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Electronic supplementary information

Development of a NanoBRET assay to validate inhibitors of Sirt2-mediated lysine deacetylation and defatty-acylation that block prostate cancer cell migration

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Supplementary Data

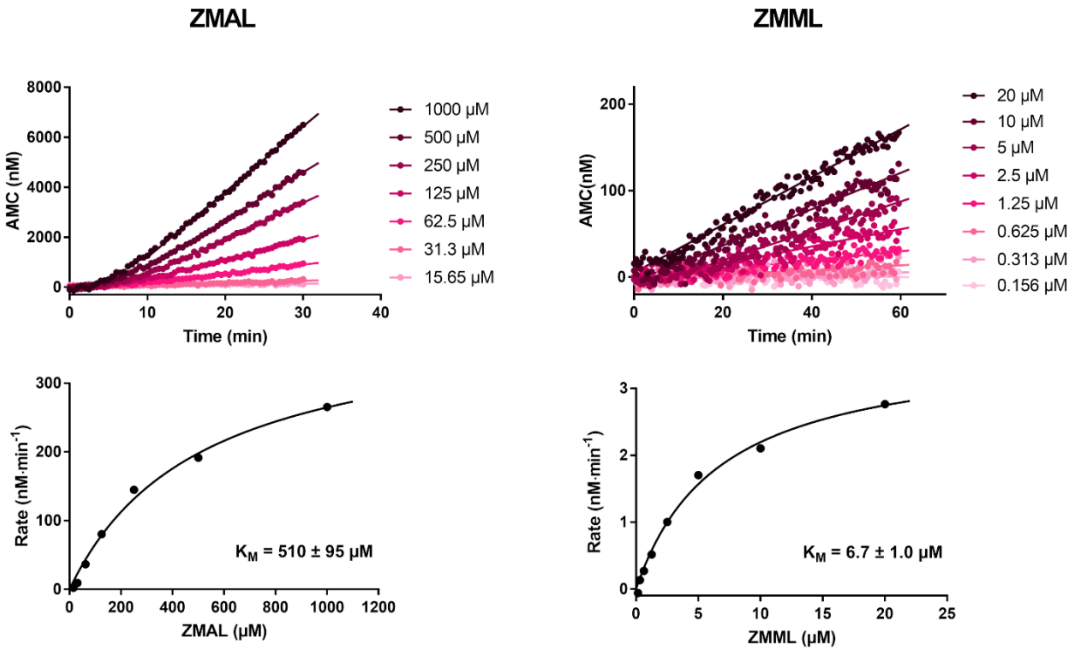


Figure S1: Determination of kinetic parameters for Sirt2-mediated deacetylation (ZMAL) and demyristoylation (ZMML). AMC concentration as a function of time (top) and Michaelis-Menten plots with the calculated K_M values are displayed for ZMAL on the left side and for ZMML on the right side.

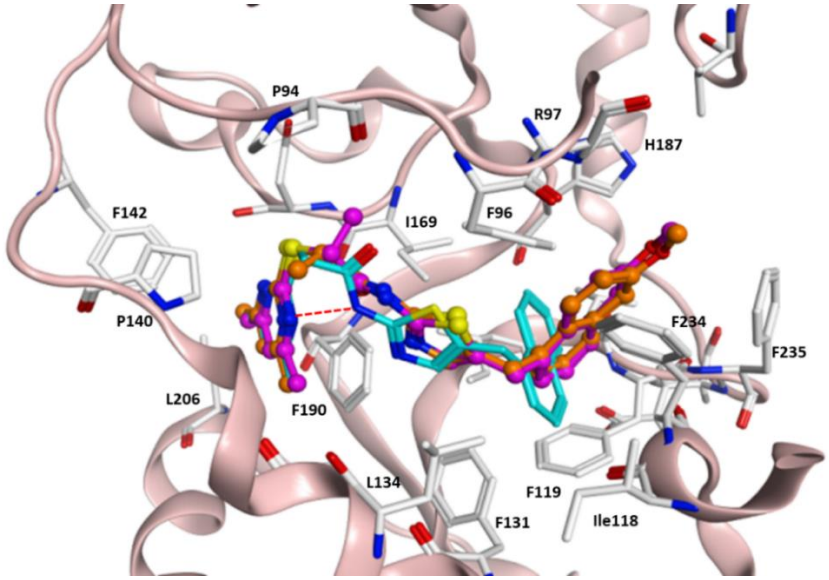


Figure S2: Docking of compound 4 and SirReal2. Docking pose of **4** *(R)*-isomer colored orange, *(S)*-isomer colored magenta) in comparison with the crystal structure of Sirt2 bound with SirReal2 (colored cyan, PDB ID 4RMH). In the case of compound **4**, the methoxy substituted naphthyl ring no longer fits well in the binding pocket due to steric effects and is unable to occupy the position of the naphthyl ring of SirReal2.

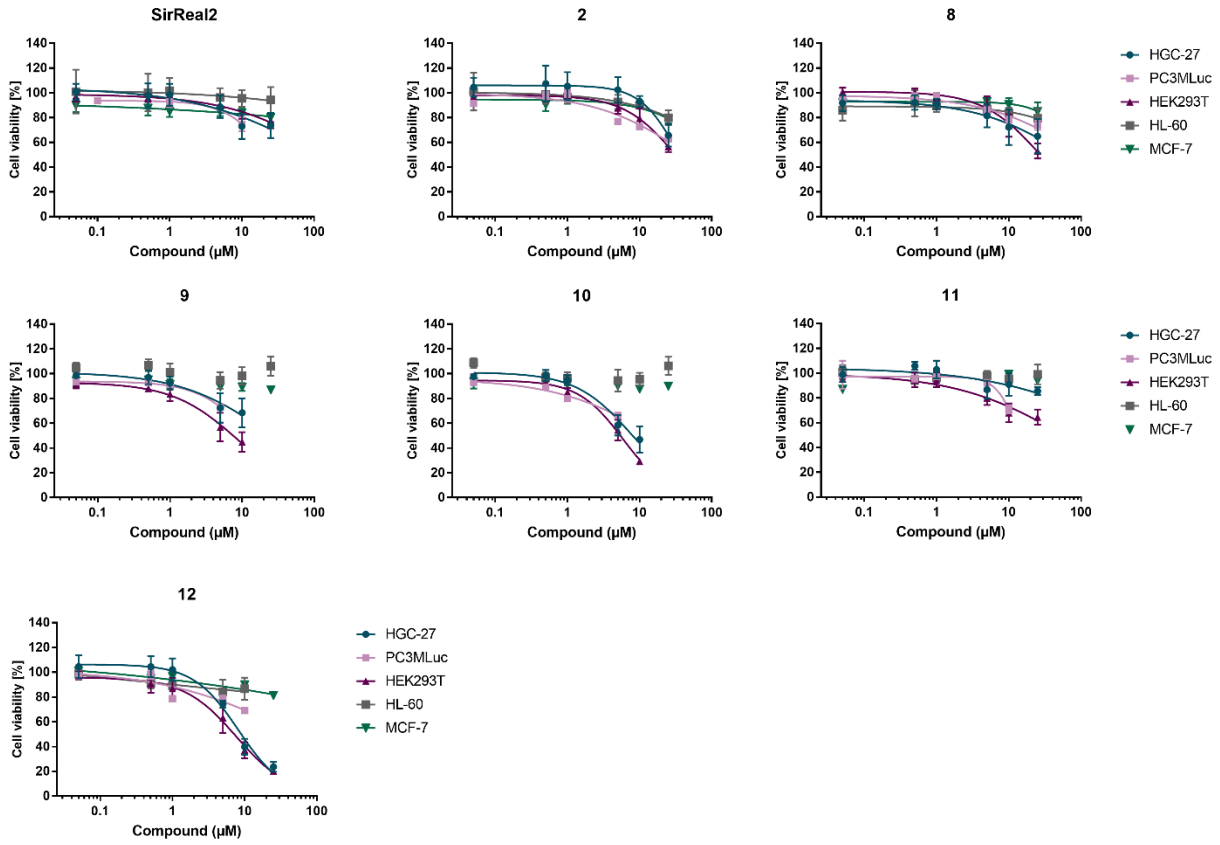


Figure S3. Results of MTS assays. Concentration-dependent cell viability curves of HGC-27, PC-3M-luc, HEK293T, HL-60 and MCF-7 cells are displayed for different Sirt2 inhibitors.

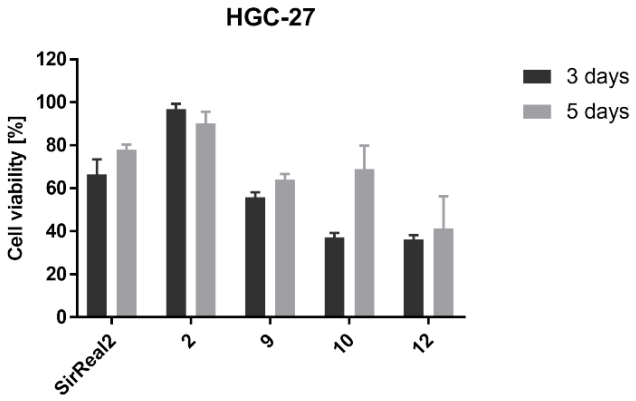


Figure S4. Time-dependent effect on cell viability in HGC-27 cells. Comparison of cell viability of HGC-27 for different Sirt2 inhibitors (10 µM) after 3 (dark grey) and 5 days (bright grey) of treatment. Values are presented as mean ± SD.

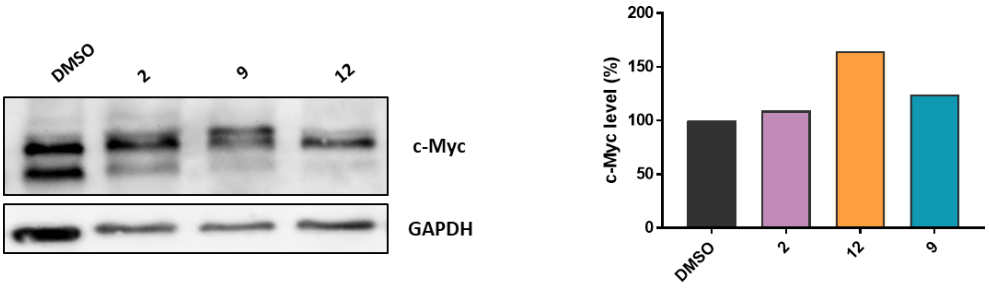


Figure S5. c-Myc protein levels after treatment with Sirt2 inhibitor (10 µM) or DMSO for 48 h. GAPDH levels were used as loading control and c-Myc protein levels of the samples treated with Sirt2 inhibitors were normalized to the DMSO treated cells.

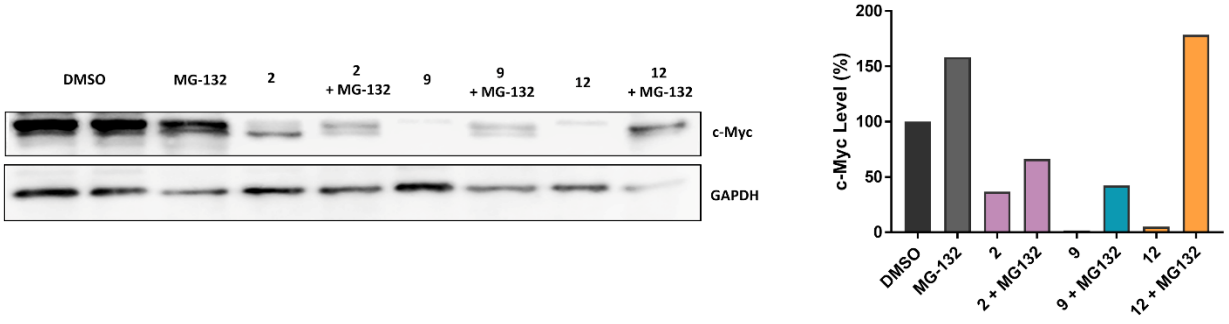


Figure S6. Proteasomal degradation of c-Myc in PC-3M-luc cells induced by Sirt2 inhibitors. Immunoblot for c-Myc protein levels after treatment with Sirt2 inhibitor (10 µM) or DMSO in the presence or absence of the proteasome inhibitor MG-132. GAPDH levels were used as loading control.

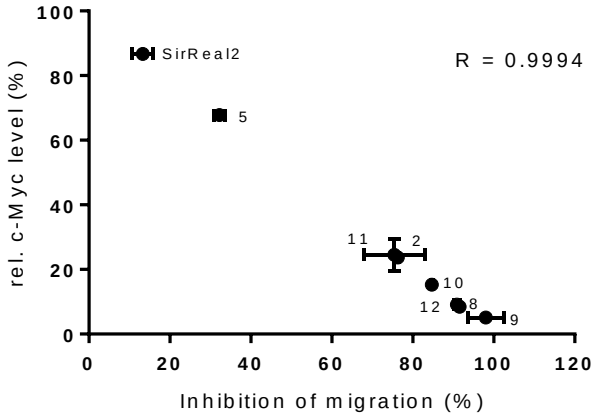


Figure S7. Correlation between inhibition of migration and reduction of c-Myc protein levels. Plot correlating inhibition of migration and reduction of c-Myc levels at 10 µM of Sirt2 inhibitor in PC-3M-luc cells.

