6, 75 °C) δ 169.54, 168.40, 154.19, 147.73, 146.64, 144.50, 141.72, 137.88, 134.02, 133.37, 131.21, 130.86, 129.58, 129.06, 127.33, 126.45, 126.12, 125.11, 122.67, 120.72, 109.98, 109.97, 108.52, 101.27, 49.64, 41.82, 27.75, 18.88, 12.20; HRMS: *m/z* $(M+H)^+$ = 512.1628 (Calculated for $C_{29}H_{26}N_3O_4S$ = 512.1639).

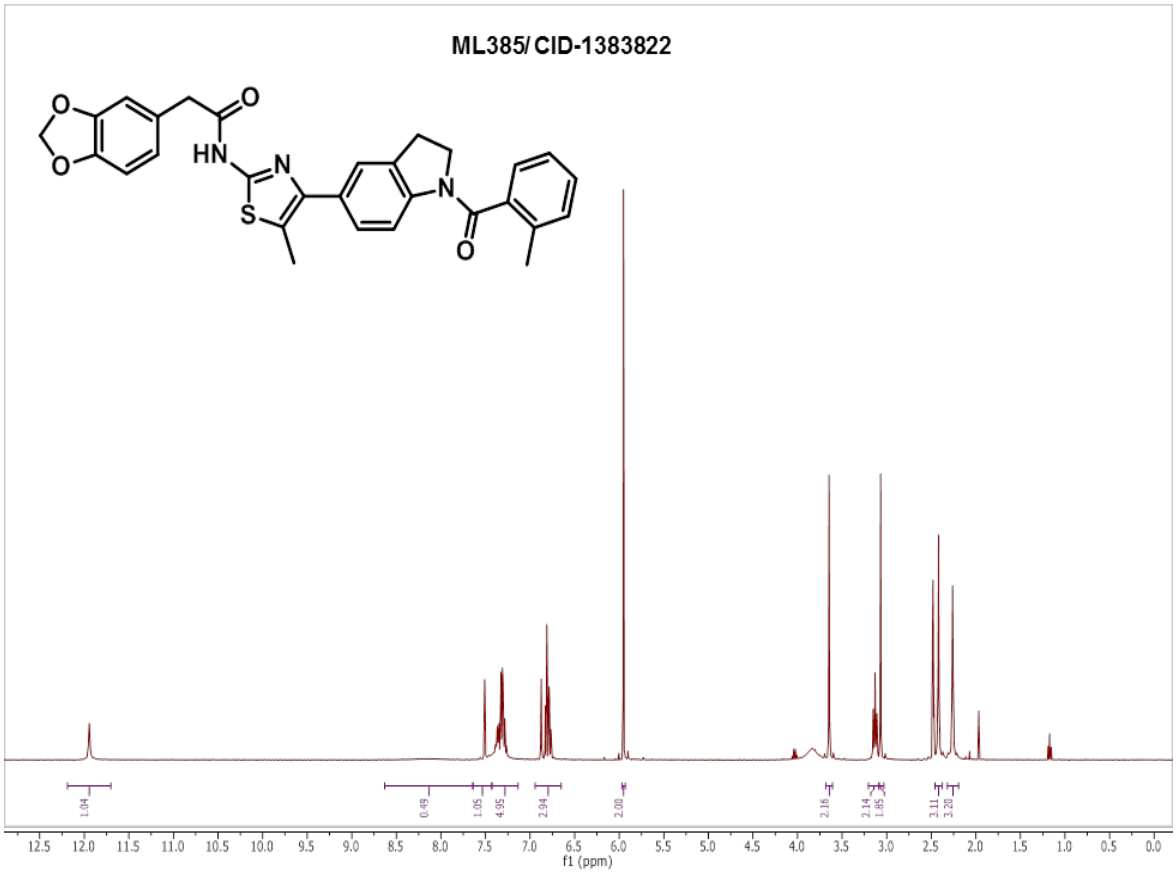


Figure 1. ^{1}H NMR spectrum of **ML385** at 75 °C in DMSO-d_6.

