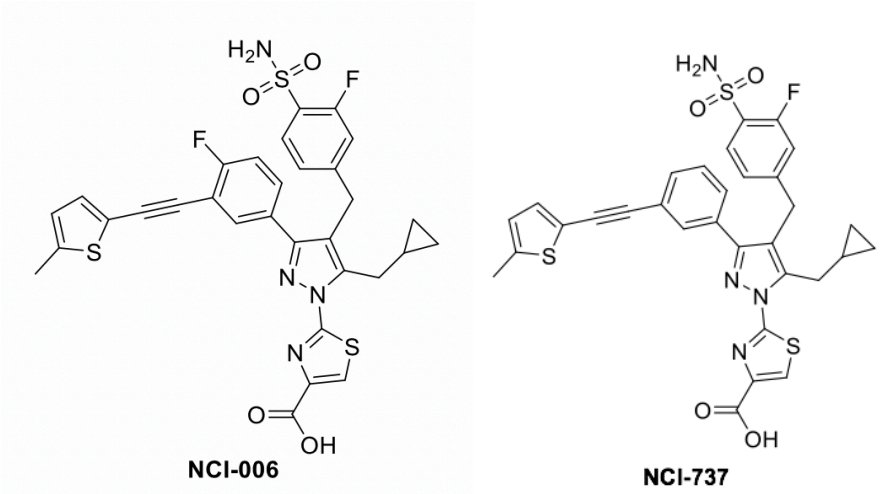
Supplemental Methods

Compound Synthesis: The LDH inhibitors used in these studies are non-competitive inhibitors, equipotent for LDHA and LDHB, and were discovered by a consortium sponsored by the National Cancer Institute (NCI) Experimental Therapeutics Program (NExT) that included NCI, the University of Pennsylvania, National Center for Advancing Translational Sciences (NCATS), Vanderbilt University, the University of New Mexico and the University of Alabama at Birmingham.

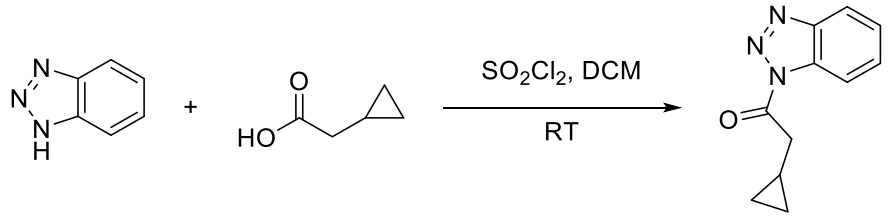
Early stage series development has been previously described (1). Structure-based design from an initial, weak HTS hit, combined with published information produced a submicromolar lead. Further optimizations focused on pyrazole substitutions, fluorinations (particularly on the phenyl sulfonamide ring), and improvements in the lipophilic phenyl/alkynyl/biphenyl area. The two lead compounds are of the same scaffold and differ by the presence of a single fluorine.



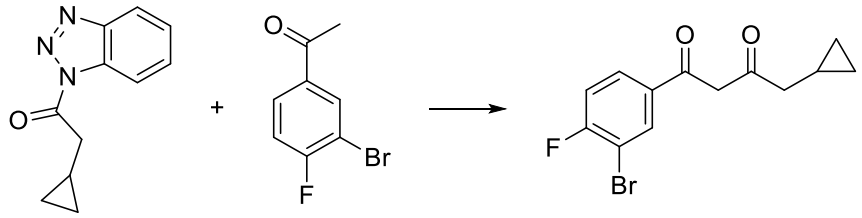
Chemical synthesis of NCI-006 and NCI-737 and determination of product purity are shown below.

Synthesis of NCGC00390006 (hereafter referred to as NCI-006)

The IUPAC name of NCI-006 is 2-(5-(cyclopropylmethyl)-3-(4-fluoro-3-((5-11 methylthiophen-2-yl)ethynyl)phenyl)-4-(3-fluoro-4-sulfamoylbenzyl)-1H-pyrazol-1-yl)thiazole-4-12 carboxylic acid, and its chemical formula is $C_{31}H_{24}F_2N_4O_4S_3$ (molecular weight = 650.7).

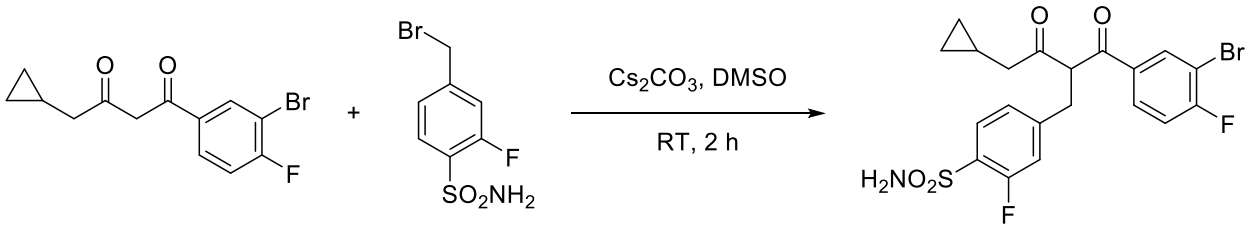


Synthesis of 1-(1H-benzo[d][1,2,3]triazol-1-yl)-2-cyclopropylethan-1-one: This compound was prepared as described in our previous paper (1). To the solution of 1H-benzo[d][1,2,3]triazole (476 g, 3995 mmol, 4 eq) in DCM (600 mL) was added thionyl chloride (72.9 ml, 999 mmol, 1eq) and stirred at RT for 0.5 h, then carefully added 2-cyclopropylacetic acid (93 ml, 999 mmol, 1 eq) upon cooling in an ice water bath (for larger scale cooling necessary due to exothermic reaction, if the reaction mixture forms thick precipitate and is difficult to stir then added more DCM) and stirred for 6 h. The reaction was filtered, and the filter cake was washed with DCM. The filtrate was added to bicarbonate solution slowly and stirred for 30 minutes then transferred to a separatory funnel. The organic layer was subsequently washed with bicarbonate solution and brine solution. The organic layer was dried under sodium sulfate and concentrated to get a thick oil. The crude product was purified on a CombiFlash system using a 340 g silica column eluting with 0-20 % EA in hexanes over 10 column volumes (divided into six batches due to large quantity). The first peak was collected, concentrated and dried to get oil which eventually solidifies into white solid (Yield 92 %). LC-MS Retention Time: = 3.51 min. $(M+H)^+ = 202$ (Standard Gradient 4% to 100% Acetonitrile 0.05% TFA over 3 minutes; Luna C18 3 micron 3 x 75mm).

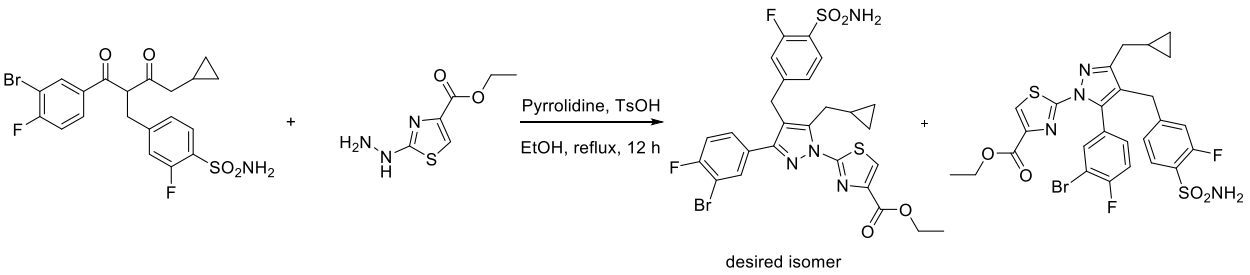


Synthesis of 1-(3-bromo-4-fluorophenyl)-4-cyclopropylbutane-1,3-dione: This compound was prepared as described in our previous paper (1). 3'-Bromo-4-fluoroacetophenone (754 mmol, 1eq) and 1-(1H-benzo[d][1,2,3]triazol-1-yl)-2-cyclopropylethan-1-one (167 g, 829 mmol, 1.2 or 1.5 eq) was charged 1000 mL DCM then added magnesium bromide diethyl etherate (487 g, 1884 mmol, 2.5 eq) in one portion in a 4 necked flask

set up with overhead stirrer. The reaction was cooled in an ice bath then added hunig's base (395 ml, 2261 mmol, and 3 eq) drop wise over 15 minutes through a dropping funnel. The reaction was stirred overnight. The reaction was placed in an ice bath then added ice cubes slowly while stirring during which heat generated (caution for exothermic reaction and slow addition is essential). The addition of ice continued until no more exothermic reaction then added 1 molar HCl dropwise under ice cooling added few ml of 6 molar HCl to acidify then extracted with DCM, the org layer was washed with brine. The org layer was dried with magnesium sulfate and concentrated. The crude product was purified on a flash system using 340 g Biotage columns eluting with gradient elution 0-30 % ethyl acetate in hexanes over 20 column volumes (divided into 8 columns due to large quantity) to get yellow liquid as a first peak. LC-MS Retention Time = 3.9 min; $(M+H)^+ = 300$ (usually as in keto enol form another peak around 3.5 min); (Standard Gradient 4% to 100% Acetonitrile 0.05% TFA over 3 minutes; Luna C18 3 micron 3 x 75mm).