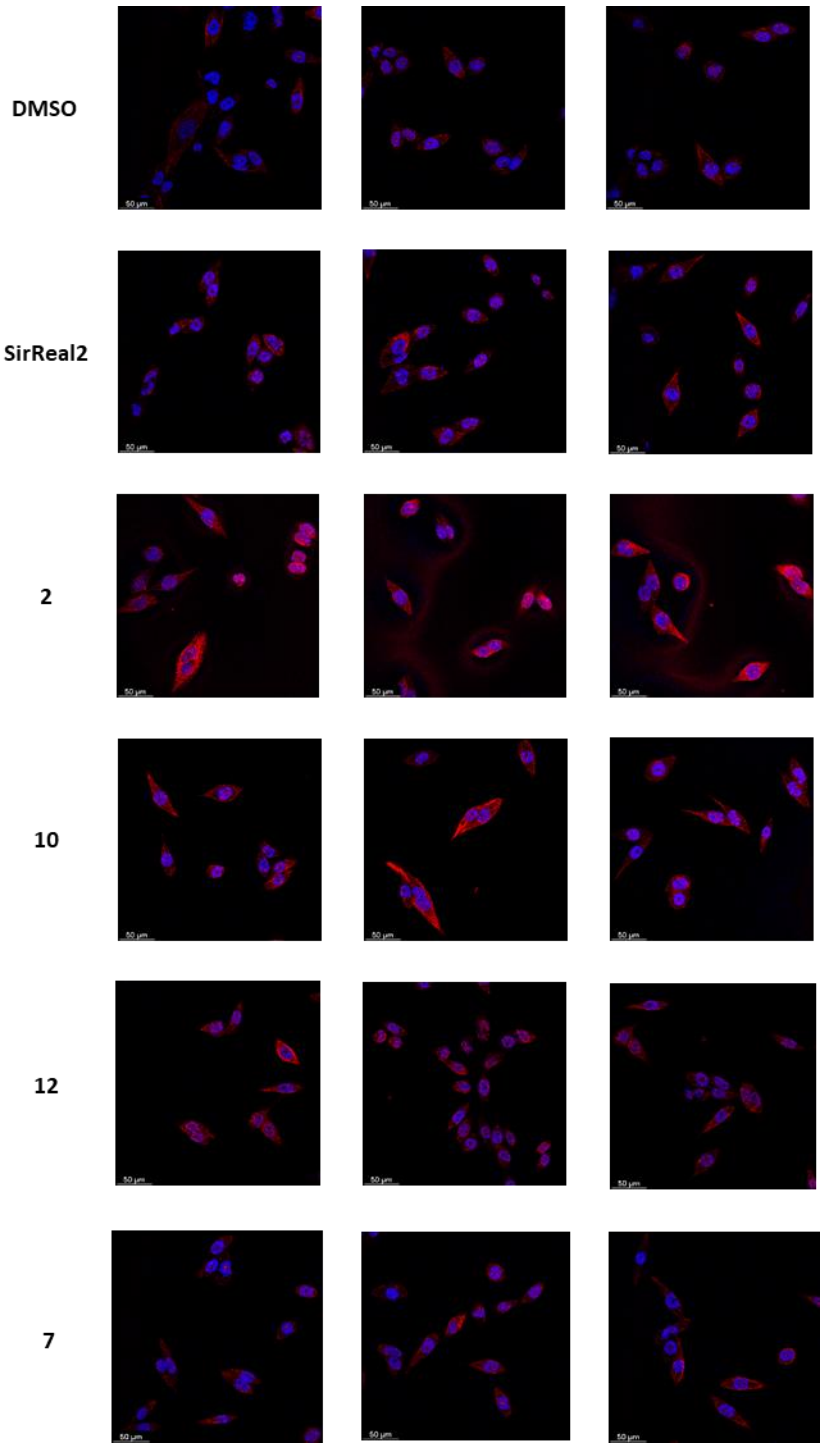


Figure S8. Immunostaining of acetylation levels of α-tubulin. Acetylation levels of α-tubulin (red) in the presence or absence of Sirt2 inhibitor (20 µM) in PC-3M-luc cells. Nuclei were DAPI-stained (blue).

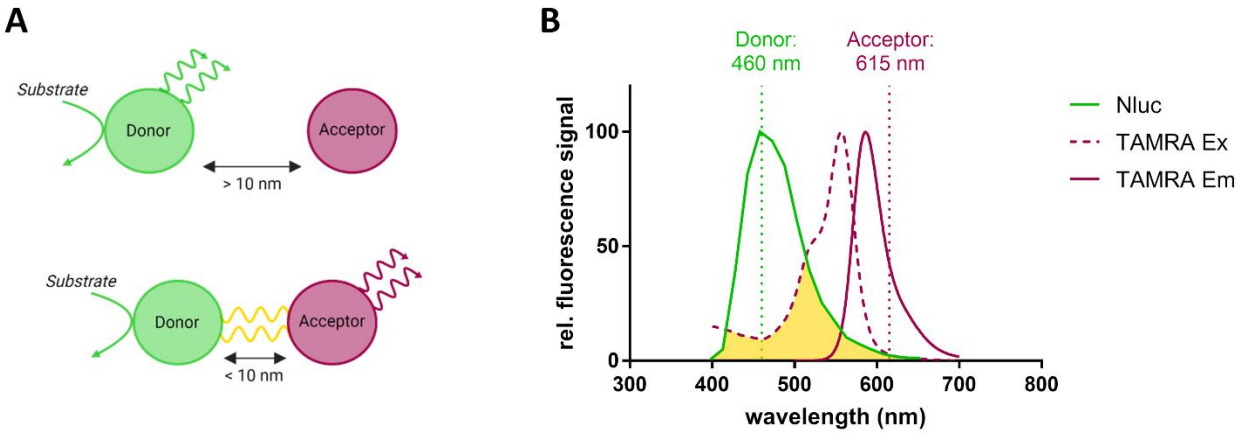


Figure S9 Principle and spectrum of the NanoBRET assay. A) Principle of the NanoBRET assay. The Donor emits light after conversion of the substrate. If an acceptor is in close proximity (< 10nm), energy is transferred from the donor to the acceptor and leads to an acceptor emission signal. B) Bioluminescence spectrum of the Nanoluciferase (Nluc) in green and excitation (broken red line) and emission (solid red line) spectrum of the acceptor (TAMRA). Energy transfer between donor and acceptor is enabled by the overlap (yellow) of the luminescence spectrum of Nluc and the excitation spectrum of TAMRA. In the NanoBRET setup, the donor signal is measured at 460 nm and the acceptor signal at 615 nm.

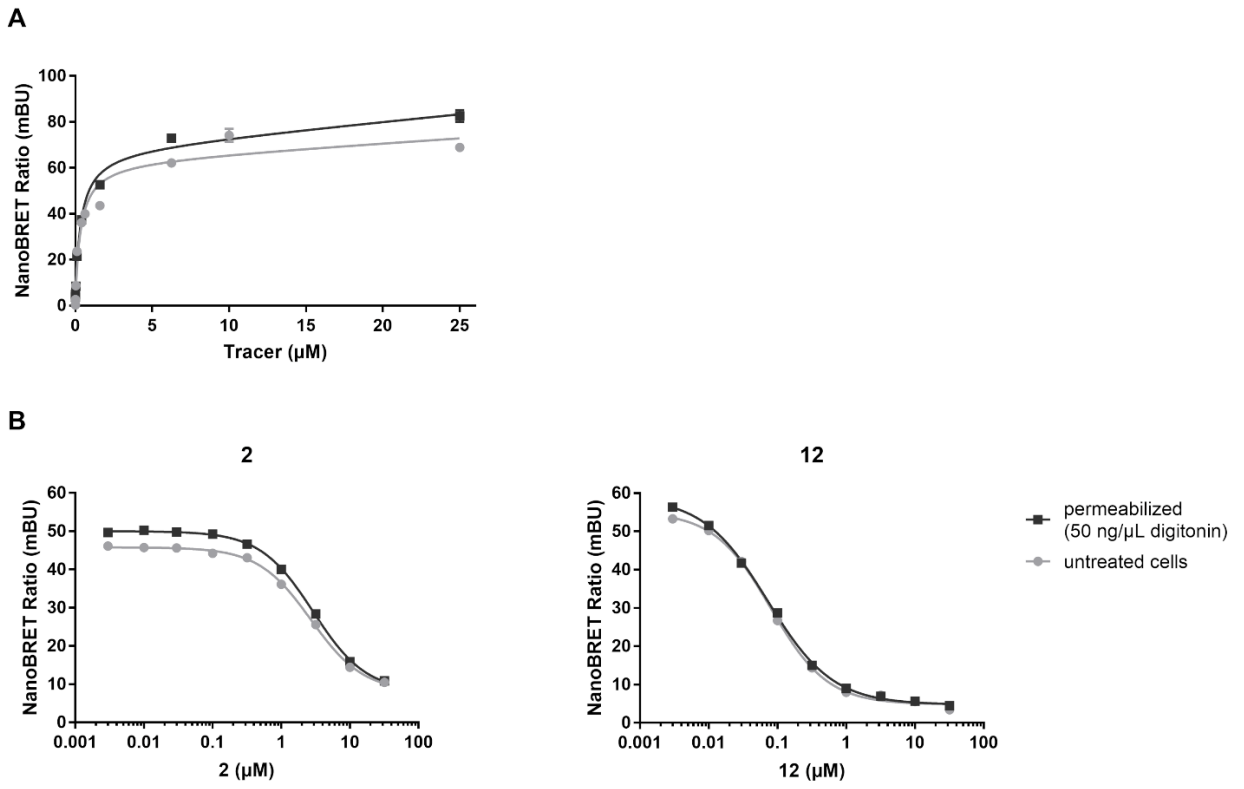


Figure S10. Cellular permeability studies using the NanoBRET assay. A) NanoBRET assay curves displaying the relative affinity of the tracer (**13**) in permeabilized (digitonin-treated, 50 ng/µL) and

non-permeabilized cells. B) NanoBRET assay curves displaying the relative affinity of **2** (left) and **12** (right) in permeabilized respectively untreated cells.

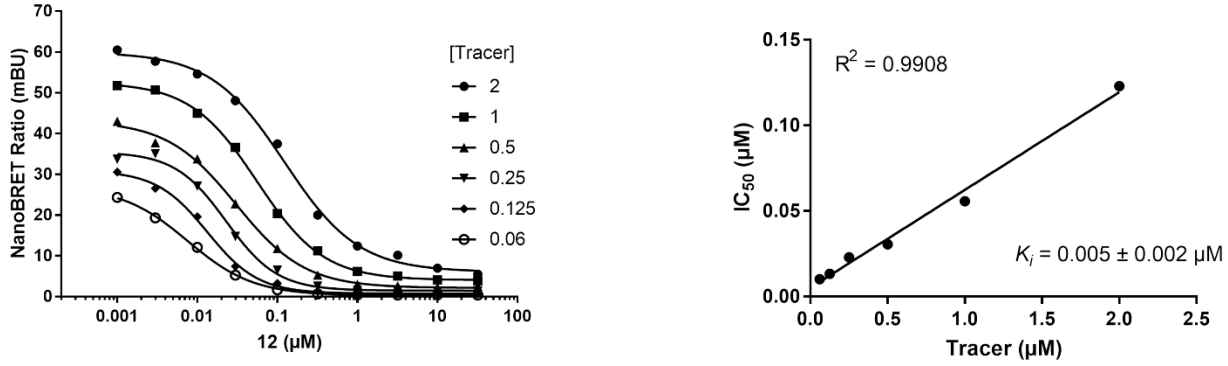


Figure S11. Determination of K_i for 12 based on Cheng-Prusoff equation using the NanoBRET assay. A) Determination of apparent IC_{50} values by fitting individual curves with the sigmoidal dose-response curve. B) Replots of apparent IC_{50} values as a function of tracer concentration.

Table S1: Kinetic parameters for Sirt2-mediated deacetylation (ZMAL) and demyristoylation (ZMML). Values for K_M, k_{cat} and k_{cat} K_M^{-1} are presented as mean ± SD (n = 3).

Substrate	K_M (µM)	k_{cat} (s^{-1})	k_{cat} K_M^{-1} ($s^{-1}M^{-1}$)
ZMAL	510 ± 95	(6.7 ± 0.8) x 10^{-3}	14.0 ± 6.1
ZMML	6.7 ± 1.0	(1.7 ± 0.1) x 10^{-5}	2.3 ± 0.3

Table S2: **Measured thermal shift assay ΔT_m values, pIC_{50} values for Sirt2 inhibition and the calculated MM-GBSA interaction energies (PDB ID 5DY5) for the studied inhibitors.**

Name	ΔT_m (°C)	pIC_{50}	**E GBSA (kcal/mol)**
1	3.75	6.36	-58.71
2	5.69	6.92	-84.07
(S)-3	3.01*	6.59*	-49.36
***(R)*-3**			-46.40
***(S)*-4**	2.70*	4.34*	-41.10

***(R)*-4**			-44.70
5	4.28	6.34	-72.77
***(S)*-6**	2.55*	5.22*	-55.35
***(R)*-6**			-52.72
7	1.40	3.21	---
8	4.26	6.80	-82.47
9	5.35	6.77	-86.53
10	4.51	6.82	-90.34
11	4.17	6.96	-84.35
12	6.63	6.92	-98.22

* Measured for the racemate.

Table S3. Results of MTS assay. Results of MTS assay for different Sirt2 inhibitors (10 µM) in HGC-27, PC-3M-luc, HEK293T, HL-60 and MCF-7 cells. Values represent % inhibition of cell viability as mean ± SD. Experiments were performed at least twice as triplicates. n.i. = no inhibition (% inhibition < 10%).