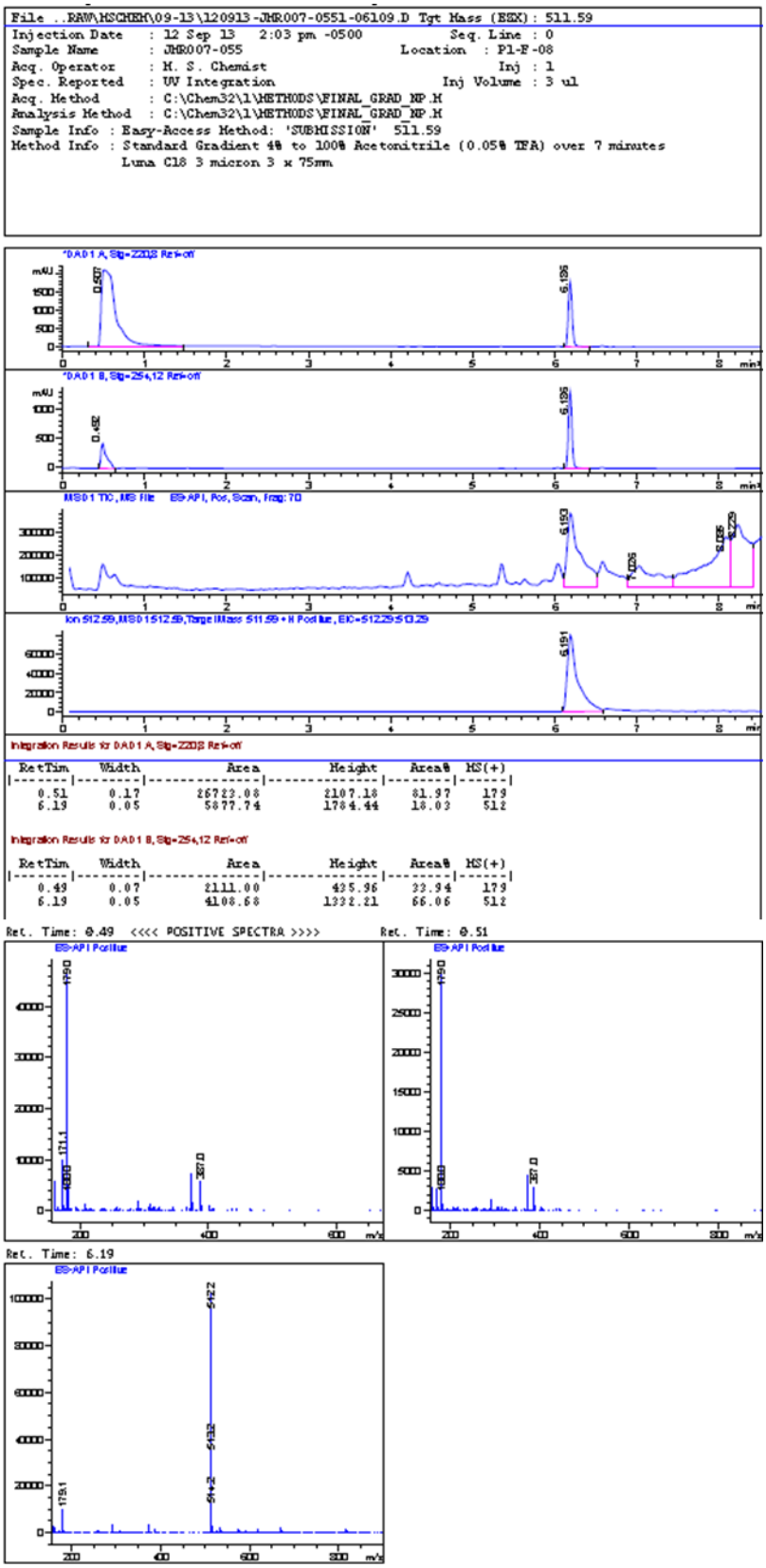
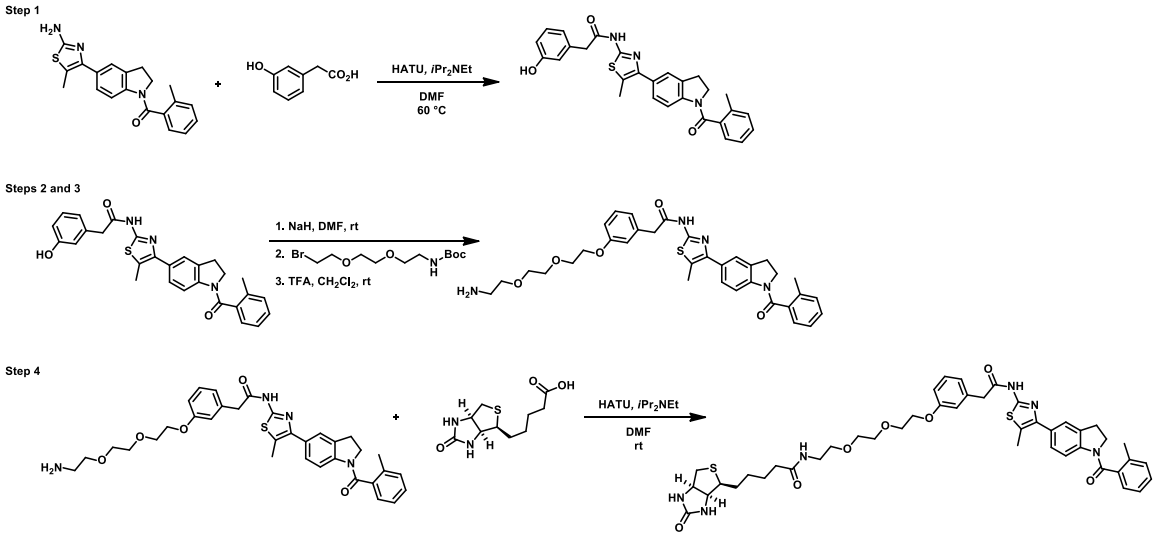