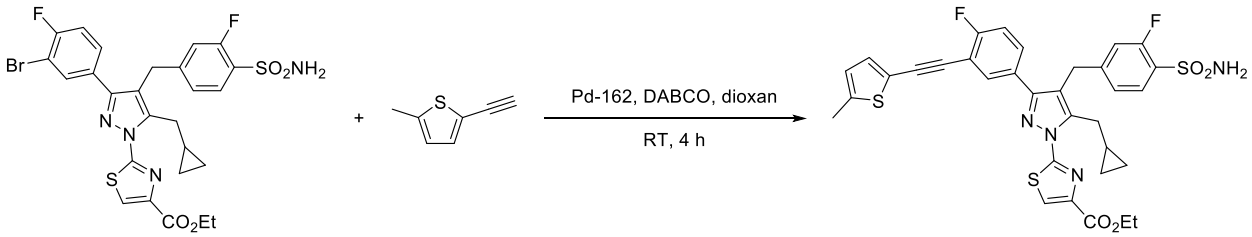


Synthesis of 4-(2-(3-bromobenzoyl)-4-cyclopropyl-3-oxobutyl)-2-fluorobenzenesulfonamide*:* A mixture of 1-(3-bromo-4-fluorophenyl)-4-cyclopropylbutane-1,3-dione (23.68 g, 79 mmol) in DMSO (100 mL) was added Cs_2CO_3 (35.2 g, 108 mmol) and stirred for 5 minutes. Added 4-(bromomethyl)-2-fluorobenzenesulfonamide (19.29 g, 72.0 mmol) portion wise upon cooling in ice water and stirred at RT for 1 h. The reaction was diluted with ethyl acetate and filtered through celite. The filtrate was washed with 1 molar HCl, and saturated ammonium chloride 2 times. The organic layer was dried over $MgSO_4$ and concentrated. The crude product was purified on isco flash system using a 220 g silica normal column eluting with 20-50 % EA in hexanes over 20 column volumes. The last peak was pooled to get a white solid. Yield 26.1 g (74.6%). LC-MS Retention Time: = 3.36 min $(M+H)^+ = 488$. (Standard Gradient 4% to 100% Acetonitrile (0.05% TFA) over 3 minutes; Luna C18 3 micron 3 x 75mm).



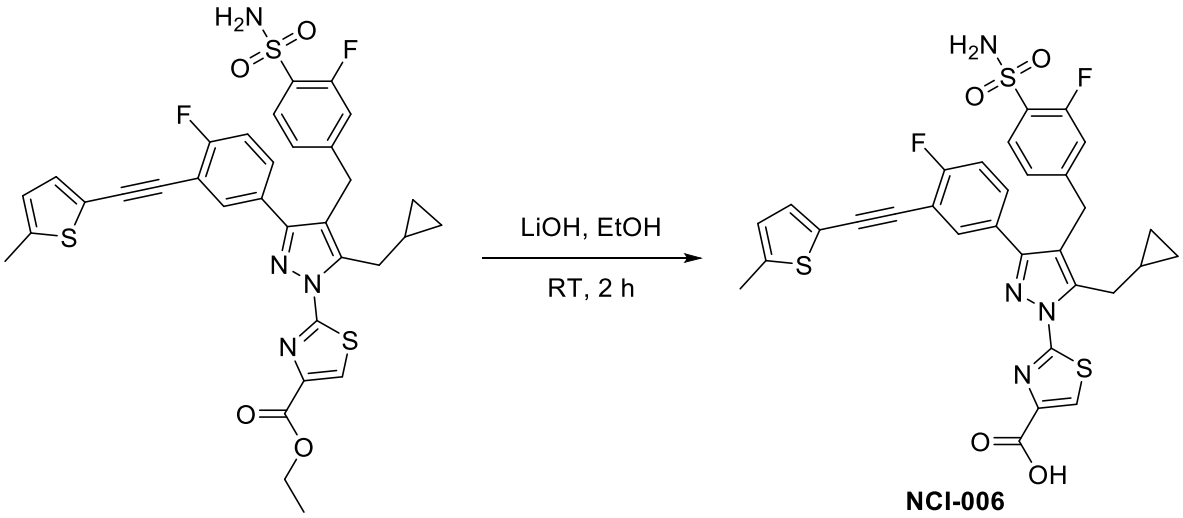
Synthesis of ethyl 2-(3-(3-bromo-4-fluorophenyl)-5-(cyclopropylmethyl)-4-(3-fluoro-4-sulfamoylbenzyl)-1H-pyrazol-1-yl)thiazole-4-carboxylate*:* A mixture of 4-(2-(3-bromo-4-fluorobenzoyl)-4-cyclopropyl-3-oxobutyl)-2-

fluorobenzenesulfonamide (25 g, 51.4 mmol, 1 eq) and tosic acid (4.89 g, 25.7 mmol, 0.5 eq) in ethanol (200 mL) was added pyrrolidine (2.126 ml, 25.7 mmol, 0.5 eq) and refluxed for 1 h. Cooled and then added ethyl 2-hydrazinylthiazole-4-carboxylate (12.51 g, 66.8 mmol) and refluxed overnight. The reaction was concentrated, and the residue was taken in DCM and immediately loaded to a silica loading cartridge. The compound was purified on an isco flash system using 330 g gold column eluting with 20-40 % ethyl acetate in hexanes over 20 column volumes. The pure product containing a mixture of 2 regioisomers were further separated on a reverse phase isco using a 415 g gold column eluting with 60-100 % ACN (0.1 TFA) in water over column volumes. The 2nd peak was pooled and concentrated the solid was stirred with a clear solution of bicarbonate. The precipitate formed was collected by filtration. The filter cake was thoroughly washed with water and air dried and finally in a vacuum desiccator under P_2O_5 to get pure white solid. Yield 11.2 g (34.2 %). LC-MS Retention Time = 6.924 min $(M+H)^+$ = 639. (Long Gradient 4% to 100% Acetonitrile (0.05% TFA) over 7 minutes, Agilent Eclipse XDB-C18 3 micron 3 x 75mm).



Synthesis of Ethyl 2-(5-(cyclopropylmethyl)-3-(4-fluoro-3-((5-methylthiophen-2-yl)ethynyl)phenyl)-4-(3-fluoro-4-sulfamoylbenzyl)-1H-pyrazol-1-yl)thiazole-4-carboxylate*:* A mixture of ethyl 2-(3-(3-bromo-4-fluorophenyl)-5-(cyclopropylmethyl)-4-(3-fluoro-4-sulfamoylbenzyl)-1H-pyrazol-1-yl)thiazole-4-carboxylate (9 g, 14.12 mmol), 2-ethynyl-5-methylthiophene (2.242 g, 18.35 mmol), [P(tBu)3] Pd(crotyl)Cl (http://jmcct.com/products-services/product_p429.html) (cat # Pd-162) (0.141 g, 0.353 mmol) and DABCO (3.17 g, 28.2 mmol) in in dioxan (30 mL) was stirred at rt for 4 h. After completion of the reaction, Pd scavenger silia DMT was added and stirred for 2 h at RT. The reaction mixture was diluted with ethyl acetate and filtered through a plug of silica. The filtrate was concentrated and purified on an isco flash system using a 330 g gold column eluting with 15-40 % ethyl acetate in hexanes over 20 column volumes. The product had some yellow color (pure by LC) which is further purified in isco flash reverse phase using a 415 g gold column eluting with 60-100 % ACN (0.1 % TFA) in water (0.1 % TFA) over 25 column volumes (elutes with around 80 % ACN). The fractions pooled and concentrated then neutralized with bicarbonate solution. The precipitate was collected by filtration and washed with water then air dried followed by vacuum drying under P_2O_5 to get white solid. Yield 8.65 g (90 %). LC-MS Retention Time = 7.111 min $(M+H)^+$ = 679. (Long Gradient 4% to 100% Acetonitrile (0.05% TFA) over 7 minutes, Agilent Eclipse XDB-C18 3 micron 3 x 75mm).^{1}H NMR (400 MHz, DMSO-d_6) δ 8.38 (s, 1H), 7.74 (dd, J = 6.9, 2.3 Hz, 1H), 7.70 – 7.58 (m, 2H), 7.57 (s, 2H), 7.37 (t, J = 9.0 Hz, 1H), 7.29 (dd, J = 3.6, 0.5 Hz, 1H), 7.16 (dd, J = 11.3, 1.5 Hz, 1H), 7.06 (dd, J = 8.1,

1.6 Hz, 1H), 6.85 (dq, *J* = 3.4, 0.9 Hz, 1H), 4.32 (q, *J* = 7.1 Hz, 2H), 4.18 (s, 2H), 3.18 (d, *J* = 6.9 Hz, 2H), 2.49 (t, *J* = 1.3 Hz, 3H), 1.33 (t, *J* = 7.1 Hz, 3H), 1.22 – 1.09 (m, 1H), 0.44 – 0.32 (m, 2H), 0.29 – 0.21 (m, 2H).