Compound	**HGC-27**	**PC-3M-luc**	**HEK293T**	**HL-60**	**MCF-7**
SirReal2	27.2 ± 10.1	12.1 ± 1.1	18.8 ± 1.6	n.i.	16.2 ± 3.5
5	44.2 ± 21.9	21.2 ± 13.8	n.i.	24.3 ± 6.9	11.0 ± 5.0
6	11.6 ± 6.6	n.i.	n.i.	n.i.	n.i.
7	29.0 ± 7.0	n.i.	11.6 ± 1.6	n.i.	n.i.
2	6.8 ± 4.4	27.4 ± 1.9	20.8 ± 8.3	9.9 ± 2.8	10.5 ± 1.7
8	40.6 ± 4.5	22.0 ± 1.4	18.4 ± 2.9	16.6 ± 4.7	n.i.
9	32.7 ± 10.8	27.0 ± 0.2	55.1 ± 8.0	7.7 ± 2.0	10.8 ± 3.1
10	53.1 ± 10.7	33.8 ± 2.1	70.9 ± 1.7	4.6 ± 1.4	n.i.
11	13.5 ± 2.9	29.2 ± 3.4	32.1 ± 7.6	4.6 ± 3.6	n.i.

12	60.3 ± 6.6	30.9 ± 2.2	63.0 ± 6.5	13.4 ± 9.0	n.i.

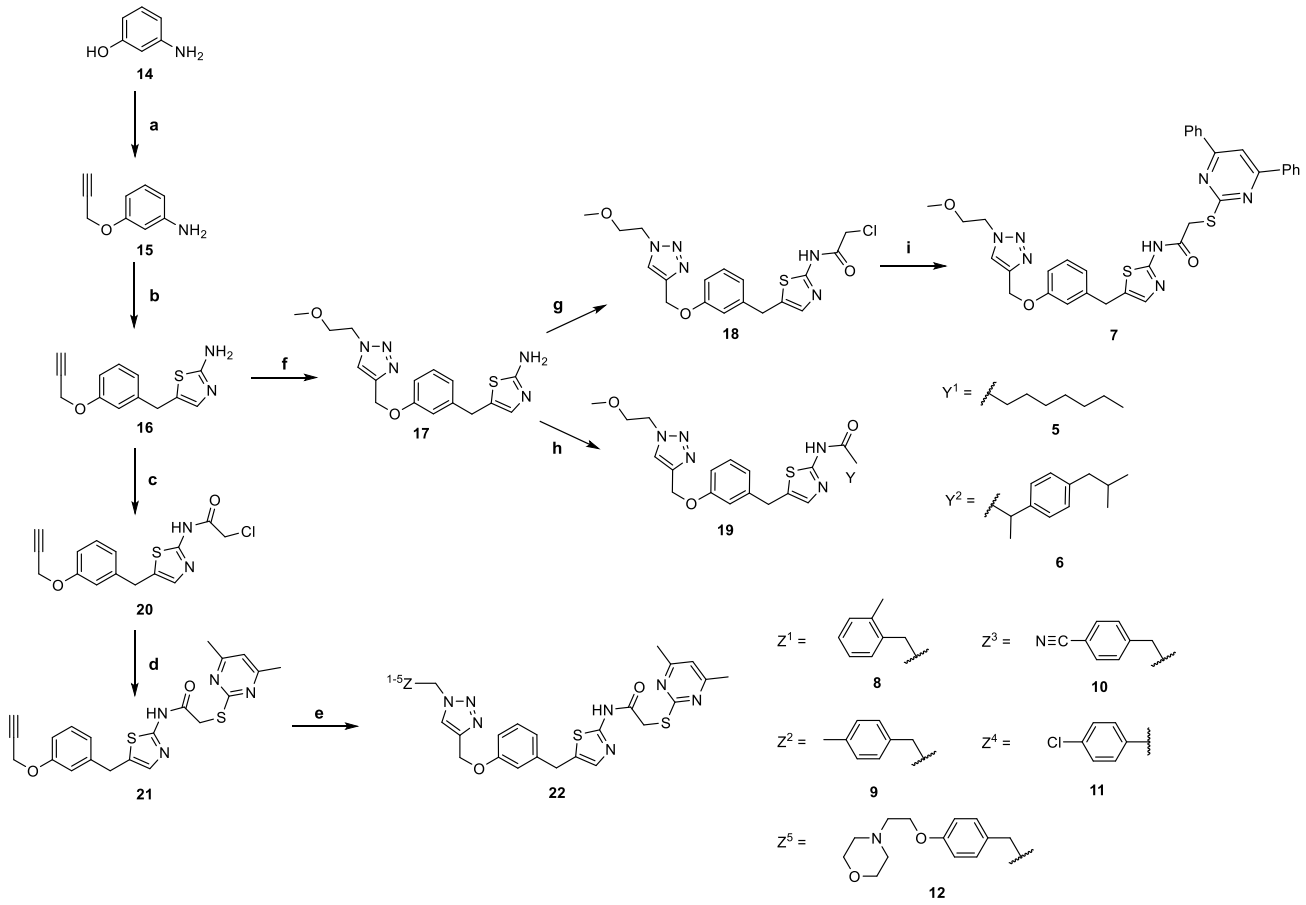
Table S4. Inhibition of colony formation. Results of colony formation assays (CFU) in PC-3M-luc cells after treatment with 25, 10 and 1 µM of selected Sirt2 inhibitors. Results are presented as mean ± SD.

Compound	Concentration	Inhibition (%)	Compound	Concentration	Inhibition (%)
SirReal2	25 µM	4.3 ± 0.4	**10**	25 µM	50.1 ± 4.8
	10 µM	13.8 ± 8.7		10 µM	30.4 ± 13.2
	1 µM	27.4 ± 8.7		1 µM	10.8 ± 4.3
2	25 µM	44.0 ± 7.8	**11**	25 µM	42.4 ± 0.8
	10 µM	15.1 ± 13.0		10 µM	29.8 ± 6.3
	1 µM	13.5 ± 10.8		1 µM	17.0 ± 9.9
9	25 µM	32.9 ± 5.4	**12**	25 µM	94.2 ± 2.6
	10 µM	28.0 ± 7.6		10 µM	8.7 ± 7.8
	1 µM	4.8 ± 3.1		1 µM	7.2 ± 7.4

Table S5: Results from in vitro fluorescence polarization binding assay. IC_{50} [µM] values (mean ± SD) are presented for different Sirt2 inhibitors. n.i. = no inhibition (% inhibition < 10% @ 10 µM)

Compound	**SirReal2**	**2**	**8**	**9**	**10**
IC_{50} [µM]	0.37 ± 0.10	0.35 ± 0.13	0.76 ± 0.16	0.16 ± 0.04	0.15 ± 0.03

Compound	**11**	**12**
IC_{50} [µM]	0.22 ± 0.04	0.07 ± 0.03

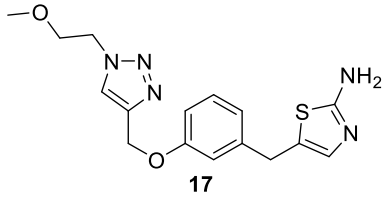


Scheme S1. Synthesis of SirReal-based Sirt2 inhibitors. Reagents and conditions: a) propargyl bromide, NaOH, acetonitrile, 20 °C, overnight, 54% yield; b) $NaNO_2$, HCl, water, 0 °C, 20 min; acrolein, $CuCl_2 \cdot 2\ H_2O$, acetone, 20 °C, 3 h; thiourea, ethanol, reflux, 2 h, 16% yield; c) chloroacetyl chloride, N,N-diisopropylethylamine, acetonitrile, 20 °C, 2 h, 99% yield; d) 4,6-dimethyl-2-methylsulfanylpyrimidine, Na_2CO_3, KI, DMSO, 20 °C, 2 h, 76% yield; e) benzyl/aryl azide, sodium ascorbate, $CuSO_4$, TBTA, water/tBuOH/DMF (1:1:1), 20 °C, 2 h; 50-59% yield f) 1-azido-2-methoxyethane, sodium ascorbate, CuSO4, TBTA, water/tBuOH/DMF (1:1:1), 20 °C, 2 h, 75% yield; g) chloroacetyl chloride, acetonitrile, N,N-diisopropylethylamine, 20 °C, 2 h, 88% yield; h) for **5**: octanoyl chloride, DIPEA, acetonitrile, 20 °C, 3.5 h, 81% yield; for **6**: Ibuprofen, EDC*HCl, DMAP, DCM, 20 °C, overnight, 97% yield; i) mercaptopyrimidine derivative, DMSO, Na_2CO_3, KI, 20 °C, 2 h, 37% yield.

Compound syntheses

The syntheses of **15**, **16**, **20** and **21** were performed according to previously published procedures.[1]

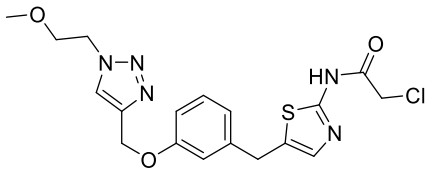
5-(3-((1-(2-methoxyethyl)-1H-1,2,3-triazol-4-yl)methoxy)benzyl)thiazol-2-amine (17)



17

16 ($8.19*10^{-4}$ mol, 200 mg) and 1-azido-2-methoxyethane ($8.19*10^{-4}$ mol, 165.4 µl, 82.8 mg, 1 equiv.) were dissolved in *tert*butanol/water (1:1, 2.2 mL) and a solution of TBTA ($8.19*10^{-5}$ mol, 43.46 mg, 0.1 equiv.) in DMF (8.2 mL) was added. A copper sulfate solution (0.1 M, 812 µL, 0.1 equiv.) and subsequently a solution of sodium ascorbate (0.2 M, 1.6 mL, 0.2 equiv.) were added and the mixture was stirred at room temperature for 2 h. After quenching the reaction with water (4 mL) all solvents were evaporated and the residue was purified by automated flash chromatography (gradient of DCM/methanol) to get 213 mg ($6.20*10^{-4}$ mol, 75 %) of **17** as a white solid. R_f: 0.62 (DCM/MeOH 95:5); ^{1}H-NMR (DMSO-D_6, 400 MHz, δ[ppm]): 8.17 (s, 1 H, triazole-H); 7.21-7.25 (m, 1 H, phenyl-H-5); 6.80-6.91 (m, 3 H, phenyl-H-2,4,6); 6.72 (bs, 3 H, amine-H_2 and aminothiazole-H); 5.11 (s, 2 H, triazole-C$\underline{H}_2$-O-Ar); 4.54 (t, 2 H, ^{3}J = 5.08, H_3C-O-CH_2-C$\underline{H}_2$-triazole); 3.87 (s, 2 H, Ar-C$\underline{H}_2$-aminothiazole); 3.74 (t, 2 H, ^{3}J = 5.08, H_3C-O-C$\underline{H}_2$-CH_2-triazole); 3.24 (s, 3 H, $\underline{H}_3$C-O-CH_2-CH_2-triazole); impurities: 3.35 (H_2O), 5.77 (DCM); ^{13}C-NMR (DMSO-D_6, 100 MHz, δ[ppm]): 158.6 (phenyl C3, aminothiazole C2); 143.0 (triazole C4); 142.6 (phenyl C1); 136.0 (aminothiazole C4); 129.9 (phenyl C5); 125.3 (triazole C5); 121.2 (phenyl C6); 115.2 (phenyl C2); 112.7 (phenyl C4); 70.5 (H_3C-O-$\underline{C}H_2$-CH_2-triazole); 61.4 (triazole-$\underline{C}H_2$-O-Ar); 58.4 ($H_3\underline{C}$-O-CH_2-CH_2-triazole); 49.7 (H_3C-O-CH_2-$\underline{C}H_2$-triazole); 32.9 (Ar-$\underline{C}H_2$-aminothiazole); LRMS m/z (ESI$^+$): $[M+H]^+$ calculated for $C_{16}H_{20}N_5O_2S^+$: 346.13, found: 346.2; HPLC (C18): retention time 13.24 min, 96.1 %.

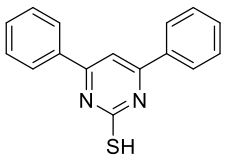
2-chloro-N-(5-(3-((1-(2-methoxyethyl)-1H-1,2,3-triazol-4-yl)methoxy)benzyl)thiazol-2-yl)acetamide (18)



18

The amine **17** (95 mg, 0.3 mmol) was dissolved in acetonitrile (5 mL) and *N,N*-diisopropyl amine (72 µL, 53 mg, 0.4 mmol, 1.5 equiv.) was added. At 0 °C chloroacetyl chloride (33 µL, 47 mg, 0.4 mmol, 1.5 equiv.) was slowly added whilst stirring. The mixture was allowed to warm up to room temperature and stirred for 2 h. Volatiles were evaporated to give a red-brown oil. Addition of water (5 mL) led to precipitation of a light brown solid. The precipitate was washed with hydrochloric acid (1 M) and water to yield 112 mg (0.27 mmol, 88%) of a beige solid after drying. R_f: 0.69 (DCM/MeOH 95:5); ^{1}H-NMR ($CDCl_3$, 400 MHz, δ[ppm]): 7.74 (s, 1 H, triazole-H); 7.24 (t, 1 H, ^3J = 8.00 Hz, phenyl H-5); 7.17 (s, 1 H, aminothiazole-H); 6.90-6.84 (m, 3 H, phenyl H-2,4,6); 5.19 (s, 2 H, triazole-CH2-O-Ar); 4.54 (t, 2 H, ^3J = 4.98 Hz, H3C-O-CH_2-$\underline{CH_2}$-triazole); 4.24 (s, 2 H, CO-CH_2-Cl); 4.06 (s, 2 H, Ar-CH_3-aminothiazole); 3.76 (t, 2 H, ^3J = 5.07 Hz, H_3C-O-$\underline{CH_2}$-CH_2-triazole); 3.25 (s, 3 H, $\underline{H_3C}$-O-CH_2-CH_2-triazole). ^{13}C-NMR (DMSO-D_6, 100 MHz, δ[ppm]): 172.4 ((C=O)-CH_2-Cl); 158.5 (aminothiazole C2); 157.5 (phenyl C3); 143.8 (triazole C4); 140.5 (phenyl C1); 133.2 (aminothiazole C4); 129.8 (phenyl C5); 123.8 (triazole C5); 121.3 (phenyl C6); 115.1 (phenyl C2); 113.0 (phenyl C4); 70.6 (H_3C-O-$\underline{C}H_2$-CH_2-triazole); 61.9 (triazole-$\underline{C}H_2$-O-Ar); 59.0 ($H_3\underline{C}$-O-CH_2-CH_2-triazole); 50.3 (H_3C-O-CH_2-$\underline{C}H_2$-triazole); 41.9 ((C=O)-$\underline{C}H_2$-Cl); 32.8 (Ar-$\underline{C}H_2$-aminothiazole). LRMS m/z (ESI$^+$): $[M+H]^+$ calculated for $C_{18}H_{21}ClN_5O_3S^+$: 422.10 and 424.1 in a 3:1 ratio, found: 422.3 and 424.3 in a 3:1 ratio.