Figure 2. LC-MS purity analysis for **ML385**.

Synthesis of AB-ML385



Scheme 2. Synthetic route to **AB-ML385**.

Preparation of *N*-(2-(2-(2-(3-(2-((5-methyl-4-(1-(2-methylbenzoyl)indolin-5-yl)thiazol-2-yl)amino)-2-oxoethyl)phenoxy)ethoxy)ethoxy)ethyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (AB-ML385):

Scheme 2, step 1: To a solution of 2-(3-hydroxyphenyl)acetic acid (40 mg, 26.0 mmol), (*N*-[(Dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide) (HATU) (119 mg, 31.2 mmol), and *N,N*-diisopropylethylamine (136 μL, 78 mmol) in DMF (2.5 mL) was added (5-(2-amino-5-methylthiazol-4-yl)indolin-1-yl)(*o*-tolyl)methanone (100 mg, 28.6 mmol). The reaction mixture was heated at 60 °C overnight. Water (20 mL) was added to the mixture and a precipitate was formed. The solid was filtered and washed with water. The crude product was purified by flash silica gel chromatography (10 to 80% EtOAc/hexanes). 2-(3-hydroxyphenyl)-*N*-(5-methyl-4-(1-(2-methylbenzoyl)indolin-5-yl)thiazol-2-yl)acetamide (0.1 g, 79%) was isolated; LCMS : *m/z* $(M+H)^+$ = 484.1.

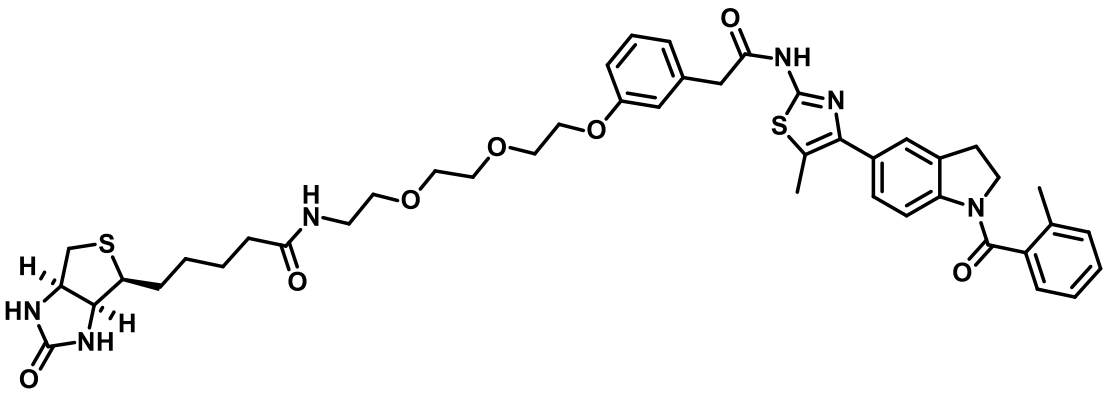
Scheme 2, step 2: To a solution of 2-(3-hydroxyphenyl)-*N*-(5-methyl-4-(1-(2-methylbenzoyl)indolin-5-yl)thiazol-2-yl)acetamide (0.1 g, 20.7 mmol) in DMF (2 mL) was added 60% sodium hydride in mineral oil (25 mg, 62 mmol). The reaction mixture evolved gas and stirred at rt for 30 min. Tert-butyl (2-(2-(2-bromoethoxy)ethoxy)ethyl)carbamate (71 mg, 22.7 mmol) was added to the reaction mixture. The resulting reaction mixture stirred overnight at rt. The reaction mixture was