Synthesis of 2-(5-(cyclopropylmethyl)-3-(4-fluoro-3-((5-methylthiophen-2-yl)ethynyl)phenyl)-4-(3-fluoro-4-sulfamoylbenzyl)-1H-pyrazol-1-yl)thiazole-4-carboxylic acid: Ethyl 2-(5-(cyclopropylmethyl)-3-(4-fluoro-3-((5-methylthiophen-2-yl)ethynyl)phenyl)-4-(3-fluoro-4-sulfamoylbenzyl)-1H-pyrazol-1-yl)thiazole-4-carboxylate (8.65 g, 12.74 mmol) in EtOH (150 mL) was added LiOH (42.5 ml, 63.7 mmol, 5 eq) and stirred at RT for 2 h. After completion of the reaction, most of the solvent was removed and the residue was diluted with water (50 mL). The reaction mixture was acidified with 1 molar HCl. The precipitate formed was stirred at RT for 1 h then collected by filtration. The filter cake was thoroughly washed with cold water. The milky precipitate was further suspended in hot water and stirred for 30 minutes and again filtered to collect the precipitate. The precipitate was washed with hot water and with cold ethanol to get the white solid which was further dried under air overnight and finally in a vacuum oven overnight at 80 ºC. Yield 7.3 g pure white solid (88 %). LC-MS Retention Time = 6.133 min $(M+H)^+$ = 651. (Long Gradient 4% to 100% Acetonitrile (0.05% TFA) over 7 minutes, Agilent Eclipse XDB-C18 3 micron 3 x 75mm). 1H NMR (400 MHz, DMSO-d6) δ 13.14 (s, 1H), 8.29 (s, 1H), 7.71 (dd, J = 6.9, 2.3 Hz, 1H), 7.66 – 7.54 (m, 4H), 7.35 (dd, J = 9.4, 8.7 Hz, 1H), 7.26 (dd, J = 3.6, 0.5 Hz, 1H), 7.14 (dd, J = 11.3, 1.6 Hz, 1H), 7.03 (dd, J = 8.1, 1.6 Hz, 1H), 6.83 (dq, J = 3.6, 1.0 Hz, 1H), 4.15 (s, 2H), 3.15 (d, J = 6.9 Hz, 2H), 2.47 – 2.45 (m, 3H), 1.20 – 1.06 (m, 1H), 0.38 – 0.29 (m, 2H), 0.24 – 0.15 (m, 2H); 13C NMR (101 MHz, DMSO-d6) δ 161.58, 160.94, 159.18, 150.73, 147.20, 147.12, 144.47, 144.44, 143.30, 133.59, 131.80, 129.81, 129.73, 129.50, 129.35, 128.51, 128.48, 128.39, 126.25, 125.90, 123.59, 123.56, 118.47, 116.75, 116.28, 116.23, 116.07, 116.02, 110.99, 84.86, 27.99, 14.90, 10.15, 4.34; HRMS (ESI) m/z $(M+H)^+$ calcd. for $C_{31}H_{25}F_2N_4O_4S$; 651.1001 found 651.1003.

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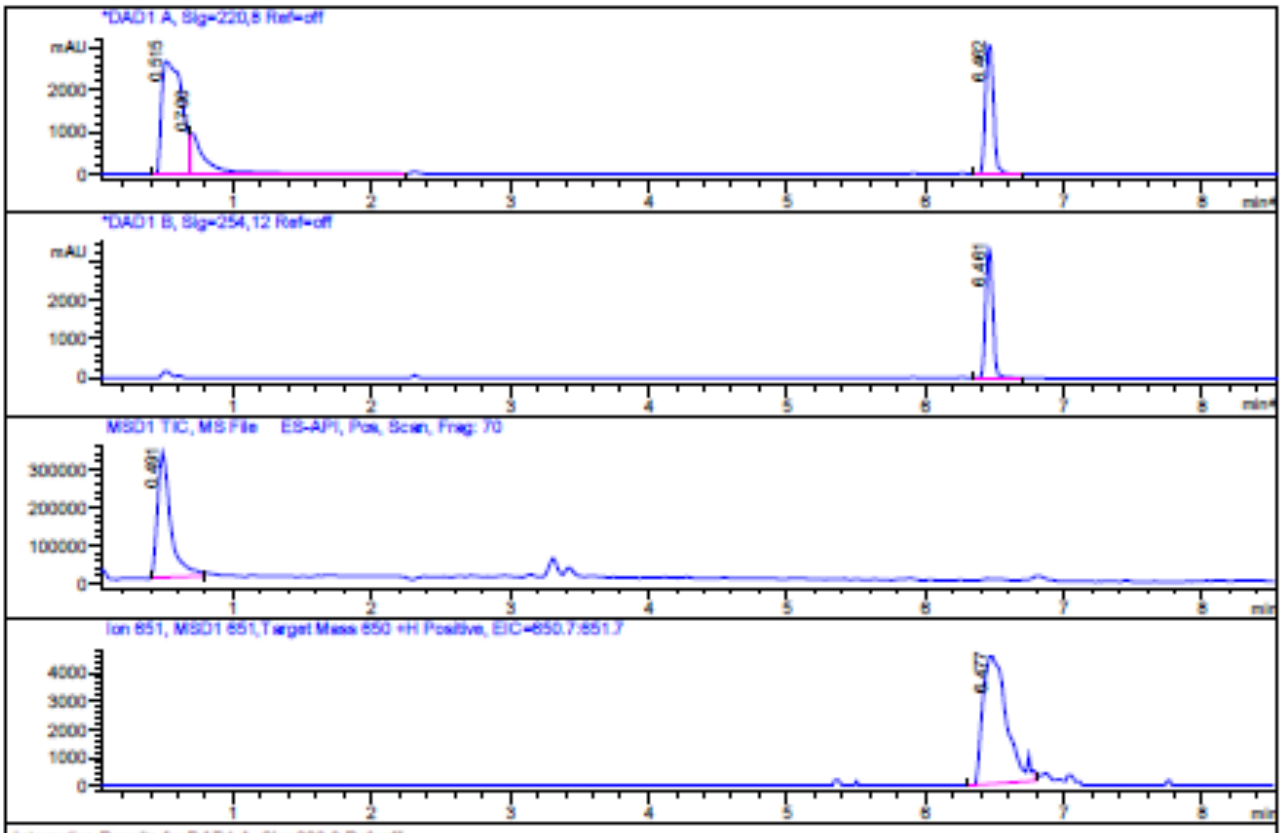

Integration Results for DAD1 A, Sig=220,8 Ref=off

RetTim	Width	Area	Height	Area%	MS(+)
0.52	0.14	27394.09	2676.13	56.91	179
0.70	0.14	8205.07	967.65	17.05	179
6.46	0.07	12533.18	3103.14	26.04	651