4,6-diphenylpyrimidine-2-thiol (23)

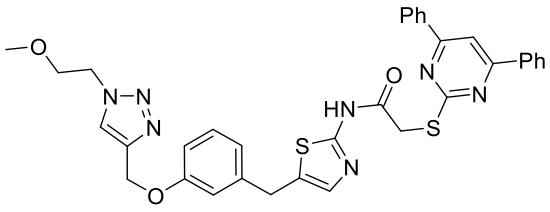


23

To a solution of sodium *tert*butyloxide (480 mg, 5 mmol, 1 equiv.) in ethanol (17 mL) thiourea (381 mg, 5 mmol) and 1,3-Diaryl-2-propen-1-one (1041 mg, 5 mmol, 1 equiv.) were added

successively. After refluxing for 1 h the solvent was evaporated, and the residue was dissolved in water (25 mL) and the mixture was acidified with acetic acid (1.7 mL). The resulting precipitate was filtered off and washed with ethanol. The crude product was dissolved in a solution of *tert*butyloxide in ethanol (3 M, 100 mL) and refluxed for 2 h to get the 283 mg (1.07 mmol, 21%) of **23** as yellow solid. R_f: 0.54 (CH/EE 9:1); ^{1}H-NMR ($CDCl_3$, 400 MHz, δ[ppm]): 8.08-8.06 (m, 4 H, 2x phenyl H2,6); 7.60-7.48 (m, 6 H, 2x phenyl H3,4,5);7.15 (s, 1 H, pyrimidine H5). LRMS m/z (ESI^+): $[M+H]^+$ calculated for $C_{16}H_{13}N_2S^+$: 265.08, found: 265.3.

2-((4,6-diphenylpyrimidin-2-yl)thio)-N-(5-(3-((1-(2-methoxyethyl)-1H-1,2,3-triazol-4-yl)methoxy)benzyl)thiazol-2-yl)acetamide (7)

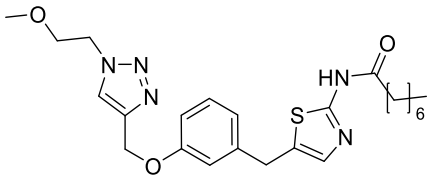


7

23 (12.1 mg, 0.05 mM, 1 equiv.) was dissolved in 183 µL DMSO. Na_2CO_3 (11.0 mg, 0.09 mM, 2 equiv.) and KI (7.6 mg, 0.05 mM, 1 equiv.) were added. The mixture was stirred for 15 min at room temperature before the alkyl chloride **18** (20 mg, 0.05 mM, 1 equiv.) was added to the reaction mixture. After stirring for 2 h 914 µL water were added. The aqueous layer was extracted with ethyl acetate (3×20 mL) and the combined organic layers were dried over Na_2SO_4 and solvents were evaporated. The crude products were purified by automated flash column chromatography (CH/EE gradient), to get 11 mg (17 µmol, 37 %) of **7** respectively. R_f: 0.36 (DCM/MeOH 95:5); ^{1}H-NMR ($CDCl_3$, 400 MHz, δ[ppm]): 10.70 (s, 1 H, aminothiazole-NH-(C=O)); 8.12-9.09 (m, 4 H, pyrimidine-(phenyl H2,6)$_2$); 7.85 (s, 1 H, pyrimidine H5); 7.71 (s, 1 H, triazole H); 7.53-7.48 (m, 6 H, pyrimidine-(phenyl H3,4,5)$_2$); 7.20 (t, 1 H, ^{3}J = 8.02 Hz, phenyl H5); 7.06 (s, 1 H, aminothiazole H); 6.88-6.80 (m, 3 H, phenyl H2,4,6); 5.16 (s, 2 H, O-CH_2-triazole); 4.52 (t, 2 H, ^{3}J = 5.09 Hz, triazole-CH_2-CH_2-O-CH_3); 4.11 (s, 2 H, phenyl-CH_2-aminothiazole); 4.00 (s, 2 H, (C=O)-CH_2-S-pyrimidine); 3.75 (t, 2 H, ^{3}J = 5.08 Hz, triazole-CH_2-CH_2-O-CH_3); 3.33 (s, 3 H, triazole-CH_2-CH_2-O-CH_3). ^{13}C-NMR ($CDCl_3$, 100 MHz, δ[ppm]): 170.0 (pyrimidine C2); 166.8 ((C=O)-CH_2-S-pyrimidine); 165.6 (pyrimidine C4,6); 158.5 (phenyl C1); 143.9 (triazole C4); 140.9 (phenyl C3); 136.1 (pyrimidine-(phenyl C4)$_2$); 134.1 (aminothiazole C2); 131.4 (aminothiazole C3); 129.7

(phenyl C5); 129.0 (pyrimidine-(phenyl C3,5)$_2$); 127.3 (pyrimidine-(phenyl C2,6)$_2$); 123.8 (triazole C5); 121.3 (phenyl C4); 115.1 (phenyl C2); 112.8 (phenyl C6); 109.4 (pyrimidine C5); 70.7 (triazole-CH_2-$\underline{C}H_2$-O-CH_3); 61.9 (O-$\underline{C}H_2$-triazole); 59.0 (triazole-CH_2-CH_2-O-$\underline{C}H_3$); 50.3 (triazole-$\underline{C}H_2$-CH_2-O-CH_3); 34.9 ((C=O)-$\underline{C}H_2$-S-pyrimidine); 32.9 (phenyl-$\underline{C}H_2$-aminothiazole). HRMS m/z (ESI$^+$): $[M+ACN+H]^+$ calculated for $C_{37}H_{36}N_7O_3S_2^+$: 691.23, found: 691.26. HPLC (C18): retention time 24.77 min, 95.8%.

N-(5-(3-((1-(2-methoxyethyl)-1H-1,2,3-triazol-4-yl)methoxy)benzyl)thiazol-2-yl)octanamide (5)

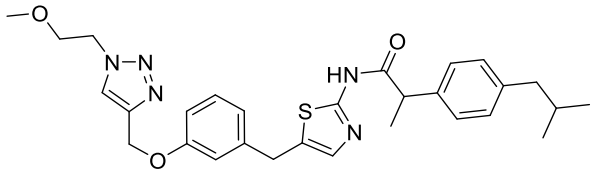


5

To a cooled solution of the amine **17** (20 mg, 0.06 mmol, 1 equiv.) in acetonitrile (2 mL) *N,N*-diisopropylethylamine (14.7 µL, 11.0 mg, 0.09 mmol, 1.5 equiv.) and octanoyl chloride (14.8 µL, 14.1 mg, 0.09 mmol, 1.5 equiv.) were added whilst stirring. After 3.5 h at 30 °C the solvent was evaporated, and water was added to the residue. The aqueous phase was washed with ethyl acetate (3 x 20 mL). Subsequently, the combined organic phase was washed with brine, dried with $MgSO_4$ and the solvent was evaporated. The product was purified via flash chromatography (DCM/MeOH 95/5). Afterwards, the product was dissolved in a sodium hydroxide solution (2 M) and the alkaline solution was again extracted with ethyl acetate (3 x 20 mL) to remove traces of the actanoic acid, giving 22.1 mg (0.05 mmol, 81 %) of **5**. R_f: 0.57 (DCM/MeOH 95:5); ^{1}H-NMR (DMSO-D_6, 400.13 MHz, δ [ppm]): 11.91 (br s, 1 H, amide-H); 8.18 (s, 1 H, triazole-H); 7.23-7.24 (m, 1 H, phenyl-H-5); 7.23 (s, 1 H aminothiazole-H); 6.84-6.93 (m, 3 H, phenyl-H-2,4,6); 5.11 (s, 2 H, triazole-C$\underline{H}_2$-O-Ar); 4.54 (dd, 2 H, ^{3}J = 5.03, 5.33 Hz, H_3C-O-CH_2-C$\underline{H}_2$-triazole); 4.04 (s, 2 H, Ar-C$\underline{H}_2$-aminothiazole); 3.73 (dd, 2 H, ^{3}J = 5.36, 5.07 Hz, H_3C-O-C$\underline{H}_2$-CH_2-triazole); 3.24 (s, 3 H, $\underline{H}_3$C-O-CH_2-CH_2-triazole); 2.37 (t, 2 H, J = 7.33, CO-C$\underline{H}_2$-CH_2-C_4H_8-CH_3); 1.55 (m, 2 H, CO-CH_2-C$\underline{H}_2$-C_4H_8-CH_3); 1.25 (s, 8 H, CO-CH_2-CH_2-$C_4\underline{H}_8$-CH_3); 0.85 (t, 3 H, J = 6.71, CO-CH_2-CH_2-C_4H_8-C$\underline{H}_3$); impurities: 3.34 (H_2O) ^{13}C-NMR (DMSO-D_6, 100.61 MHz, δ [ppm]): 171.46 q (octanoyl-$\underline{C}_1$); 158.61 (phenyl C-3); 157.34 (aminothiazole C-2); 142.92 (triazole C-4); 142.36 (phenyl C-1); 134.94 (aminothiazole C-4); 131.15 (aminothiazole C-5); 130.05 (phenyl C-5); 125.31 (triazole C-5); 121.31 (phenyl C-4); 115.36 (phenyl C-2); 112.81 (phenyl C-6); 70.53 (H_3C-O-$\underline{C}H_2$-

CH_2-triazole); 61.38 (triazole-$\underline{C}H_2$-O-Ar); 58.34 ($H_3\underline{C}$-O-CH_2-CH_2-triazole); 49.65 (H_3C-O-CH_2-$\underline{C}H_2$-triazole); 35.20 (octanoyl-$\underline{C}_2$); 32.32 (Ar-$\underline{C}H_2$-aminothiazole); 31.51 (octanoyl-$\underline{C}_6$); 28.88 (octanoyl-$\underline{C}_4$); 28.78 (octanoyl-$\underline{C}_5$); 25.14 (octanoyl-$\underline{C}_3$); 22.47 (octanoyl-$\underline{C}_7$); 14.35 (octanoyl-$\underline{C}_8$); the carbon atoms of the octanoyl residue were assigned according to Paukstelis *et al*.[136] LMRS m/z (ESI^+): $[M+Na]^+$ calculated for $C_{24}H_{33}N_5NaO_3S^+$: 494.21, found: 494.2, $[M+Li]^+$ calculated for $C_{24}H_{33}LiN_5O_3S^+$: 478.23, found: 478.2; HPLC (C18): retention time 23.60 min, 96.08%.

2-(4-isobutylphenyl)-N-(5-(3-((1-(2-methoxyethyl)-1H-1,2,3-triazol-4-l)methoxy)benzyl)thiazol-2-yl)propenamide (6)

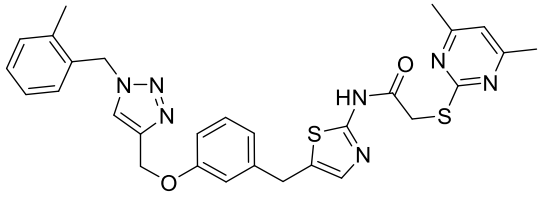


6

A mixture of ibuprofen (29.8 mg, 0.15 mmol, 2.5 equiv.), EDCl (27.9 mg, 0.15 mmol, 2.5 equiv.) and DMAP (14.2 mg, 0.12 mmol, 2 equiv.) in DCM (3 mL) was stirred for 30 min, before **17** (20 mg, 0.06 mmol, 1 equiv.), dissolved in DCM (2 mL), was added. The reaction mixture was stirred overnight and then quenched with water. The aqueous phase was extracted with DCM (3 x 10 mL) and the combined organic phases were washed with brine, dried with $MgSO_4$ and evaporated. The residue was purified via flash chromatography (DCM/MeOH 95/5) to give 29.9 mg (0.06 mmol, 97 %) of **6**. R_f: 0.76 (DCM/MeOH 95:5); ^{1}H-NMR (DMSO-D_6, 400.13 MHz, δ [ppm]): 12.13 (br s, 1 H, amide-H); 8.16 (s, 1 H, triazole-H); 7.23-7.25 (m, 4 H, aminothiazole-H, phenyl-H-5, ibuprofen-Ar-H2,6); 7.09-7.11 (m, 2 H, ibuprofen-Ar-H3,5); 6.82-6.91 (m, 3 H, phenyl-H-2,4,6); 5.11 (s, 2 H, triazole-C$\underline{H}_2$-O-Ar); 4.53 (dd, 2 H, ^{3}J = 5.01, 5.39 Hz, H_3C-O-CH_2-C$\underline{H}_2$-triazole); 4.03 (s, 2 H, Ar-C$\underline{H}_2$-aminothiazole); 3.90 (q, 1 H, ^{3}J = 6.94 Hz, ibuprofen-Ar-C$\underline{H}$(CH_3)-CO) 3.72 (dd, 2 H, ^{3}J = 5.34, 5.01 Hz, H_3C-O-C$\underline{H}_2$-CH_2-triazole); 3.23 (s, 3 H, $\underline{H}_3$C-O-CH_2-CH_2-triazole); 2.39 (d, 2 H, ^{3}J = 7.30 Hz, ibuprofen-Ar-C$\underline{H}_2$-CH(CH_3)$_2$); 1.79 (m, 1 H, ibuprofen-Ar-CH_2-C$\underline{H}$(CH_3)$_2$); 1.38 (d, 3 H, ^{3}J = 6.88 Hz, ibuprofen-Ar-CH(C$\underline{H}_3$)-CO); 0.84 (d, 6 H, ^{3}J = 6.66, ibuprofen-Ar-CH_2-CH(C$\underline{H}_3$)$_2$); impurities: 3.35 (H_2O) ^{13}C-NMR (DMSO-D_6, 100.61 MHz, δ [ppm]): 172.57 (ibuprofen-$\underline{C}$O); 158.59 (phenyl C-3); 157.31 (aminothiazole C-2); 142.92 (triazole C-4); 142.30 (phenyl C-1); 140.22 (ibuprofen-Ar-C-4); 138.62 (ibuprofen-Ar-C-1); 135.05 (aminothiazole C-4); 131.48 (aminothiazole C-5); 130.06 (phenyl C-5); 129.44 (2 C, ibuprofen-Ar-C-2,6); 127.46 (2 C, ibuprofen-Ar-C-3,5);