quenched by the addition of water and extracted with ethyl acetate (2x). The combined organic layers were washed with sat. Na_2SO_4 (3x) and then with brine, dried with Na_2SO_4, and concentrated *in vacuo.* The residue was purified by flash silica gel chromatography (20 to 100% EtOAc/hexanes) to afford tert-butyl (2-(2-(2-(3-(2-((5-methyl-4-(1-(2-methylbenzoyl)indolin-5-yl)thiazol-2-yl)amino)-2-oxoethyl)phenoxy)ethoxy)ethoxy)ethyl)carbamate (0.1 g, 68%); LCMS : *m/z* $(M+H)^+$ = 715.2.

Scheme 2, step 3: To a solution of tert-butyl (2-(2-(2-(3-(2-((5-methyl-4-(1-(2-methylbenzoyl)indolin-5-yl)thiazol-2-yl)amino)-2-oxoethyl)phenoxy)ethoxy)ethoxy)ethyl)carbamate (0.12 g, 16.8 mmol) in dichloromethane (2 mL) was added trifluoroacetic acid (0.1 mL, 130 mmol). The reaction mixture stirred at rt for 3 hrs. The solvent was removed and the crude product (2-(3-hydroxyphenyl)-*N*-(5-methyl-4-(1-(2-methylbenzoyl)indolin-5-yl)thiazol-2-yl)acetamide) was used in the following step without purification; LCMS : *m/z* $(M+H)^+$ = 615.2.

Scheme 2, step 4: To a solution of 5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoic acid (31 mg, 12.6 mmol), HATU (53 mg, 13.8 mmol), and *N,N*-diisopropylethylamine (100 μL, 56.6 mmol) in DMF (1.4 mL) was added 2-(3-hydroxyphenyl)-*N*-(5-methyl-4-(1-(2-methylbenzoyl)indolin-5-yl)thiazol-2-yl)acetamide (85 mg, 13.8 mmol). The reaction mixture was stirred at rt for 3 hrs and subsequently purified by reverse phase chromatography utilizing a gradient of 6 to 100% acetonitrile/water (0.1% TFA) to provide *N*-(2-(2-(2-(3-(2-((5-methyl-4-(1-(2-methylbenzoyl)indolin-5-yl)thiazol-2-yl)amino)-2-oxoethyl)phenoxy)ethoxy)ethoxy)ethyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (**AB-ML385**) (73.3 mg, 61%).

Characterization of AB-ML385



*Purity >95% as determined by LC/MS and ^{1}H NMR analyses.

N-(2-(2-(2-(3-(2-((5-methyl-4-(1-(2-methylbenzoyl)indolin-5-yl)thiazol-2-yl)amino)-2-oxoethyl)phenoxy)ethoxy)ethoxy)ethyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide: LC-MS Retention Time: t_1 = 5.491 min; ^{1}H NMR (400 MHz, DMSO-d_6, 75 °C) δ 11.99 (s, 1H), 7.55 – 7.25 (br m, 6H), 7.22 (t, *J* = 7.9 Hz, 1H), 6.92 (d, *J* = 2.1 Hz, 1H), 6.89 (d, *J* = 7.6 Hz, 1H), 6.83 (dd, *J* = 8.2, 2.6 Hz, 1H), 6.11 (br s, 2H), 4.37 – 4.22 (m, 1H), 4.19 – 4.02 (m, 3H), 3.84 (br s, 3H), 3.74 (dd, *J* = 5.7, 4.0 Hz, 2H), 3.71 (s, 2H), 3.59 (dd, *J* = 6.1, 4.0 Hz, 2H), 3.53 (dd, *J* = 6.0, 3.7 Hz, 2H), 3.42 (t, *J* = 6.0 Hz, 2H), 3.23 – 3.16 (m, 2H), 3.13 (t, *J* = 8.6 Hz, 2H), 3.10 – 3.04 (m, 1H), 2.83 (d, *J* = 5.2 Hz, 1H), 2.59 (dd, *J* = 12.4, 1.1 Hz, 1H), 2.42 (s, 3H), 2.26 (s, 3H), 2.06 (t, *J* = 7.4 Hz, 2H), 1.63 (m, 1H), 1.57 – 1.38 (m, 3H), 1.38 – 1.21 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6, 75 °C) δ 172.56, 169.32, 163.04, 159.08, 154.19, 144.51, 137.88, 136.92, 134.03, 133.38, 131.23, 130.87, 129.79, 129.59, 127.34, 126.46, 126.13, 125.12, 121.99, 120.75, 116.34, 113.43, 70.44, 70.11, 69.73, 69.51, 67.85, 61.64, 59.85, 55.73, 49.75, 42.26, 40.18, 39.07, 35.67, 28.56, 28.53, 27.73, 25.64, 18.88, 12.2; HRMS:*m/z* $(M+H)^+$ = 841.3414 (Calculated for $C_{44}H_{53}N_6O_7S_2$ = 841.3412).