Integration Results for DAD1 B, Sig=254,12 Ref=off

RetTim	Width	Area	Height	Area%	MS(+)
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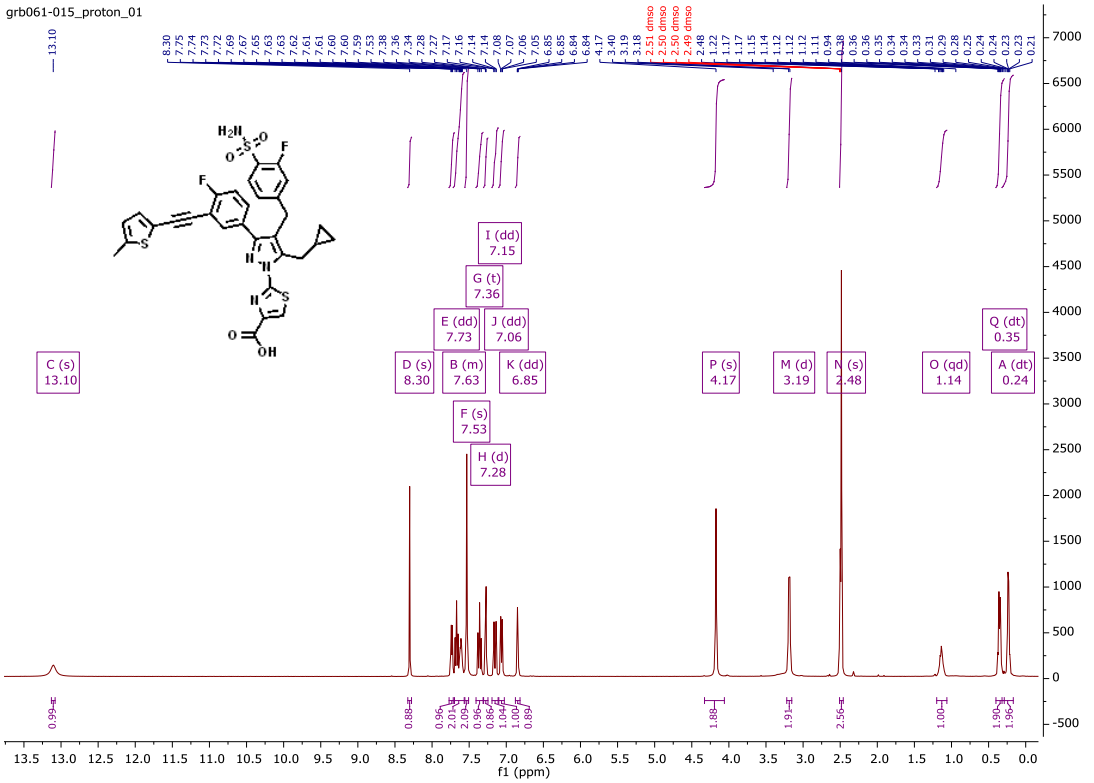
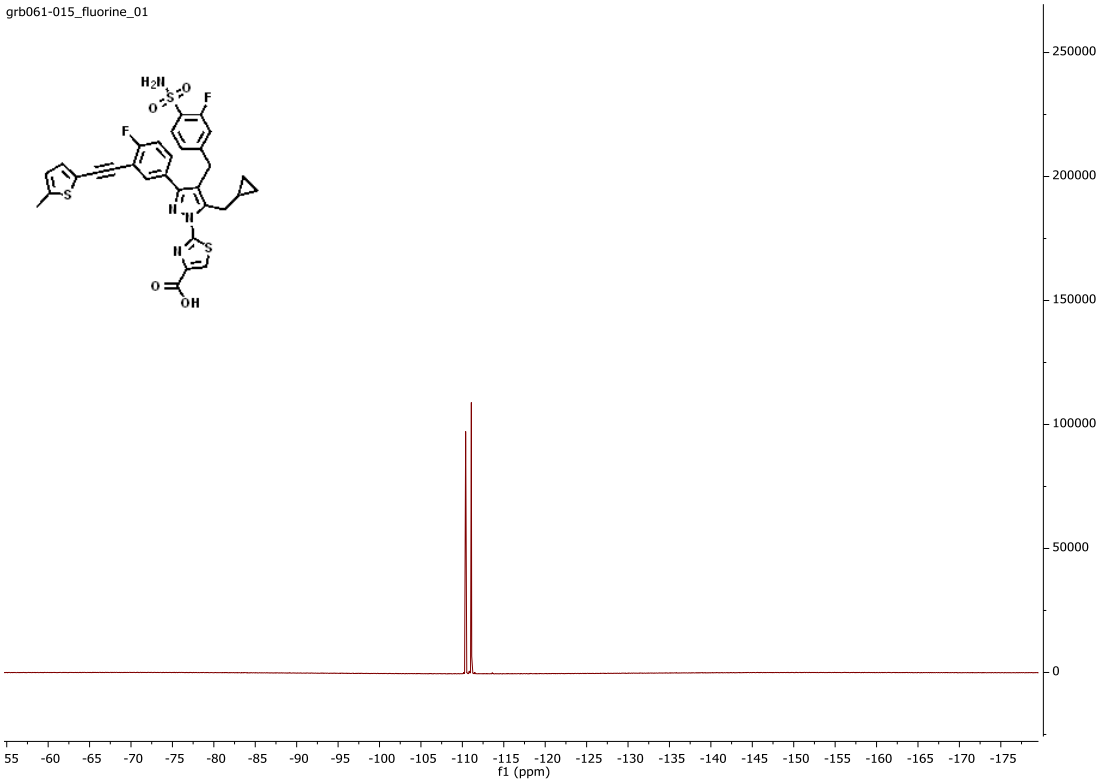
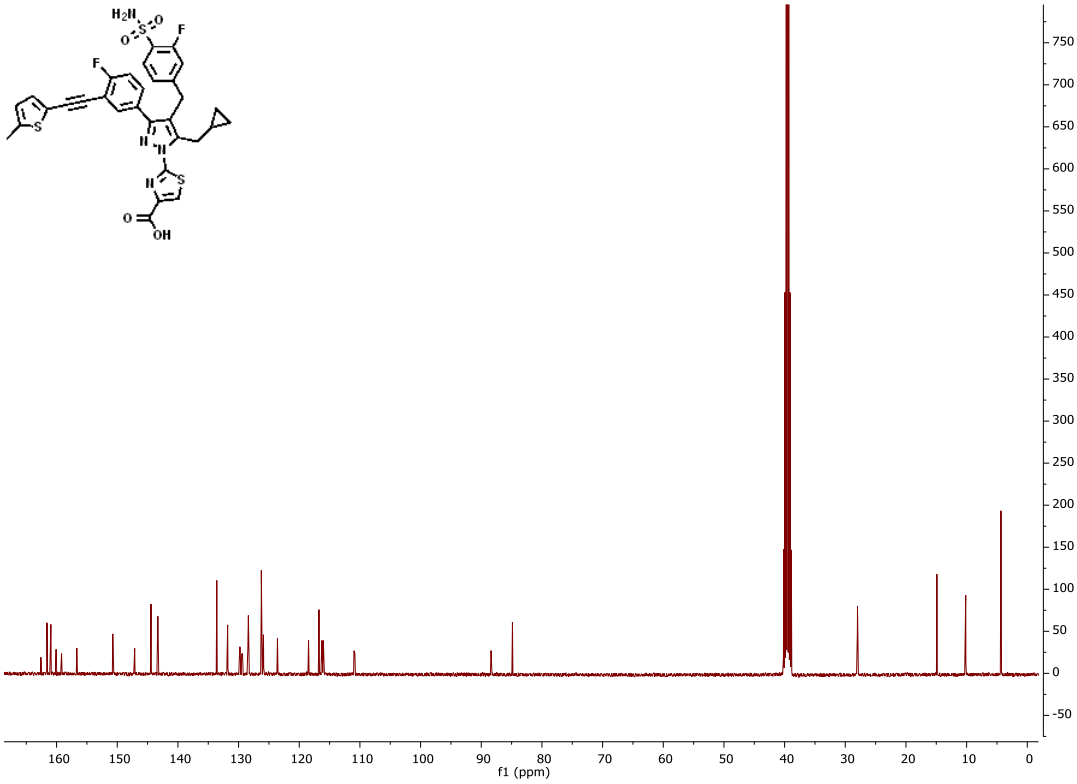
Proton NMR and Fluorine NMR



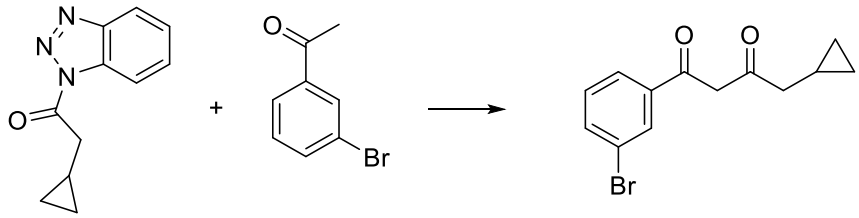


^{13}C NMR spectra

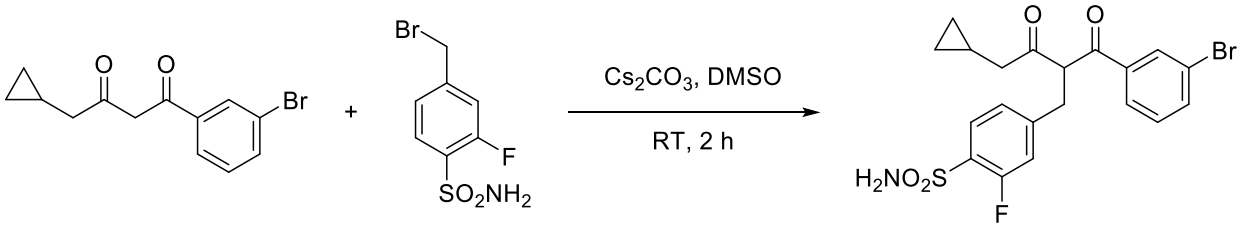


Synthesis of NCGC00420737 (hereafter referred to as NCI-737)

The IUPAC name of NCI-737 is 2-(5-(cyclopropylmethyl)-4-(3-fluoro-4-sulfamoylbenzyl)-3-(3-((5-methylthiophen-2-yl)ethynyl)phenyl)-1H-pyrazol-1-yl)thiazole-4-carboxylic acid, and its chemical formula is $C_{31}H_{26}FN_4O_4S_3$ (molecular weight = 632.7).

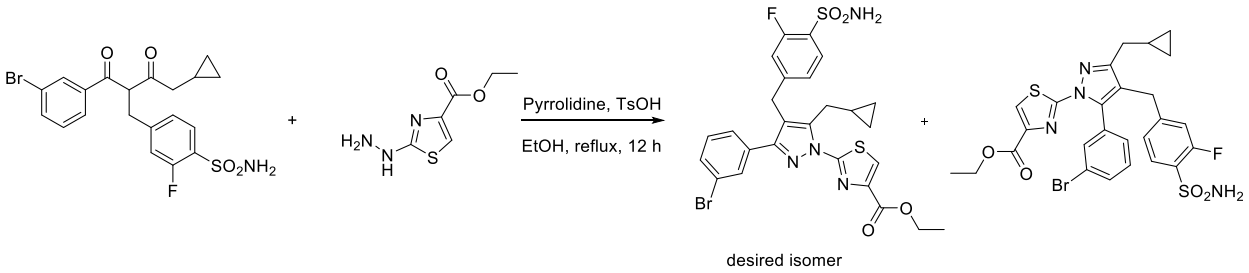


Synthesis of 1-(3-bromophenyl)-4-cyclopropylbutane-1,3-dione: 3'-Bromoacetophenone (100 ml, 754 mmol, 1eq) and 1-(1H-benzo[d][1,2,3]triazol-1-yl)-2-cyclopropylethan-1-one (167 g, 829 mmol, 1.1 eq) was charged 1000 mL DCM then added magnesium bromide diethyl etherate (487 g, 1884 mmol, 2.5 eq) in one portion in a 4 necked flask set up with overhead stirrer. The reaction was cooled in an ice bath then added hunig's base (395 ml, 2261 mmol, and 3 eq) drop wise over 15 minutes through a dropping funnel. The reaction was stirred overnight. The reaction was placed in an ice bath then added ice cubes slowly while stirring during which heat generated. The addition of ice continued until no more exothermic reaction then added 1 molar HCl dropwise under ice cooling added few ml of 6 molar HCl to acidify then extracted with DCM, the organic layer was washed with brine. The organic layer was dried with magnesium sulfate and concentrated. The crude product was purified on a flash system using 340 g Biotage columns eluting with gradient elution (0-30 % ethyl acetate in hexanes over 20 column volumes (used 8 of them) to get yellow liquid as a first peak. LC-MS Retention Time = 3.95 and 3.55 min; $(M+H)^+$ = 281 (Standard Gradient 4% to 100% Acetonitrile 0.05% TFA over 3 minutes; Luna C18 3 micron 3 x 75mm).