125.30 (triazole C-5); 121.29 (phenyl C-4); 115.32 (phenyl C-2); 112.85 (phenyl C-6); 70.52 (H_3C-O-$\underline{C}H_2$-CH_2-triazole); 61.38 (triazole-$\underline{C}H_2$-O-Ar); 58.33 ($H_3\underline{C}$-O-CH_2-CH_2-triazole); 49.64 (H_3C-O-CH_2-$\underline{C}H_2$-triazole); 44.62 (ibuprofen-Ar-$\underline{C}H(CH_3)$-CO); 44.58 (ibuprofen-Ar-$\underline{C}H_2$-$CH(CH_3)_2$); 32.31 (Ar-$\underline{C}H_2$-aminothiazole); 30.01 (ibuprofen-Ar-CH_2-$\underline{C}H(CH_3)_2$); 22.59 (2 C, ibuprofen-Ar-CH_2-$CH(\underline{C}H_3)_2$); 18.63 (ibuprofen-Ar-$CH(\underline{C}H_3)$-CO); the carbon atoms of the ibuprofen residue were assigned according to Marathias *et al.*[137] LMRS m/z (ESI^+): $[M+Na]^+$ calculated for $C_{29}H_{35}N_5NaO_3S^+$: 556.22, found: 556.2, $[M+Li]^+$ calculated for $C_{29}H_{35}LiN_5O_3S^+$: 540.25, found: 540.2; HPLC (C18): retention time 25.33 min, 96.90%.

2-((4,6-dimethylpyrimidin-2-yl)thio)-N-(5-(3-((1-(2-methylbenzyl)-1H-1,2,3-triazol-4-yl)methoxy)benzyl)thiazol-2-yl)acetamide (8)

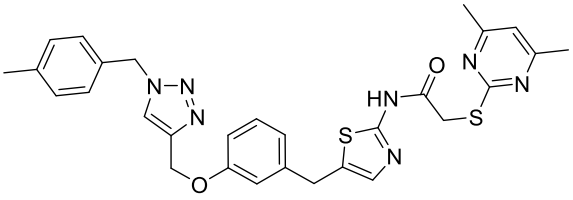


8

21 (0.08 mmol, 34 mg) and 1-azidomethyl-2-methylbenzole (0.08 mmol, 160 µl, 0.5 M in TBME, 1 equiv.) were dissolved in *tert*butanol/water (1:1, 1.4 mL) and a solution of TBTA ($8*10^{-3}$ mmol, 4.00 mg, 0.1 equiv.) in DMF (0.8 mL) was added. A copper sulfate solution (0.1 M, 80 µL, 0.1 equiv.) and subsequently a solution of sodium ascorbate (0.2 M, 160 µL, 0.2 equiv.) were added and the mixture was stirred at room temperature for 2 h. After quenching the reaction with water (2 mL) all solvents were evaporated and the residue was purified by automated flash chromatography (gradient of DCM/methanol) to get a 19.5 mg (0.03 mmol, 43%) of **8** as a white solid. ^{1}H-NMR (acetone-D_6, 400.16 MHz, δ [ppm]): 11.51 (s, 1H, amide-H); 7.93 (s, 1H, triazole H-5); 7.30-7.14 (m, 6H, benzyl H-3,4,5,6 & aminothiazole H-4 & Phenyl H-5); 6.99 (s, 1H, pyrimidine H-5); 6.94 (s, 1H, phenyl H-2); 6.89 (d, ^3J = 7.5 Hz, 2H, phenyl H-4,6); 5.65 (s, 2H, Ar-CH_2-N); 5.15 (s, 2H, triazole-CH_2-O-Ar); 4.08 (s, 4H, S-CH_2-CO-N & Ar-CH_2-aminothiazole); 2.41 (s, 6H, 2x CH_3-pyrimidine); 2,35 (s, 3H, CH_3-benzyl). ^{13}C-NMR (acetone-D_6, 100.61 MHz, δ [ppm]): 169.6 (pyrimidine C-2); 167.7 (pyrimidine C-4,6); 166.9 (Ar-NH-CO-CH_2-S-Ar); 158.7 (phenyl C-3); 143.6 (triazole C-1); 141.9 (Phenyl C-1); 136.6 (benzyl C-4); 134.9 (aminothiazole C-4); 134.0 (benzyl C-1); 130.5 (phenyl C-5); 128.9 (benzyl C-6); 123.6 (triazole C-5); 120.9 (phenyl C-6); 116.3

(pyrimidine C-5); 115.0 (phenyl C-2); 112.7 (phenyl C-4); 61.4 (triazole-CH_2-O-Ar); 51.3 (Ar-CH_2-triazole), 34.0 (CO-CH_2-S); 32.3 (Ar-CH_2-aminothiazole); 22.7 (2x CH_3-pyrimidine); 18,15 (CH_3-benzyl). LMRS m/z (ESI$^+$): $[M+Na]^+$ calculated for $C_{29}H_{29}N_7NaO_2S_2^+$: 594.17, found: 594.2; HPLC (C18): retention time 22.8 min, 98.7%.

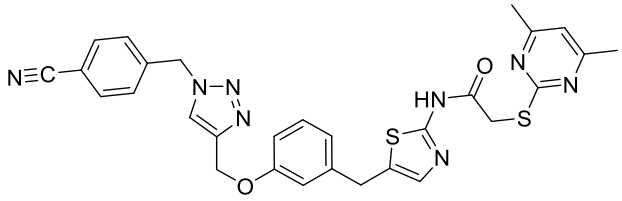
2-((4,6-dimethylpyrimidin-2-yl)thio)-N-(5-(3-((1-(4-methylbenzyl)-1H-1,2,3-triazol-4-yl)methoxy)benzyl)thiazol-2-yl)acetamide (9)



9

21 (0.08 mmol, 34 mg) and 1-azidomethyl-4-methylbenzole (0.08 mmol, 192 µl, 0.5 M in TBME, 1.2 equiv.) were dissolved in *tert*butanol/water (1:1, 1.4 mL) and a solution of TBTA ($8*10^{-3}$ mmol, 4.00 mg, 0.1 equiv.) in DMF (0.8 mL) was added. A copper sulfate solution (0.1 M, 80 µL, 0.1 equiv.) and subsequently a solution of sodium ascorbate (0.2 M, 160 µL, 0.2 equiv.) were added and the mixture was stirred at room temperature for 2 h. After quenching the reaction with water (2 mL) all solvents were evaporated and the residue was purified by automated flash chromatography (gradient of DCM/methanol) to get 27.0 mg (0.05 mmol, 59%) of **9** as a white solid. ^{1}H-NMR (DMSO-D_6, 400.16 MHz, δ [ppm]): 12.23 (s, 1 H, amide-H); 8.22 (s, 1 H, triazole H-5); 7.26 (1 H, aminothiazole H-4); 7.24-7.15 (m, 5 H, benzyl H-2,3,5,6 and Phenyl H-5); 6.93 (s, 1 H, pyrimidine H-5); 6.91-6.80 (m, 3 H, phenyl H-2,4,6); 5.54 (s, 2 H, Ar-CH_2-N); 5.09 (s, 2 H, triazole-CH_2-O-Ar); 4.09 (s, 2 H, S-CH_2-CO-N); 4.03 (s, 2 H, Ar-CH_2-aminothiazole); 2.28 (s, 9H, 2x CH_3-pyrimidine and CH_3-benzyl). ^{13}C-NMR (DMSO-D_6, 100.61 MHz, δ [ppm]): 169.3 (pyrimidine C-2); 167.4 (pyrimidine C-4,6); 167.2 (Ar-NH-CO-CH_2-S-Ar); 158.6 (phenyl C-3); 157.3 (aminothiazole C-2); 143.3 (triazole C-1); 142.3 (phenyl C-1); 137.9 (benzyl C-4); 135.2 (aminothiazole C-4); 133.4 (benzyl C-1); 131.5 (aminothiazole C-5); 130.0 (phenyl C-5); 129.7 (benzyl C3,5); 128.4 (benzyl C-2,6); 124.9 (triazole C-5); 121.3 (phenyl C-6); 116.5 (pyrimidine C-5); 115.3 (phenyl C-2); 112.9 (phenyl C-4); 61.4 (triazole-CH_2-O-Ar); 53.0 (Ar-CH_2-triazole), 34.5 (CO-CH_2-S); 32.3 (Ar-CH_2-aminothiazole); 23.7 (2x CH_3-pyrimidine); 21,1 (CH_3-benzyl). LMRS m/z (ESI$^+$): $[M+Na]^+$ calculated for $C_{29}H_{29}N_7NaO_2S_2^+$: 594.17, found: 594.2; HPLC (C18): retention time 22.9 min, 96.4%.

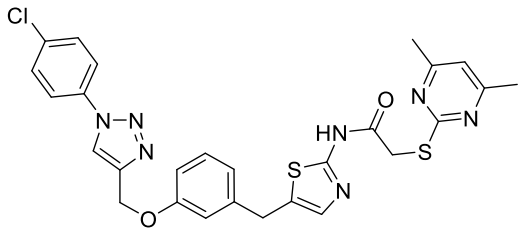
2-((4,6-dimethylpyrimidin-2-yl)thio)-N-(5-(3-((1-(4-cyanobenzyl)-1H-1,2,3-triazol-4-yl)methoxy)benzyl)thiazol-2-yl)acetamide (10)



10

21 (0.08 mmol, 34 mg) and 1-azidomethyl-2-methylbenzol (0.08 mol, 160 µl, 0.5 M in TBME, 1 equiv.) were dissolved in *tert*butanol/water (1:1, 1.4 mL) and a solution of TBTA ($8*10^{-3}$ mmol, 4.00 mg, 0.1 equiv.) in DMF (0.8 mL) was added. A copper sulfate solution (0.1 M, 80 µL, 0.1 equiv.) and subsequently a solution of sodium ascorbate (0.2 M, 160 µL, 0.2 equiv.) were added and the mixture was stirred at room temperature for 2 h. After quenching the reaction with water (2 mL) all solvents were evaporated and the residue was purified by automated flash chromatography (gradient of DCM/methanol) to get 23.2 mg (0.04 mmol, 50%) of **10** as a white solid. ^{1}H-NMR (DMSO-D_6, 400.16 MHz, δ [ppm]): 12.23 (s, 1 H, amide-H); 8.32 (s, 1 H, triazole H-5); 7.89-7.84 (m, 2 H, benzyl H-3,5); 7.45 (d, ^{3}J = 8.5 Hz, 2 H, benzyl H-2,6); 7.26 (1 H, aminothiazole H-4); 7.25-7.20 (m, 1 H, phenyl H-5); 6.94 (s, 1 H, pyrimidine H-5); 6.92-6.82 (m, 3 H, phenyl H-2,4,6); 5.73 (s, 2 H, Ar-CH_2-N); 5.12 (s, 2 H, triazole-CH_2-O-Ar); 4.09 (s, 2 H, S-CH_2-CO-N); 4.03 (s, 2 H, Ar-CH_2-aminothiazole); 2.28 (s, 6H, 2x CH_3-pyrimidine). ^{13}C-NMR (DMSO-D_6, 100.61 MHz, δ [ppm]): 169.3 (pyrimidine C-2); 167.4 (pyrimidine C-4,6); 167.3 (Ar-NH-CO-CH_2-S-Ar); 158.5 (phenyl C-3); 157.3 (aminothiazole C-2); 143.6 (triazole C-1); 142.3 (phenyl C-1); 141.9 (benzyl C-1); 135.2 (aminothiazole C-4); 133.2 (benzyl C-2,6); 131.5 (aminothiazole C-5); 130.1 (phenyl C-5); 129.1 (benzyl C3,5); 125.4 (triazole C-5); 121.4 (phenyl C-2); 119.0 (-C≡N); 116.5 (pyrimidine C-5); 115.4 (phenyl C-2); 112.9 (phenyl C-4); 61.4 (triazole-CH_2-O-Ar); 52.6 (Ar-CH_2-triazole), 34.5 (CO-CH_2-S); 32.2 (Ar-CH_2-aminothiazole); 23.7 (2x CH_3-pyrimidine). LMRS m/z (ESI$^+$): $[M+Na]^+$ calculated for $C_{29}H_{26}N_8NaO_2S_2^+$: 605.15, found: 605.2; HPLC (C18): retention time 21.2 min, 99.9%.