Determination of ML385 concentration in tumor samples: Pre-weighed frozen tumor samples were placed in 48 well plates and stored at -80 °C until the analysis. A UPLC-MS/MS method was developed to determine ML385 concentration in tumor samples. Mass spectrometric analysis was performed on a Waters Xevo TQ-S triple quadrupole instrument using electrospray ionization in positive mode with the selected reaction monitoring (SRM). The SRM for ML385 was 512.2/350.1 at the collision energy of 28 V. The separation was performed on an Acquity BEH C18 column (50 x 2.1 mm, 1.7 µ) using a Waters Acquity UPLC system with 0.6 mL/min flow rate. The column temperature was maintained at 60 °C. The mobile phase A was 0.1% formic acid in water and the mobile phase B was 0.1% formic acid in acetonitrile. The UPLC gradient method was 20% B (0-0.2 min), 20% B to 95% B (0.2-1.2 min), 95% B (1.2-1.8 min), and 20% B (1.8-2.0 min). The total run time was 2.5 minute. Tumor samples were

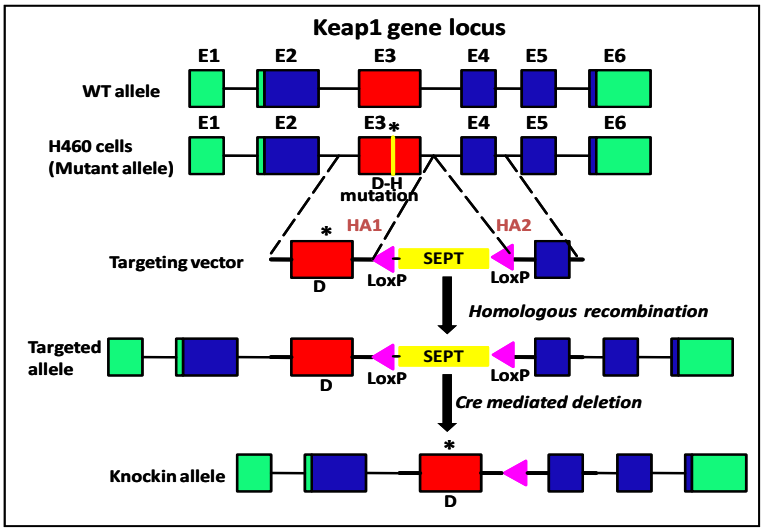
homogenized with 4 volumes of water. The calibration standards (1.0-1000 ng/mL) were prepared in the control blank tumor homogenate. Fifty-microliter tumor homogenate was mixed with 200 μL internal standard in acetonitrile to precipitate proteins in a 96-well plate and 1.0 μL supernatant was injected for the UPLC-MS/MS analysis.

Establishment of tumor xenografts and treatment: A549 cells (5×10^6) and H460 cells (1.5×10^6) were injected subcutaneously into the flank of athymic nude mice and tumor dimensions were measured by caliper at an interval of 3-5 days[3]. The tumor volumes were calculated using the following formula: [length (mm) × width (mm) × width (mm) × 0.52]. Once the tumor volumes were approximately 50-100mm^3, mice were randomly allocated into 4 groups: vehicle, ML385, carboplatin, ML385+carboplatin. Vehicle, carboplatin (5 mg/kg daily Monday to Friday)[3], ML385 (30 mg/kg daily Monday to Friday) and a combination of ML385 and carboplatin were administered intraperitoneally for three weeks. At the end of treatment period, mice were sacrificed and tumor, blood, lung and liver samples were collected.