Synthesis of 4-(2-(3-bromobenzoyl)-4-cyclopropyl-3-oxobutyl)-2-fluorobenzenesulfonamide: 1-(3-Bromophenyl)-4-cyclopropylbutane-1, 3-dione (92.5 g, 329 mmol) in DMSO (300 mL) was added Cs_2CO_3 (129 g, 395 mmol) and stirred at RT for 10 minutes. To the above mixture was 4-(bromomethyl)-2-

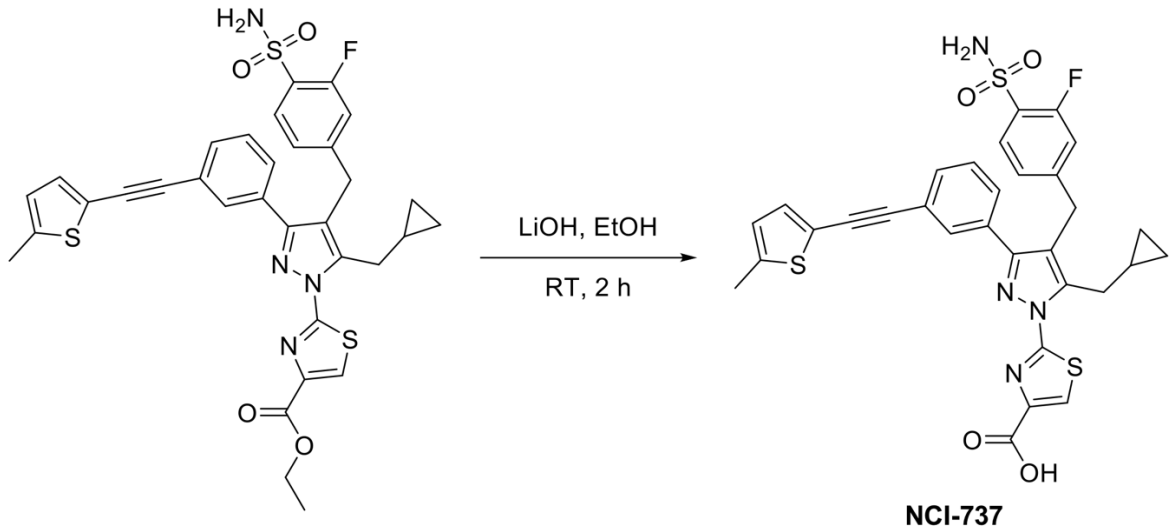
fluorobenzenesulfonamide (88 g, 329 mmol) portion wise upon cooling in ice water bath then allowed to stir at RT for 1 h. The reaction was diluted with ethyl acetate and filtered to remove the solids. The filtrate was quenched with 100 mL of 1 molar HCl and extracted with ethyl acetate. The aqueous layer was again extracted twice with ethyl acetate then the combined org layer was washed with saturated ammonium chloride solution 3 times. The combined org layer was dried with Magnesium sulfate and concentrated. The crude material was taken in DCM and loaded to 5 silica loading cartridges then purified in isco flash system using 330 g gold columns (distributed into 5 columns) The product was collected and dried to get 121.5 g of solid (79 % yield). LC-MS Retention Time: = 3.388 min $(M+Na)^+$ = 490. (Standard Gradient 4% to 100% Acetonitrile (0.05% TFA) over 3 minutes; Luna C18 3 micron 3 x 75mm).



desired isomer

Synthesis of ethyl 2-(3-(3-bromophenyl)-5-(cyclopropylmethyl)-4-(3-fluoro-4-sulfamoylbenzyl)-1H-pyrazol-1-yl)thiazole-4-carboxylate: A mixture of 4-(2-(3-bromobenzoyl)-4-cyclopropyl-3-oxobutyl)-2-fluorobenzenesulfonamide (25 g, 53.4 mmol), Ts-OH (5.08 g, 26.7 mmol) in ethanol (100 mL) was added pyrrolidine (2.207 ml, 26.7 mmol) then refluxed for 1 h. The reaction was cooled and added ethyl 2-hydrazinylthiazole-4-carboxylate, 2HBr (22.36 g, 64.1 mmol) and refluxed further overnight. The reaction was concentrated, and the residue was taken in DCM and immediately loaded to a silica loading cartridge. The compound was purified on an isco flash system using 330 g gold column eluting with 20-40 % ethyl acetate in hexanes over 20 column volumes. The pure product containing a mixture of 2 regioisomers were further separated on a reverse phase isco using a 415 g gold column eluting with 60-100 % ACN (0.1 TFA) in water over 20 column volumes. The 2nd pk was pooled and concentrated the solid was stirred with a clear solution of bicarbonate. The precipitate formed was collected by filtration. The filter cake was thoroughly washed with water and air dried and finally in a vacuum desiccator under P_2O_5 to get pure white solid. Yield 10.8 g (32.7 %). LC-MS Retention Time = 6.62 min $(M+H)^+$ = 621. (Long Gradient 4% to 100% Acetonitrile (0.05% TFA) over 7 minutes, Agilent Eclipse XDB-C18 3 micron 3 x 75mm).

Synthesis of ethyl 2-(5-(cyclopropylmethyl)-4-(3-fluoro-4-sulfamoylbenzyl)-3-(3-((5-methylthiophen-2-yl)ethynyl)phenyl)-1H-pyrazol-1-yl)thiazole-4-carboxylate: A mixture of ethyl 2-(3-(3-bromophenyl)-5-(cyclopropylmethyl)-4-(3-fluoro-4-sulfamoylbenzyl)-1H-pyrazol-1-yl)thiazole-4-carboxylate (15 g, 24.21 mmol), [P(tBu)3] Pd(crotyl)Cl (http://jmcct.com/products-services/product_p429.html) (cat # Pd-162) (0.351 g, 1.211 mmol), 2-ethynyl-5-methylthiophene (4.14 g, 33.9 mmol) and DABCO (5.43 g, 48.4 mmol) in dioxan (50 mL) was bubbled with argon for 10 minutes and stirred at RT overnight. The reaction was diluted with ethyl acetate and added Pd scavenger silia DMT and stirred for 2 h at RT then filtered through a plug of silica. The filtrate was concentrated and purified on isco flash system using 220 g gold column eluting with 20-40 % ethyl acetate in hexanes. The product had some color which is purified in isco reverse phase using a 415 g gold column eluting with 60-100 % ACN in water (0.1% TFA) over 25 column volumes (in 2 batches). The pure fraction was pooled and concentrated then poured onto a clear bicarbonate solution. The precipitate was collected and vacuum dried under P_2O_5 to get 12.9 of white solid (81 %). %). LC-MS Retention Time = 7.48 min $(M+H)^+ = 661$. (Long Gradient 4% to 100% Acetonitrile (0.05% TFA) over 7 minutes, Agilent Eclipse XDB-C18 3 micron 3 x 75mm).



NCI-737

Synthesis of 2-(5-(cyclopropylmethyl)-4-(3-fluoro-4-sulfamoylbenzyl)-3-(3-((5-methylthiophen-2-yl)ethynyl)phenyl)-1H-pyrazol-1-yl)thiazole-4-carboxylic acid: Ethyl 2-(5-(cyclopropylmethyl)-4-(3-fluoro-4-sulfamoylbenzyl)-3-(3-((5-methylthiophen-2-yl)ethynyl)-phenyl)-1H-pyrazol-1-yl)thiazole-4-carboxylate (10.9 g, 16.50 mmol) in ethanol (150 mL) was treated with 1.5 molar LiOH (55.0 ml, 82 mmol, 5 eq) and the reaction was

stirred at RT for 2 h. After removing most of the solvent, the reaction was diluted with 100 mL of water acidified with 1 molar HCl. The precipitate formed was collected by filtration and washed with water. The wet precipitate was suspended in hot water and sonicated for 2 h followed by stirring for another 1 h (Alternatively the precipitate can be stirred overnight). The nice precipitate formed was collected by filtration, washed with water and a mixture of cold water/ethanol mixture (1/1) and air dried. Finally, the compound was dried under high vacuum under P_2O_5 overnight to get pure product as white solid 10.2 g (98 %). LC-MS Retention Time = 6.402 min $(M+H)^+$ = 632. (Long Gradient 4% to 100% Acetonitrile (0.05% TFA) over 7 minutes, Agilent Eclipse XDB-C18 3 micron 3 x 75mm). 1H NMR (400 MHz, DMSO-d6) δ 13.18 (s, 1H), 8.32 (s, 1H), 7.74 – 7.62 (m, 2H), 7.56 (s, 0H), 7.52 (dt, J = 7.7, 1.4 Hz, 1H), 7.48 – 7.40 (m, 1H), 7.24 (d, J = 3.5 Hz, 1H), 7.17 (dd, J = 11.3, 1.6 Hz, 1H), 7.07 (dd, J = 8.1, 1.6 Hz, 1H), 6.83 (dq, J = 3.4, 1.1 Hz, 1H), 4.18 (s, 2H), 3.18 (d, J = 6.9 Hz, 2H), 2.47 (d, J = 1.1 Hz, 3H), 1.23 – 1.09 (m, 1H), 0.39 – 0.31 (m, 2H), 0.27 – 0.20 (m, 2H). 13C NMR (101 MHz, DMSO-d6) δ 161.70, 161.11, 159.24, 156.73, 151.55, 147.42, 147.34, 144.53, 144.45, 142.72, 133.19, 132.20, 131.08, 129.80, 129.50, 129.36, 129.26, 128.46, 127.59, 126.24, 126.11, 123.67, 123.64, 122.51, 119.11, 116.86, 116.36, 116.14, 91.67, 83.60, 28.10, 15.01, 10.27, 4.45. HRMS (ESI) m/z $(M+H)^+$ calcd. for $C_{31}H_{26}FN_4O_4S_3$; 633.1095 found 633.1092.