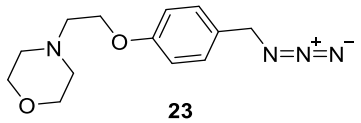
2-((4,6-dimethylpyrimidin-2-yl)thio)-N-(5-(3-((1-(4-chlorophenyl)-1H-1,2,3-triazol-4-yl)-methoxy)-benzyl)-thiazol-2-yl)-acetamide (11)



11

21 (0.08 mmol, 34 mg) and 1-azido-4-chlorobenzene (0.10 mmol, 160 µl, 0.5 M in TBME, 1 equiv.) were dissolved in *tert*butanol/water (1:1, 1.4 mL) and a solution of TBTA ($8*10^{-3}$ mmol, 4.00 mg, 0.1 equiv.) in DMF (0.8 mL) was added. A copper sulfate solution (0.1 M, 80 µL, 0.1 equiv.) and subsequently a solution of sodium ascorbate (0.2 M, 160 µL, 0.2 equiv.) were added and the mixture was stirred at room temperature for 2 h. After quenching the reaction with water (2 mL) all solvents were evaporated and the residue was purified by automated flash chromatography (gradient of DCM/methanol) to get 18.4 mg (0.03 mmol, 40%) of **11** as a white solid. ^{1}H-NMR (DMSO-D_6, 400.16 MHz, δ [ppm]): 12.23 (s, 1 H, Amide-H); 8.97 (s, 1 H, triazole H-5); 7.99-7.92 (m, 2 H, benzyl H-2,6); 7.73-7.63 (m, 2 H, benzyl H-3,5); 7.29-7.22 (m, 2 H, phenyl H5 and aminothiazole H-4); 6.99-6.86 (m, 4 H, phenyl H-2,4,6 and pyrimidine H-5); 5.21 (s, 2 H, triazole-CH_2-O-Ar); 4.08 (s, 2 H, S-CH_2-CO-N); 4.06 (s, 2 H, Ar-CH_2-aminothiazole); 2.28 (s, 6 H, 2x CH_3-pyrimidine). ^{13}C-NMR (DMSO-D_6, 100.61 MHz, δ [ppm]): 169.3 (pyrimidine C-2); 167.4 (pyrimidine C-4,6); 167.2 (Ar-NH-CO-CH_2-S-Ar); 158.6 (phenyl C-3); 157.3 (aminothiazole C-2); 144.5 (triazole C-1); 142.4 (phenyl C-1); 135.8 (benzyl C-1); 135.2 (aminothiazole C-4); 133.4 (benzyl C-4); 131.4 (aminothiazole C-5); 130.3 (benzyl C-3,5); 130.1 (phenyl C-5); 123.3 (triazole C-5); 122.3 (benzyl C-2,6); 121.5 (phenyl C-6); 116.5 (pyrimidine C-5); 115.4 (phenyl C-2); 112.9 (phenyl C-4); 61.3 (triazole-CH_2-O-Ar); 34.5 (CO-CH_2-S); 32.3 (Ar-CH_2-aminothiazole); 23.7 (2x CH_3-pyrimidine). LMRS m/z (ESI$^+$): $[M+Na]^+$ calculated for $C_{27}H_{24}N_7NaO_2S_2Cl^+$: 600.10, found: 600.1; HPLC (C18): retention time 24.1 min, 98.3%.

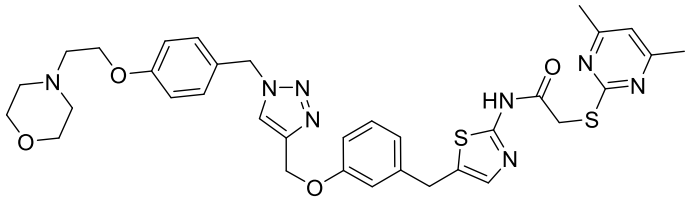
4-(2-(4-(azidomethyl)phenoxy)ethyl)morpholine (23)



23

(4-(2-morpholinoethoxy)phenyl)methanol[2] (1.26 mmol, 300 mg, 1 equiv.), sodium azide (1.52 mmol, 99 mg, 1.2 equiv.) and triphenylphosphane (1.39 mmol, 365 mg, 1.1 equiv.) were dissolved in a mixture of CCl_4/DMF (1/4, 10 mL). The mixture was heated to 90 °C and refluxed for 3 h under N_2-atmosphere. After cooling down, diethylether (10 mL) was added and the organic phase was washed with water (10 mL). The ether was evaporated, and the remaining mixture cooled to 0 °C and filtered to remove crystallized triphenylphosphanoxide. The organic phase was dried with $MgSO_4$ and the solvents were evaporated. The product was purified via flash chromatography (DCM/MeOH; 1-10 % MeOH gradient) to give 102 mg (0.389 mmol, 31%) of **23** as a yellow oil. R_f: 0.52 (DCM/MeOH 95/5). ^{1}H-NMR (DMSO-D_6, 400.16 MHz, δ [ppm]): 7.33-7.25 (m, 2 H, Aryl-H 2,6); 7.00-6.93 (m, 2 H, Aryl-H 3,5), 4.35 (s, 2 H, Aryl-C$\underline{H_2}$-N_3); 4.08 (t, J 5.8 Hz, 2 H, O-C$\underline{H_2}$-CH_2); 3.61-3.52 (m, 4 H, morpholine-H 2,6); 2.68 (t, J = 5.8 Hz, 2 H, N-C$\underline{H_2}$-CH_2), 2.43 (m, 4 H, morpholine-H 3,5). ^{13}C-NMR (DMSO-D_6, 100.61 MHz, δ [ppm]): 158.40 (Aryl-C 1); 132.04 (Aryl-C 4); 130.08 (2 C, Aryl-C 2,6); 114.63 (2 C, Aryl-C 3,5); 66.18 (2 C, morpholine-C 2,6); 65.31 (O-$\underline{C}H_2$-CH_2); 57.01 (N-$\underline{C}H_2$-CH_2); 53.64 (2 C, morpholine-C 3,5); 53.18 (N_3-$\underline{C}H_2$-Aryl).

2-((4,6-dimethylpyrimidin-2-yl)thio)-N-(5-(3-((1-(4-(2-morpholinoethoxy)benzyl)-1H-1,2,3-triazol-4-yl)methoxy)benzyl)thiazol-2-yl)acetamide (12)



12

21 (0.38 mmol, 162 mg, 1 equiv.) and **23** (0.38 mol, 100 mg, 1 equiv.) were dissolved in *tert*butanol/water (1:1, 10 mL). A copper sulfate solution (0.1 M, 382 µL, 0.1 equiv.) and subsequently a solution of sodium ascorbate (0.2 M, 763 µL, 0.2 equiv.) were added and the mixture was stirred at room temperature for 2 h. After quenching the reaction with water (2 mL) all solvents were evaporated and the residue was purified by automated flash chromatography

(gradient of DCM/methanol) to get 105 mg (0.15 mmol, 40 %) of **12** as a white solid. Yield: 40%, R_f: 0.68 (DCM/MeOH 95:1), ^{1}H-NMR (DMSO-D_6, 400.16 MHz, δ [ppm]): 12.22 (s, 1 H, amide-H); 8.20 (s, 1 H, triazole H-5); 7.27 (m, 2 H, benzyl H2,6); 7.25 (1 H, aminothiazole H-4); 7.23-7.18 (m, 1 H, phenyl H-5); 6.95-6.90 (m, 3 H, pyrimidine H-5 and benzyl H-3,5); 6.90-6.85 (m, 2 H, phenyl H-2,6); 6.82 (d, ^{3}J = 7.6 Hz, 1 H, phenyl H-4); 5.49 (s, 2 H, Ar-CH_2-N); 5.07 (s, 2 H, triazole-CH_2-O-Ar); 4.08 (s, 2 H, S-CH_2-CO-N); 4.05 (t, ^{3}J = 5.8 Hz, 2 H, O-CH_2-CH_2-morpholin); 4.02 (2, 2 H, Ar-CH_2-aminothiazole); 3.61-3.50 (m, 4 H, morpholine H-2,6); 2.66 (t, ^{3}J = 5.8 Hz, 2 H, O-CH_2-CH_2-morpholine); 2.48-2.40 (m, 4 H, morpholine H-3,5); 2.27 (s, 6H, 2x CH_3-pyrimidine). ^{13}C-NMR (DMSO-D_6, 100.61 MHz, δ [ppm]): 168.9 (pyrimidine C-2); 167.0 (pyrimidine C-4,6); 166.9 (Ar-NH-CO-CH_2-S-Ar); 158.4 (benzyl C1); 158.2 (phenyl C-3); 156.9 (aminothiazole C-2); 142.9 (triazole C-1); 141.9 (phenyl C-1); 134.8 (aminothiazole C-4); 131.0 (aminothiazole C-5); 129.6 (benzyl C3,5 and phenyl C-5); 128.0 (benzyl C-4); 124.3 (triazole C-5); 120.9 (phenyl C-2); 116.1 (pyrimidine C-5); 114.9 (phenyl C-2); 112.5 (phenyl C-4); 66.2 (morpholine C-2,6); 65.3 (O-$\underline{C}H_2$-CH_2-morpholine); 61.0 (triazole-CH_2-O-Ar); 57.0 (O-CH_2-$\underline{C}H_2$-morpholine); 53.6 (morpholine C-3,5); 52.4 (Ar-CH_2-triazole), 34.1 (CO-CH_2-S); 31.9 (Ar-CH_2-aminothiazole); 23.3 (2x CH_3-pyrimidine). LMRS m/z (ESI$^+$): $[M+Na]^+$ calculated for $C_{34}H_{38}N_8NaO_4S_2^+$: 709.23, found: 709.2, $[M+H]^+$ calculated for $C_{34}H_{39}N_8O_4S_2^+$: 687.25, found: 687.3; HPLC (C18): retention time 16.7 min, 99.5%.

Determination of kinetic parameters for ZMAL and ZMML

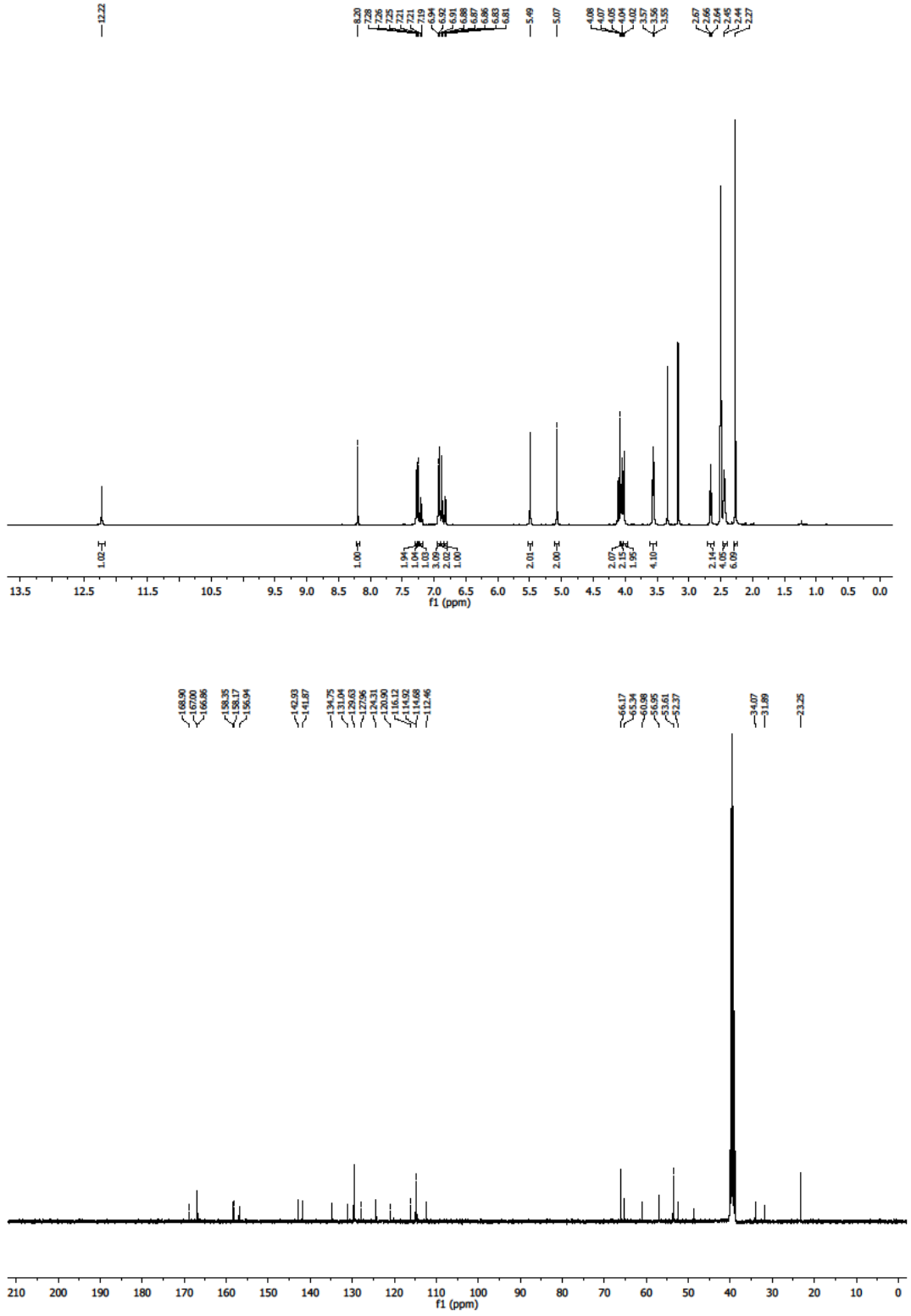
Rate experiments to determine kinetic parameters were performed in a homogeneous fluorescence-based experiment. A Tris-based buffer (25 mM Tris–HCl, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, pH = 8.0) was used for the ZMAL assay and a HEPES buffer (25 mM N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid (HEPES), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.015% Triton X-100, pH = 8.0) for the assay with ZMML. In black 96-well plates (OptiPlateTM - 96F, black, 96 well, Pinch bar design, PerkinElmer, USA) the Sirt2 enzyme (1 µM for ZMAL, 3.5 µM for ZMML) was mixed with a 2-fold dilution series of ZMAL or ZMML (prepared from 12.6 mM stock solution in DMSO and diluted with assay buffer; ZMAL: 15.65 – 1000 µM, ZMML: 0.156 – 20 µM), trypsin (20 ng/µL) and NAD^+ (500 µM) in a total assay volume of 60 µL. Measurements were started immediately after the addition of NAD^+ and the fluorescence intensity was measured every 30 seconds for 30 min (ZMAL) or 60 min (ZMML) at 20 °C with a microplate reader (λ_{ex} = 390 nm, λ_{em} = 460 nm, BMG POLARstar Optima, BMG Labtech, Germany). Experiments were performed as triplicates. The increasing fluorescence intensity was plotted as a function of time and the resulting initial rates were fit to the Michaelis-Menten equation using GraphPad 7.0 to obtain K_M and k_{cat}.