For orthotopic lung tumor model, A549 (1×10^6) and H460 cells (1×10^6) were dilute 1:1 in matrigel (30μl) injected directly into the lungs. Ten days post cell implantation, mice were imaged. Mice with visible localized lung tumor were randomly divided into 4 groups: vehicle, ML385, carboplatin, ML385+carboplatin. Vehicle, carboplatin (5 mg/kg daily Monday to Friday), ML385 (30 mg/kg daily Monday to Friday) and a combination of ML385 and carboplatin were administered intraperitoneally for two weeks. High resolution lung CT images were acquired in 512 projections (270 μA, 75 kVp) and data were reconstructed using the ordered subsets-expectation maximization algorithm. Volume rendered whole lung images were generated using Amira 5.3.0 software (Visage Imaging Inc., Carlsbad, CA). For each mouse, pretreatment available lung volume was defined as 100% and compared with post-treatment lung volumes. All experimental protocols conducted on the mice were performed in accordance with NIH guidelines and were approved by the Johns Hopkins University Animal Care and Use Committee.

Generation of somatic Keap1 knock-in in lung cancer cell (H460) with complete loss of Keap1 activity: Somatic knock-in of wild-type Keap1 in H460 cells created using the protocol described by Rago et al (2007)[4].

H460 cell line is a hypotriploid cell line with the modal chromosome number as 57. These cells harbor a G-C point mutation in Keap1 gene that results in aspartic acid to histidine substitution at 236 amino acid position and complete loss of wild type Keap1 activity. We designed the targeting construct to revert the histidine substitution back to aspartic acid found in wild-type Keap1 allele. For construction of targeting vector, the two homology arms (HA1 and HA2) were amplified from genomic DNA (H460 cell line DNA) using specific primers and high fidelity Taq polymerase. The PCR primers for HA1 had NotI and Xba1 restriction sites in their forward and reverse primers, respectively. Similarly, the PCR primers for HA2 arm had SalI and NotI restriction sites in their forward and reverse primers, respectively.



HA1-Forward primer- AAAAAAGCGGCCGC CATCACAATGTACGCGGTTCCT
HA1-Reverse primer- AAAAAATCTAGA CCCAGGCTAGTCTCCTGGACTC
HA2-Forward primer: AAAAAAGTCGAC TAGAGTAAGACCCTGTCTCTCTTGC
HA2- Reverse primer: AAAAAAGCGGCCGC TCCTGGGTTCAAGCAGTTATTCAG

The PCR product was gel purified and cloned in pCR-TOPO vector (Invitrogen) as per the manufacturer's instructions. To knock-in the wild type aspartic acid allele into the Keap1 intervening region (IVR) domain, we introduced a single base substitution using site directed mutagenesis kit from Stratagene. The homology arms were digested with the appropriate pair of restriction enzymes.

Preparation of AAV shuttle vector and SEPT cassette: AAV shuttle plasmid (pAAV-MCS), the trans-elements required for packaging of virus (pAAV-RC and pHelper) and SEPT vector was kindly provided by Dr. Fred Bunz (Johns Hopkins University). The SEPT cassette along with the two homology arms were ligated into AAV shuttle plasmid and transformed. The recombinants were screened by restriction digestion and further

verified by sequencing. The introduction of single base substitution in the HA1 was confirmed by sequencing before proceeding further

Production of AAV virus and infection of H460 cells: The recombinant AAV plasmid containing homology arms and SEPT element and the plasmids that contain the trans-elements required for packaging (pAAV-RC and pHelper) were mixed in 1:1:1 ratio and transfected into AAV 293 cells using Lipofectamine 2000 to produce virus. For infecting H460 cells with AAV virus, cells were plated at 50-70% confluency and infected with virus directly. Forty-eight hours after infection, cells were trypsinized and distributed to twenty 96-well plates in medium containing geniticin at a concentration 750 µg/ml. The wells containing single colonies were identified and the clones were expanded.

PCR screen for homologous recombinant clones and excision of neo cassette: For screening the recombinant clones, we used forward primer that anneals in the SEPT cassette and the reverse primer that is outside the HA2. Primers that span the upstream HA1 were used to confirm the positive clones. An aliquot of the positive clones were frozen and the remaining cells were expanded. The clones were seeded at 30 to 50% confluency in 25 cm2 flask and infected with adenovirus-Cre for 24 hrs. After infection, cells were washed, trypsinized, and plated at very low density in a series of 96-well plates. Individual colonies were expanded and screened. Clones that had underdone SEPT excision regained sensitivity to Geniticin. The SEPT excision was further verified by PCR based screening. Knockin of the WT-Keap1 allele was confirmed by sequencing. To target the 2nd allele, same protocol of AAV virus infection, selection and screening was carried out using the somatic knockin clone heterozygous for WT-KEAP1 allele.

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