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Spec. Reported    : UV Integration                Inj Volume : 3 ul
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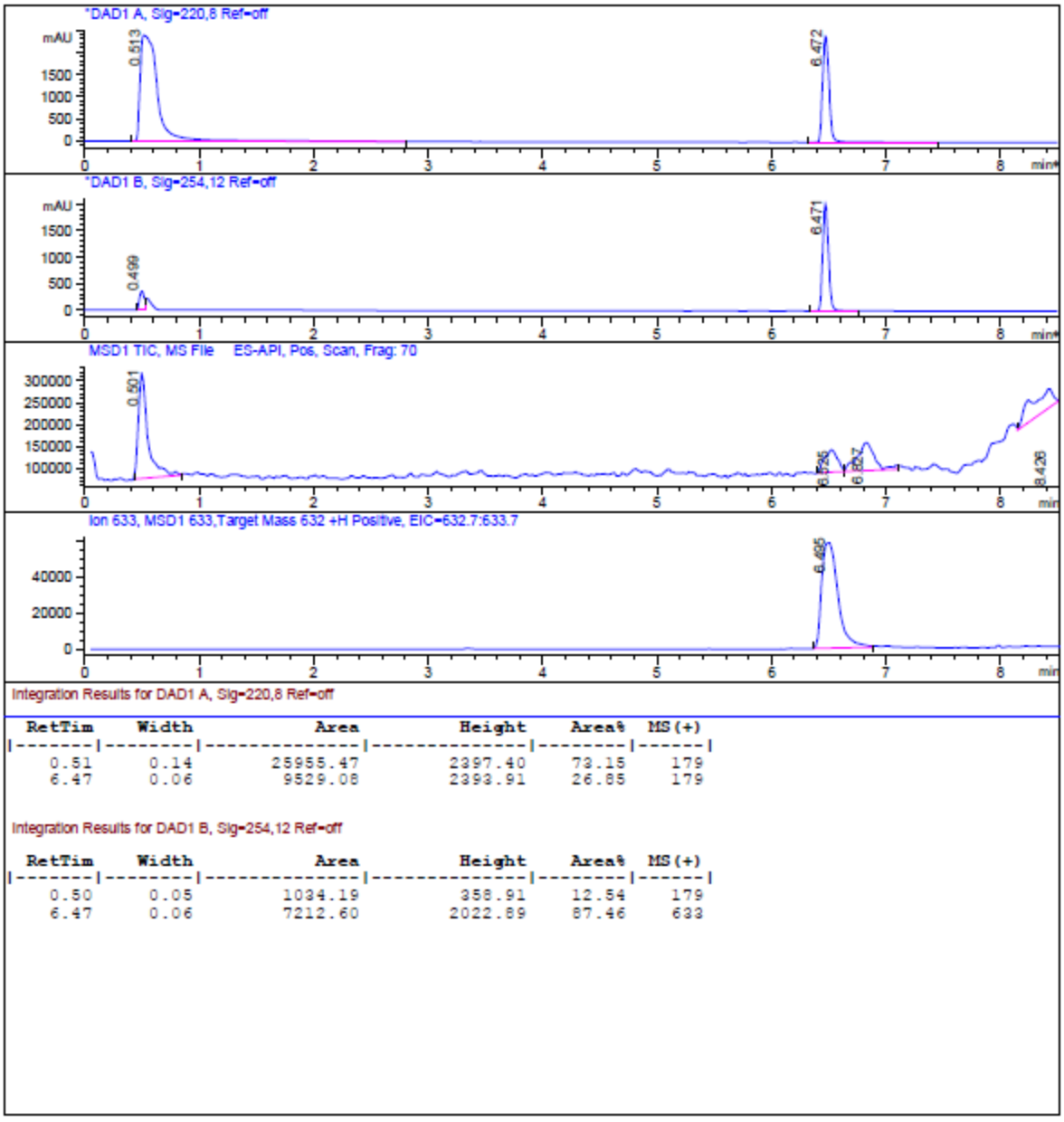

Integration Results for DAD1 A, Sig=220,8 Ref=off

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6.47	0.06	9529.08	2393.91	26.85	179