Luminescence and fluorescence spectra

For the Nluc spectrum, HEK293T cells were transiently transfected with the fusion protein plasmid and a luminescence scan was performed using a Tecan Spark 10M multimode microplate reader. The excitation spectrum of the tracer (TAMRA-labeled SirReal) was measured using a UV-1800 Shimadzu Sepctrophotometer at a concentration of 1 µM (dissolved in dH_2O). With the same concentration, the emission spectrum was detected with a FP-8300 Jasco Spectrofluorometer at a fixed emission at 460 nm. All spectra were normalized to a maximum fluorescence signal of 100.

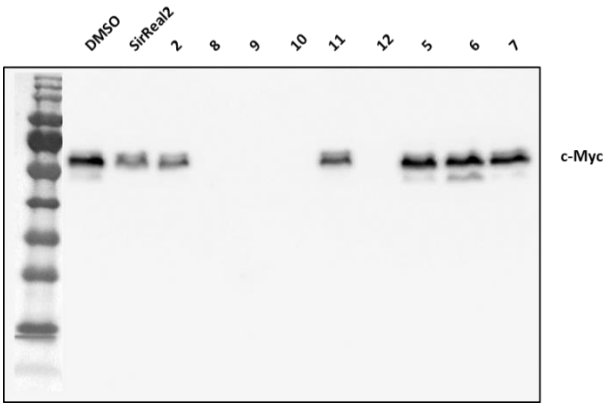
NMR spectra

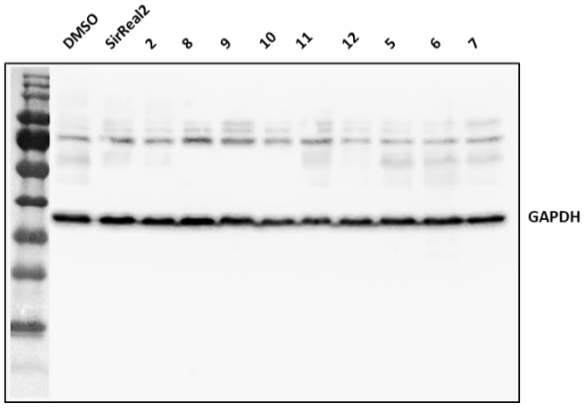
Exemplary ^{1}H NMR spectra (top) and ^{13}C NMR spectra (bottom) of compound **12**

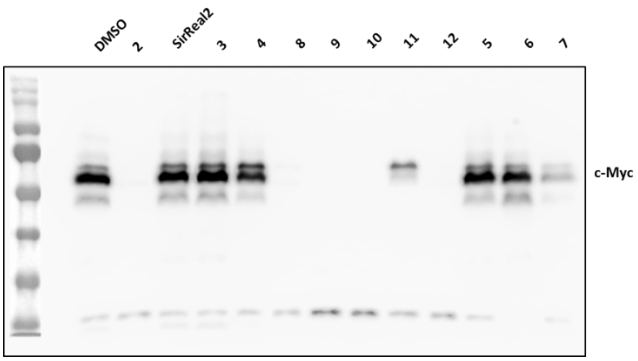


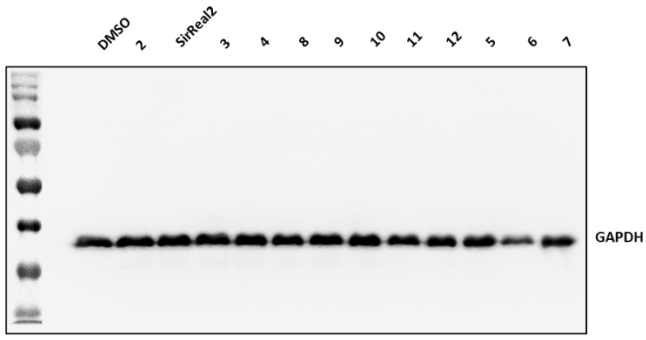
Western Blot images

Effects on c-Myc level by different Sirt2 inhibitors:

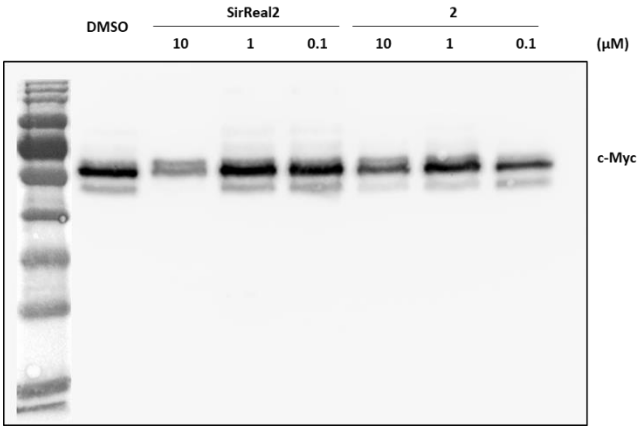


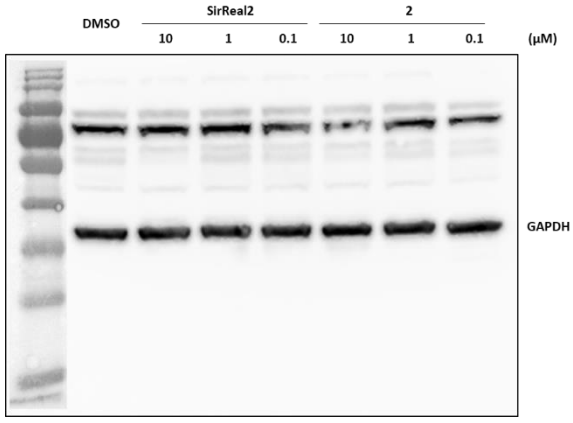


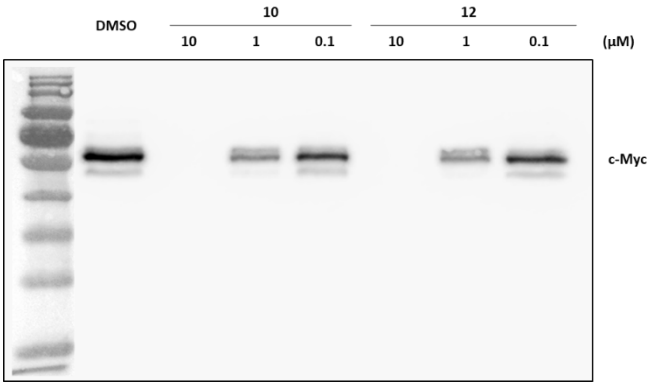




Concentration-dependent effect on c-Myc levels:



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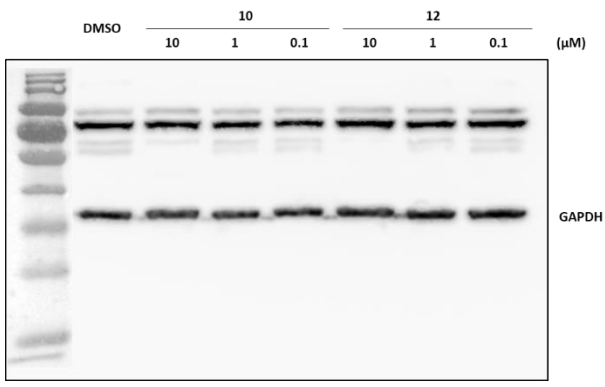
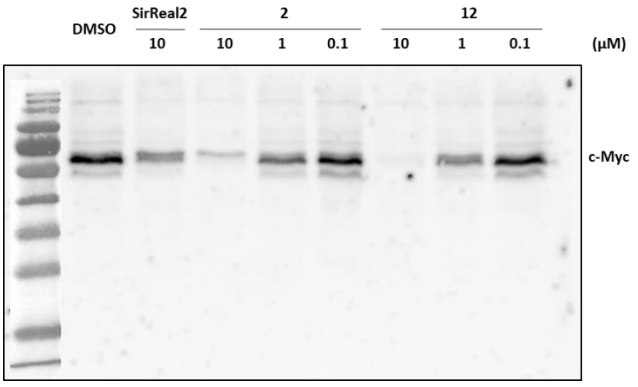
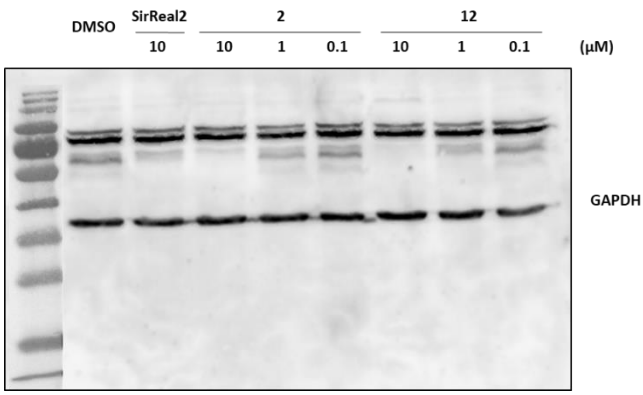
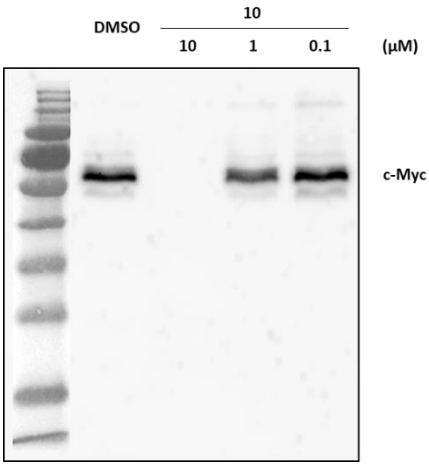
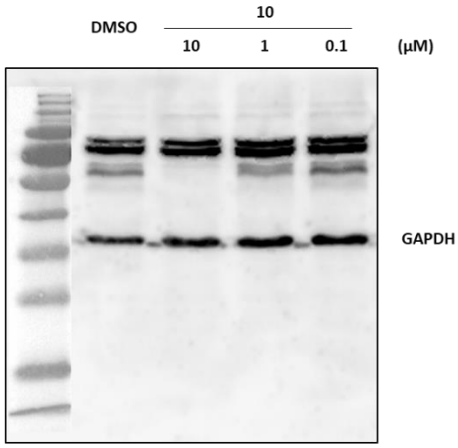
10
12
10
1
0.1
10
1
0.1
(μM)
c-Myc