Integration Results for DAD1 B, Sig=254,12 Ref=off

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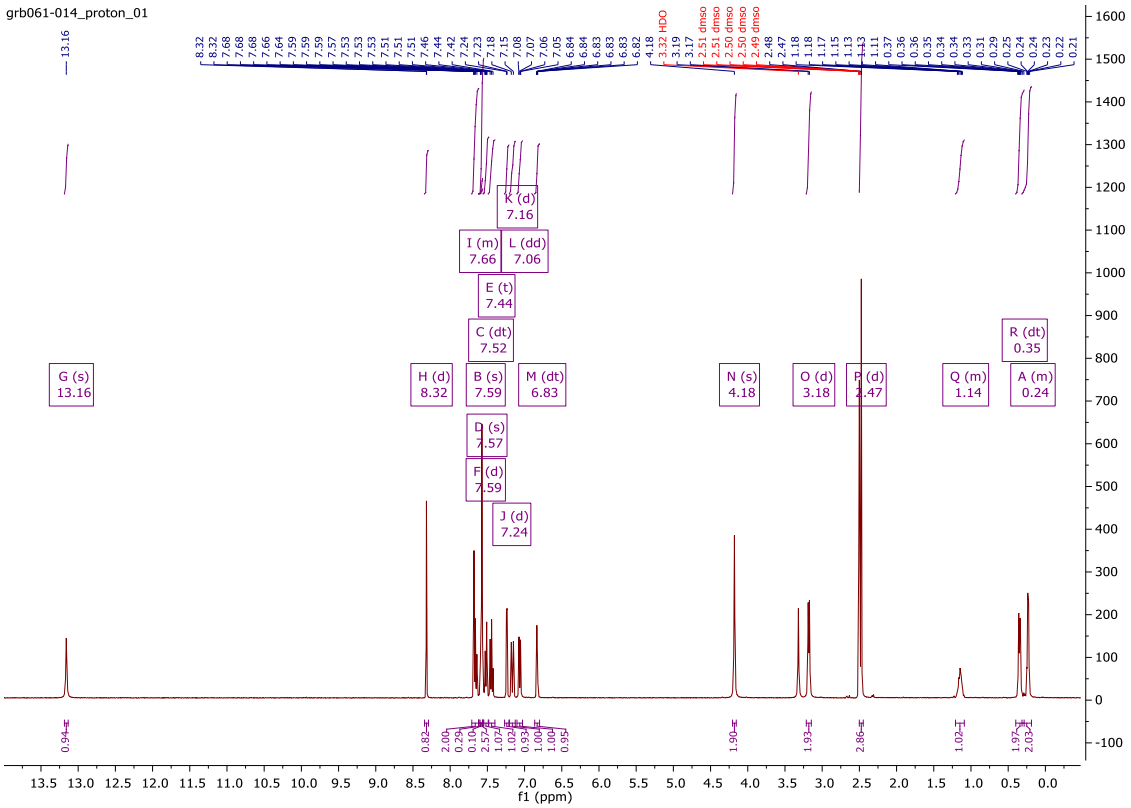
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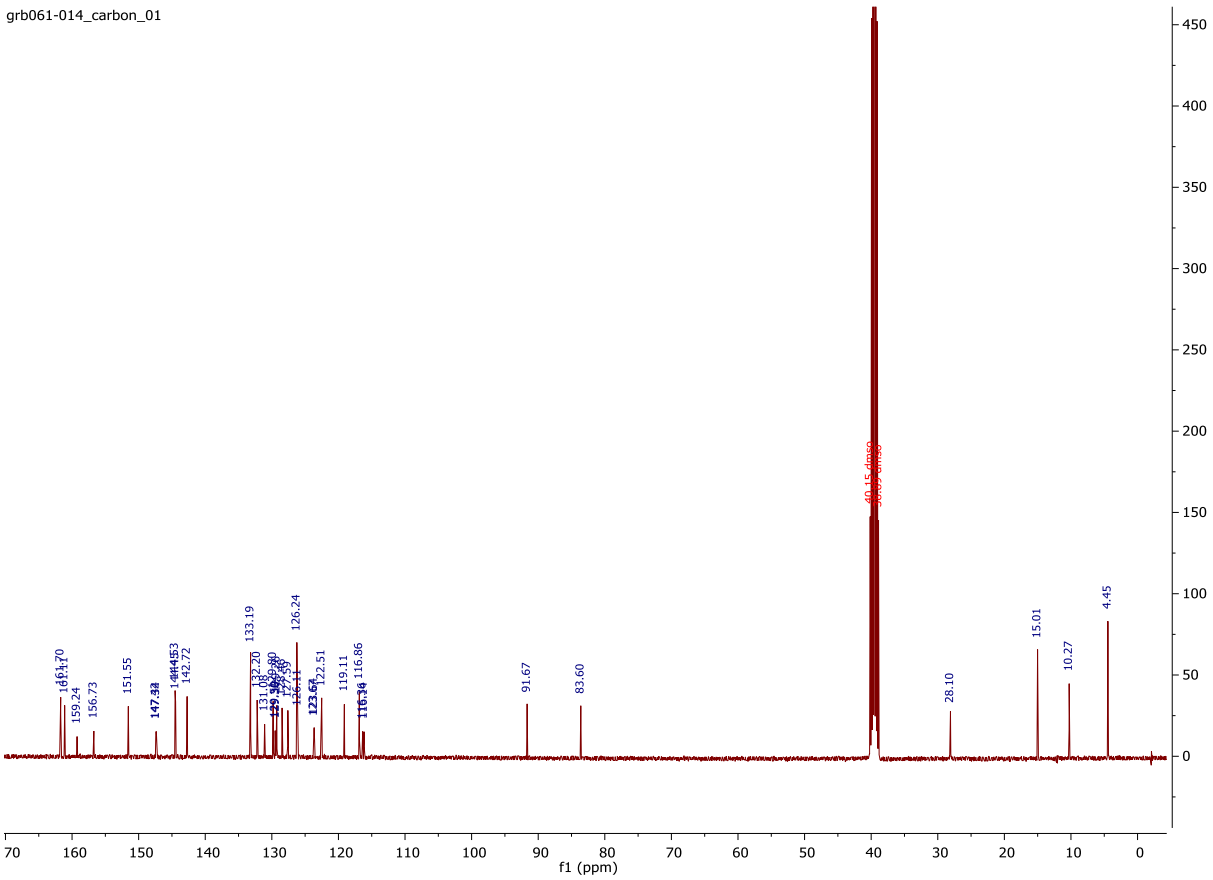
G (s) 13.16
H (d) 8.32
B (s) 7.59
M (dt) 6.83
D (s) 7.57
F (d) 7.59
J (d) 7.24
K (d) 7.16
I (m) 7.66
L (dd) 7.06
E (t) 7.44
C (dt) 7.52
N (s) 4.18
O (d) 3.18
P (d) 2.47
Q (m) 1.14
R (dt) 0.35
A (m) 0.24
f1 (ppm)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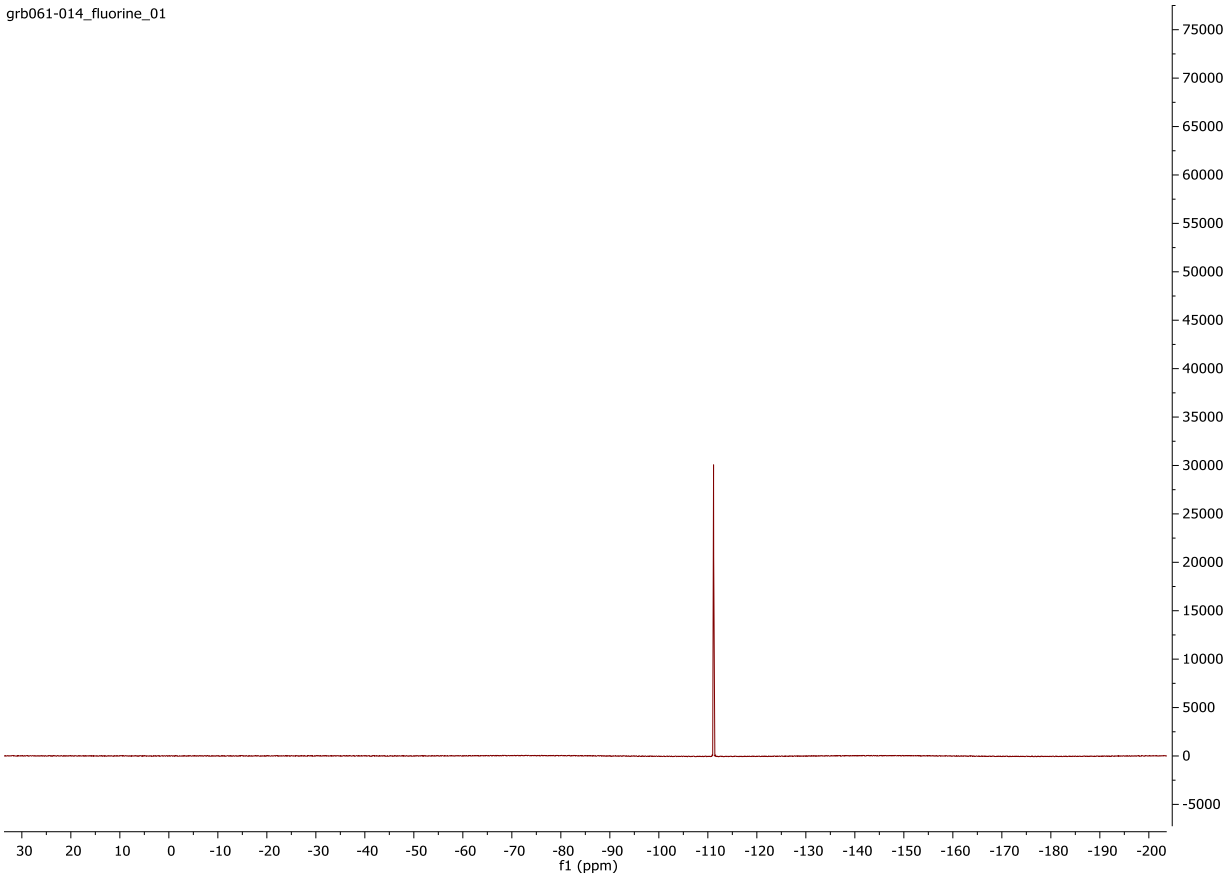
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30 20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140 -150 -160 -170 -180 -190 -200
f1 (ppm)

Protein Analysis

Blots were incubated with primary antibodies against cleaved PARP (Cell Signaling, Danvers, MA) at 1:1,250, cleaved caspase 7 (Cell Signaling) at 1:1,000, LDHA (Proteintech Group, Inc., Rosemont, IL) at 1:1,000, LDHB (Proteintech Group, Inc.) at 1:1,000, Fli1 (Abcam, Cambridge, MA) at 1:250, and NROB1 (Cell Signaling) at 1:1,000. Beta-actin (Abcam) at 1:15,000 and GAPDH (Santa Cruz, Dallas, TX) at 1:10,000 were used as loading controls.

siRNA Studies

Lipofectamine RNAi Max (ThermoFisher, Waltham, MA) was used to deliver siLDHA #3 (CAGCTGATTTATAATCTTCTA), #5 (AATCTGGATTCAGCCCGATTC, or #7 (AAGTCCAATATGGCAACTCTA), siLDHB #2, (CACCGCGTGATTGGAAGTGGA), #5 (AAGATTGTAGTGGTAACTGCA), #6 (ACAGTCCTGATTGCATCATAA), or #7 (ATGGTGGTTGAAAGTGCCTAT), and siFli1 #6 (ACCCACGTGCCTTCACACTTA) or #8 (AGTCGTGTAACAGTACTGCAA) (Qiagen, Germantown, MD) to 250,000 cells in six-well plates, 24 hours after seeding. 3 μL of 10 μM siRNA was used. Protein was harvested 72 hours post-transfection in 1X RIPA buffer (Santa Cruz) for western blot.

For proliferation assays with siRNA, cells were plated at 5,000-12,000 cells/well in six-well plates and siRNA was delivered to cells as described above, 72 hours after plating. Cell growth was analyzed using the IncuCyte live cell analysis system. At day 7 or 8 days post-transfection, cells were stained with NeatStain (AstralDiagnostics, West Deptford, NJ) at and images were acquired.

Extracellular Flux Analysis

Unless otherwise specified, cells were plated at 25,000 cells/well in XF 96-well plates (Agilent) and incubated overnight at 37°C. The following day, cells were treated with the indicated concentrations of LDHi or DMSO (control) for 6 hours. Assay media was prepared according to the manufacturer's instructions; however, pyruvate was excluded unless otherwise indicated.

To generate the energy plot, cells were plated as described above. Eight hours after plating, cells were treated with DMSO (control) or 100 nM NCI-737 for 24 hours. Assays to measure basal ECAR and basal OCR were performed on treated and untreated cells.

For assays comparing the activity of NCI-737 and NCI-006, cells were plated at 20,000 cells/well in XF 96-well plates and incubated for 24 hours at 37°C. Media was changed to XF-DMEM (5.5 mM glucose, 1 mM pyruvate and 4 mM glutamine, pH 7.4) 1 hour prior to performing the assay at 37 °C. The experiment started with

4 measurements of basal OCR or ECAR, then injection of LDH inhibitors (NCI-006 or NCI-737), followed by OCR/ECAR measurements for 16 minutes or 2 hours.

^{13}C Glucose Tracing

Sample preparation and LCMS analysis

Cells were plated at 5 million cells/10-cm plate. The following morning, media was removed and replaced with media containing 11mM glucose that was either [U-$^{13}C_6$] D-glucose (Cambridge Isotope Laboratories, Inc, Tewksbury, MA) or unlabeled. At the same time, cells were treated with NCI-737 at 250 nM or DMSO (control) and plates were incubated for 6 hours before all media was removed. 3 mL of PBS wash solution was dispensed to each plate, aspirated, and discarded. Each plate was briefly washed with 1.5 mL of chilled Milli-Q water to remove PBS and quench cells. The duration and volume of the wash was sufficiently reduced to avoid disruption of the osmotic stability, while effectively removing excess PBS salts that impact LC/MS signal suppression and data quality. After removing excess fluid, 1 mL chilled Milli-Q water was dispensed, and samples were placed on dry ice to snap-freeze and fully quench activity. Samples were then stored at – 80℃ prior to collection. Upon collection, samples were partially thawed on ice bed and then detached with cell scraper. For each sample, an 850 mL portion was aspirated, transferred to 15-mL conical tube, placed on dry ice to snap-freeze, and stored at – 80°C. Then, samples were thawed in an ice water bath and sonicated at 30 amps for 30 seconds to prepare a homogenous solution. Lysate solutions were vortexed (mid-speed) for 10 seconds, and a 60 µL aliquot was set aside for Bradford protein assay. 750 µL of chilled HPLC grade methanol was added to remaining sample, vortexed, and incubated overnight at -20°C. Next, 500 µL of chilled HPLC grade chloroform was added to each sample, vortexed, and incubated in ice for 30 minutes on mixer. Upon the formation of two layers using the Bligh and Dyer biphasic extraction, samples were centrifuged at 12,000 rpm for 20 min at 4 °C. Finally, 1400 µL of the top polar phase was transferred to separate, sterilized 1.7 mL microtubes and concentrated under N_2 gas to complete dryness at RT before storage at - 80°C. For LC/MS-based metabolic analysis, extracts were reconstituted in 60% methanol (aq) reagent at volumes that were corrected based on protein values obtained from Bradford assay, in order to ensure that metabolite pools were relatively consistent for each group.

As previously described, sample extracts were resolved and analyzed using ESI negative mode on the Agilent 6500 QTOF-MS coupled with the Infinity II 1290 LC system to collect quantitative data on labeled and unlabeled TCA cycle and glycolytic intermediates (3). Metabolites were separated using two different liquid chromatography applications, including: 1) AdvanceBio Glycan Mapping 2.1 x 100 mm, 2.7 µm and AdvanceBio Glycan 2.1 X 5 mm, 2.7 µm Guard UHPLC columns, and 2) Acquity BEH Amide 2.1 x 100 mm, 1.7 µm UPLC column with Acquity 0.2 µm in-line filter. Metabolites separation occurred over two binary gradients composed of LC/MS grade solvents and additives, as follows: 1) solvent A—12% acetonitrile (aq) pH 6.85, 10 mM ammonium acetate and 0.01% InfinityLab deactivator additive, and solvent B—90% acetonitrile (aq) pH 6.85 and 10 mM

ammonium acetate; 2) solvent A—water, 0.1% formic acid and 0.01% InfinityLab deactivator additive, and solvent B—95% acetonitrile, 5% methanol and 0.1% formic acid. The LC/MS samples were resolved using the LC/MS conditions as previously described (3). Agilent Masshunter Profinder B.10.0 was used to bin metabolites of interest using a targeted approach with a validated in-house personal compound data library (PCDL) and mass accuracy limits of 0-5 mDa. Follow-on isotopic analysis was performed to align and quantify each precursor ion along with their isotopes. Agilent Vista Flux software was employed to conduct ^{13}C isotopologue and natural abundance comparative analysis for labeled media-substrate experiments.

Sample preparation and NMR analysis

0.5 mL of cell media was collected after 6 hours of incubation in media containing ^{13}C-glucose, spun down, and the clear supernatant was transferred to a new tube and mixed with 1 mL ice-cold methanol. Subsequently, samples were incubated for 1 hour at -20°C and spun down at 12,000 rpm for 30 minutes. The supernatant was collected and dried under a stream of N_2. The dried sediment was reconstituted in 180 uL of pH 7 50 mM phosphate buffer in D_2O (containing d-TSP as internal standard), then transferred to a 3 mm-NMR tube for analysis. All the spectra were acquired at 25 °C on a Bruker Avance III 600 MHz spectrometer (Structural Biophysics Laboratory, NCI, Frederick, Maryland, USA) equipped with a cryogenically cooled probe. Single pulse ^{1}H NMR experiments were performed using the *noesygppr1d* (TopSpin 3.5, Bruker Biospin) pulse sequence for water suppression. For each spectrum, 32 scans were acquired, with a relaxation delay of 3.5 s, a spectral width of 10,800 Hz, and a time domain of 32K points. Spectra were referenced to the TSP internal standard signal (s, $\delta = 0.00$ ppm), zero-filled to 64K points, phased and baseline-corrected using ACD Labs Spectrus Processor 2016, and an exponential line broadening function of 0.30 Hz was applied. For quantification, ^{1}H NMR resonance signals were normalized to the TSP singlet located at 0.00 ppm and corrected to the total protein content as obtained from the Bradford assay.

***In Vivo* Studies**

For experiments with oral or tail vein agent dosing, four- to six-week-old female Fox Chase SCID beige mice (CB17.B6-Prkdcscid Lystbg/Crl) were purchased from Charles River Laboratories (Wilmington, MA). Mice were randomized when palpable tumors developed, at which point treatment with agents began. Mice were fed with a defined gel meal (Nutra-Gel, NCI, Sterile, # S4950) for the entire course of all animal experiments.

For experiments with oral dosing, treatments began on day 14 for TC71 and TC32 tumor-bearing mice and on day 28 for EW8 tumor-bearing mice. Agents were administered by oral gavage at the established maximum tolerated daily doses (MTD) given either once or divided to twice daily. For experiments with IV administration, agents were delivered via tail vein injections for short dosing periods (3 consecutive days or less or every other day for up to 3 weeks) or via indwelling jugular venous catheters (JVCs) for longer consecutive day dosing periods. For JVC experiments, ten- to 12-week-old female Fox Chase SCID beige mice (CB17.B6-Prkdcscid Lystbg/Crl) with

pre-implanted 22-gauge polyurethane JVCs with single-channel vascular access buttons (Instech Laboratories, Plymouth Meeting, PA) were purchased from Charles River Laboratories. Treatment with NCI-737 began between days 7 and 10 for TC71 tumor-bearing mice.

For all experiments, toxicity assessments included evaluation of overall appearance, weekly body weight measurement, blood sampling for complete blood count and chemistry panels, and full necropsies on selected mice. Tumors were measured twice weekly with calipers for assessment of treatment efficacy. Tumor volume was calculated by the following formula: V (mm^3) = (Dxd^2)/6 x 3.14, where D is the longest tumor axis and d is the shortest tumor axis. Tumors were harvested on day three and at study endpoints for assessments of drug level and target inhibition.

Histopathologic Image Analysis

SVS image files were viewed using Aperio ImageScope and imported to Definiens Developer software. Image analysis was performed using a customized rule set written with Developer XD version 2.1.1 (Definiens, Munich, Germany) based on Definiens Cognition Network Technology®. From each whole slide image, automated tissue recognition and segmentation of tumor parenchyma, necrosis, blood, and non-tumor tissue was performed. Average percent tumor necrosis ± SEM was plotted for each experimental group. A Student's t-test was used to determine statistical significance. $P < 0.05$ was considered significant.

Hyperpolarized MRS

[1-^{13}C] pyruvate (30 μL) containing 15 mmol/L OXO63 and 2.5 mmol/L gadolinium chelate was hyperpolarized using the Hypersense DNP polarizer. The hyperpolarized sample was rapidly dissolved in 4.5 mL of a superheated alkaline buffer and injected IV (12 μL/g body weight). Hyperpolarized ^{13}C MRI studies were performed on a 3T scanner (MRSolutions, Guildford, UK) using a 17-mm home-built ^{13}C solenoid coil placed inside of a saddle coil for 1H. The ^{13}C MR spectra were acquired every second with a spectral width of 3330 Hz, repetition time of 1000ms and flip angle of 10°. Chemical shift value of each signal used in this study is [1-^{13}C] Pyruvate: 170.6 ppm, [1-^{13}C] Lactate: 182.7 ppm, [1-^{13}C] Alanine: 176 ppm and [1-^{13}C] Bicarbonate: 163.5 ppm, respectively. Initial ^{13}C MRI evaluation was performed in a mouse harboring either a TC71 or EW8 xenograft tumor (average size 1-1.5 cm in diameter), 1 day before IV injection of NCI-737 at 60 mg/kg ("pre-treatment"), and again 30 minutes after NCI-737 injection ("post-treatment"), thus serving as its own control.

During magnetic resonance spectroscopic imaging (MRSI) measurements, the breathing rate of the mouse was monitored with a pressure transducer (SA Instruments Inc., Stony Brook, NY, USA) and was maintained at 80 ± 10 breaths/min. Core body temperature was monitored using a nonmagnetic rectal temperature probe (FISO, Quebec, QC, Canada) and was maintained at 36 ± 1 ℃ with a flow of warm water.

MRSI data were analyzed using MATLAB (MathWorks, Natick, MA, USA) and ImageJ 1.49v (NIH, Bethesda, MD, USA). Statistical analyses were carried out using GraphPad Prism 8 (San Diego, CA, USA) software. Differences were considered to be statistically significant for P-values < 0.05. All results are expressed as mean ± SEM. T-test was performed to evaluate the statistical difference between two groups.

References

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3. Ruiz-Rodado V, Lita A, Dowdy T, Celiku O, Cavazos-Saldana A, Wang H, et al. Metabolic Plasticity of IDH1- *Mutant* Glioma Cell Lines Is Responsible for Low Sensitivity to Glutaminase Inhibition (May 14, 2019). Cell Reports-D-19-01819. Available at SSRN: https://ssrn.com/abstract=3387658.