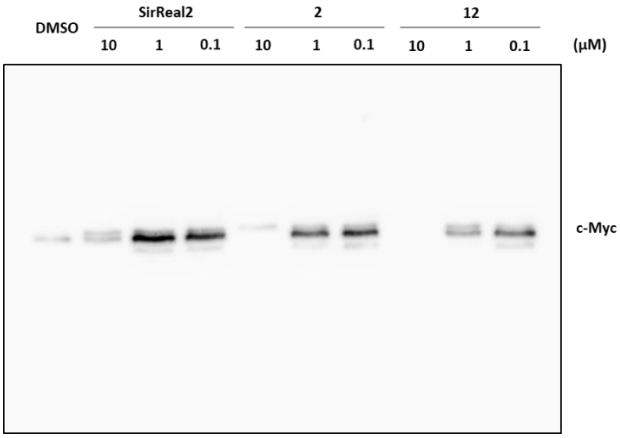

DMSO
10
12
10
1
0.1
10
1
0.1
(μM)
GAPDH

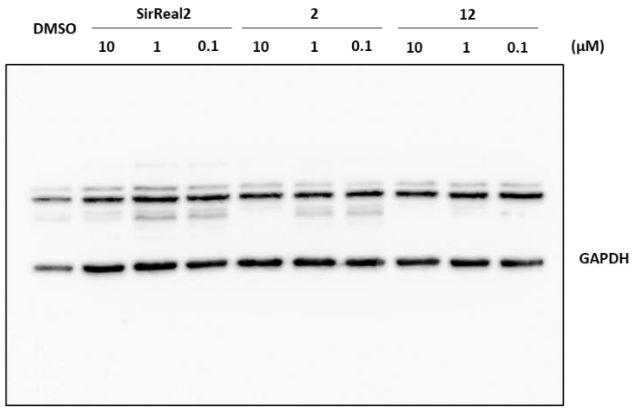

DMSO
SirReal2
2
12
10
10
1
0.1
10
1
0.1
(μM)
c-Myc

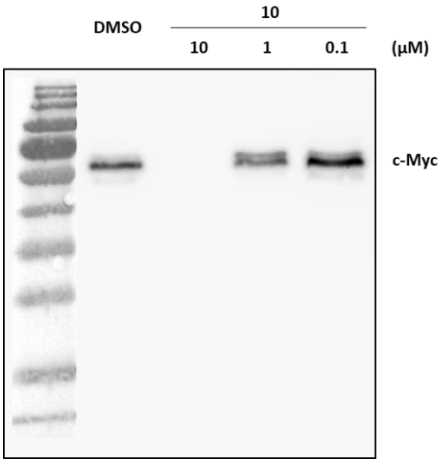

DMSO
SirReal2
2
12
10
10
1
0.1
10
1
0.1
(μM)
GAPDH

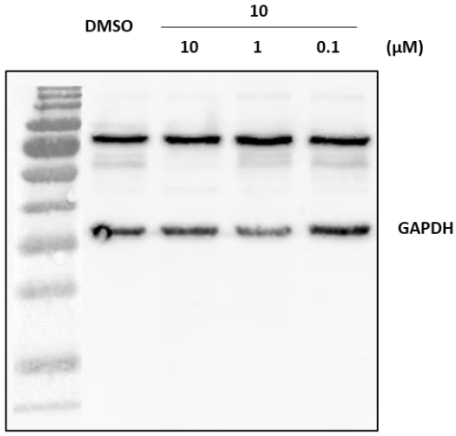

DMSO
10
10
1
0.1
(μM)
c-Myc


DMSO
10
10
1
0.1
(μM)
GAPDH

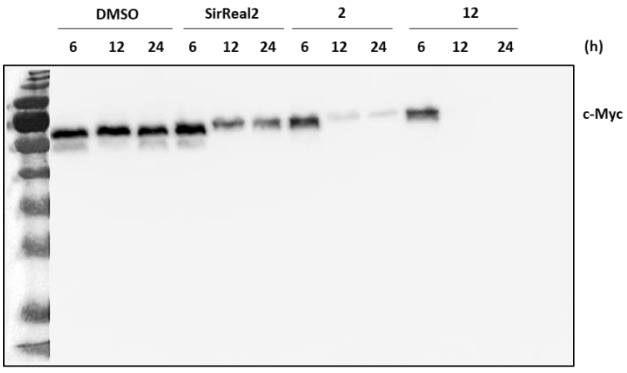


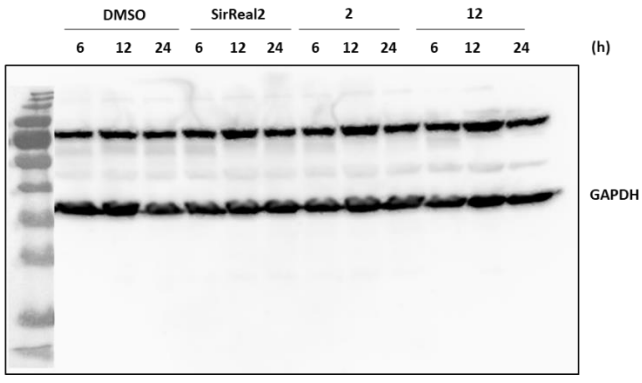


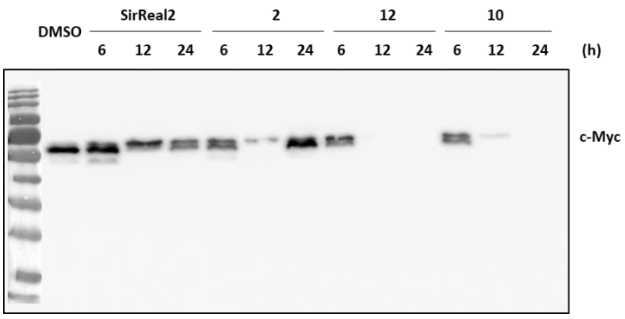


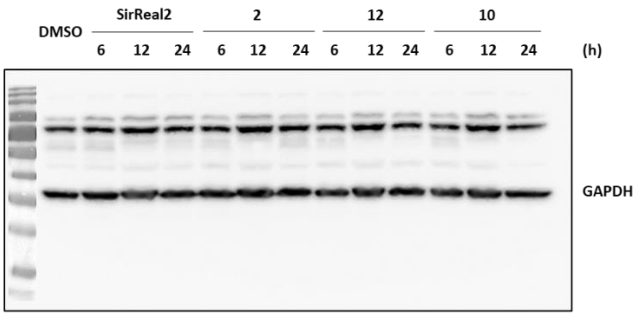


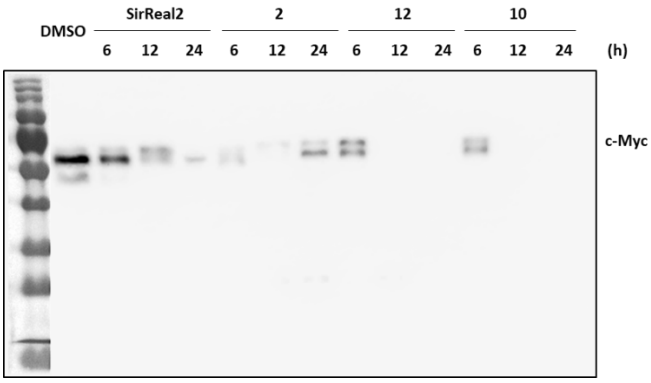
Time-dependent effect on c-Myc levels:

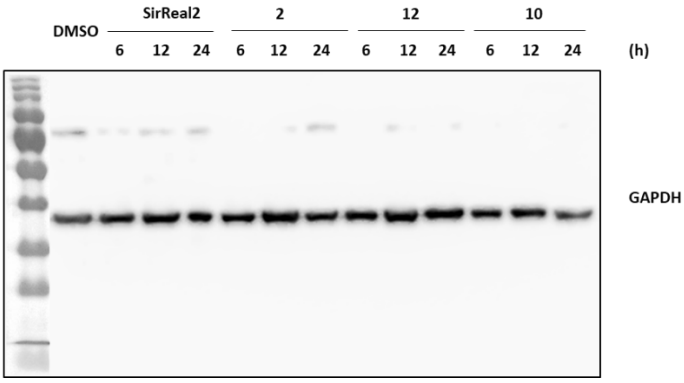




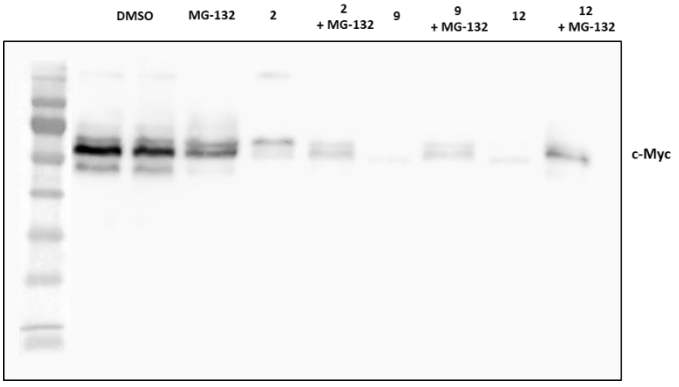


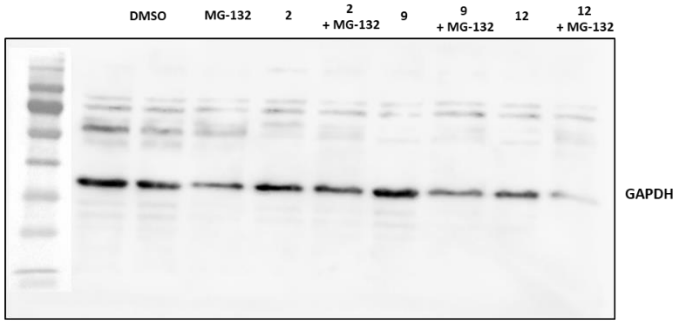






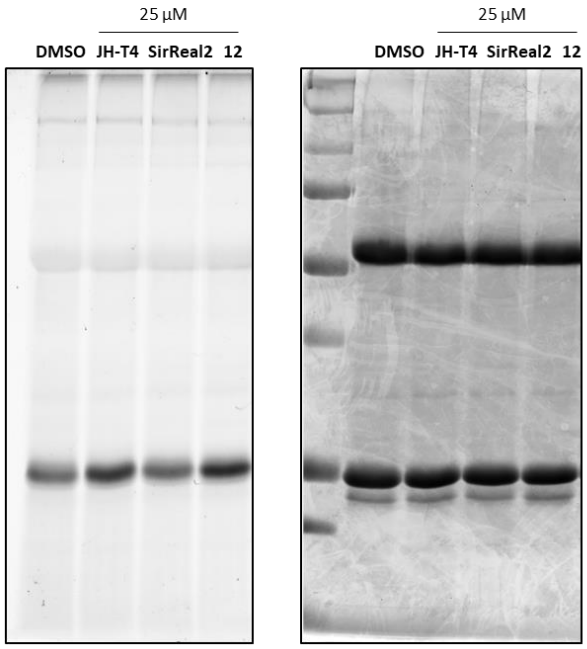
Proteasomal degradation of c-Myc:





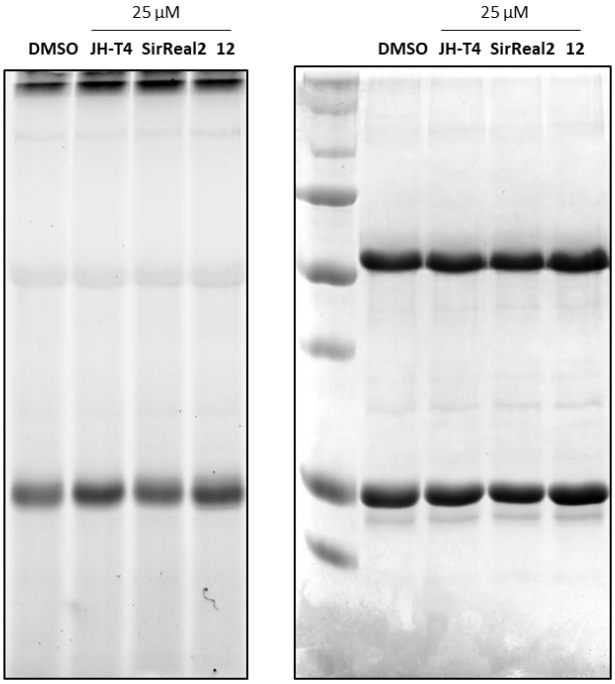
Detection of KRas4a fatty acylation levels:

Experiment 1



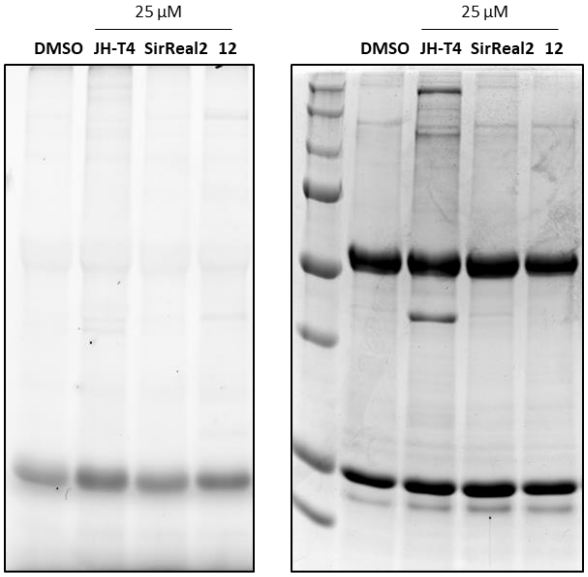
Fluorescence scan CBB-stained gel

Experiment 2



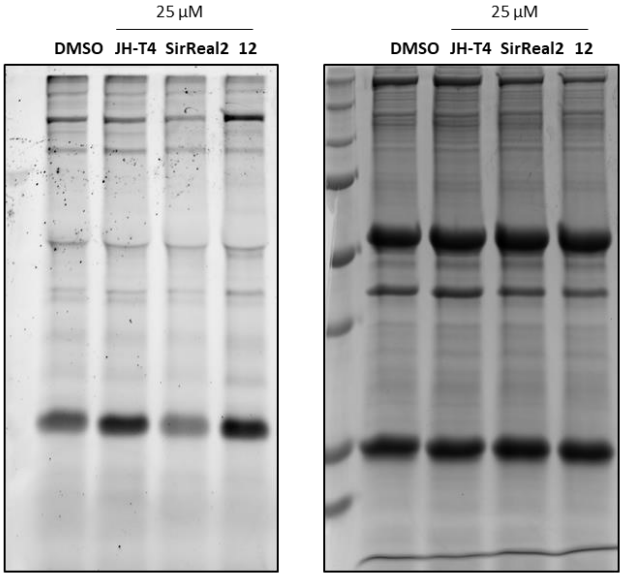
Fluorescence scan CBB-stained gel

Experiment 3



Fluorescence scan CBB-stained gel

Experiment 4



Fluorescence scan CBB-stained gel

1 References

1 M. Schiedel, T. Rumpf, B. Karaman, A. Lehotzky, S. Gerhardt, J. Ovádi, W. Sippl, O. Einsle and M. Jung, *Angew Chem Int Ed Engl*, 2016, **55**, 2252–2256.

2 S. S. Bisht, N. Dwivedi, V. Chaturvedi, N. Anand, M. Misra, R. Sharma, B. Kumar, R. Dwivedi, S. Singh, S. K. Sinha, V. Gupta, P. R. Mishra, A. K. Dwivedi and R. P. Tripathi, *Eur J Med Chem*, 2010, **45**, 5965–5978.