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Novel highly selective inhibitors of ubiquitin specific protease 30 (USP30) accelerate mitophagy

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ABSTRACT

Mitophagy is one of the processes that cells use to maintain overall health. An E3 ligase, parkin, ubiquitinates mitochondrial proteins prior to their degradation by autophagasomes. USP30 is an enzyme that de-ubiquitinates mitochondrial proteins; therefore, inhibiting this enzyme could foster mitophagy. Herein, we disclose the structure–activity relationships (SAR) within a novel series of highly selective USP30 inhibitors. Two structurally similar compounds, MF-094 (a potent and selective USP30 inhibitor) and MF-095 (a significantly less potent USP30 inhibitor), serve as useful controls for biological evaluation. We show that MF-094 increases protein ubiquitination and accelerates mitophagy.

Mitochondria have a complex life cycle that includes many processes, including: biogenesis, fusion, fission, interaction with endoplasmic reticulum and organelle specific degradation by mitophagy.[1] Loss of the mitochondrial membrane electrochemical potential initiates the mitophagy process. The depolarization is followed by stabilization of protein kinase PINK1 on damaged mitochondria;[2] this is followed by recruitment of the E3 ubiquitin ligase parkin to phosphorylated ubiquitin,[3] which results in increased ubiquitination of multiple mitochondrial proteins and engulfment by the autophagosome.[4] Parkin-mediated mitophagy is an important process in mitigating cellular damage in many tissues, for example: 1) the liver induced toxicity caused by alcohol and acetaminophen[5] and 2) the kidney damage caused by cisplatin[6] use. Additionally, parkin loss of function is a causal event in autosomal recessive juvenile onset parkinsonism.[7]

The ubiquitination of cellular proteins through the action of E3 ubiquitin ligases is opposed by a family of de-ubiquitinating enzymes (DUBs).[8] The DUB USP30, a cysteine protease,[9] is localized to mitochondria and has been shown to oppose the action of parkin-mediated ubiquitination in clearing damaged mitochondria.[10,11] USP30 is specific for isopeptide bonds between the lysine-6 ε-amino group of ubiquitin and the c-terminus of the next member of the polyubiquitin chain.[12,13] In HeLa cells, USP30 has been shown to target the lysine-6 linked ubiquitin chains on specific outer membrane proteins while leaving others intact.[12]

Previously reported inhibitors of USP30 include dodecapeptides and aptamers[14,15] and the triterpene 15-oxaspiramilactone (Fig. 1).[9] Recently some *N*-cyano pyrrolidines (Fig. 1) and other heterocycles as USP30 inhibitors have been reported in the patent literature.[16] Some of the *N*-cyano pyrrolidines in the earlier patent applications were reported to be dual inhibitors of USP30 and UCLH1 (ubiquitin C-terminal hydrolases 1) but selectivity data on the other compounds have not been reported. These structures bear some resemblance to the known Cathepsin C inhibitors and are likely covalent inhibitors with a cysteine residue of the protein forming an adduct with the *N*-cyano group.[17]

High-throughput screen led to the identification of racemic phenylalanine derivative **1** as a USP30 inhibitor. Compound **1** has an IC_{50} <1 μM and does not inhibit USP's 1, 8 and 9 at 10 μM concentration (Fig. 1).[18–20] We report herein the results of a structure–activity relationship (SAR) study of the family of analogs derived from **1** leading to identification of several potent and highly selective inhibitors of USP30. These inhibitors are expected to be non-covalent inhibitors of USP30.

A rapid SAR exploration of **1** was enabled by the modular synthetic approach shown in Fig. 2 and Tables 1–3. Analogs of **1** were prepared using a general synthetic route (or with minor

Abbreviations: USP, ubiquitin specific peptidase; DUB, deubiquitinase; biotin-UbVME, biotin-ubiquitin-vinylmethyl ester; BrdU, bromodeoxyuridine.

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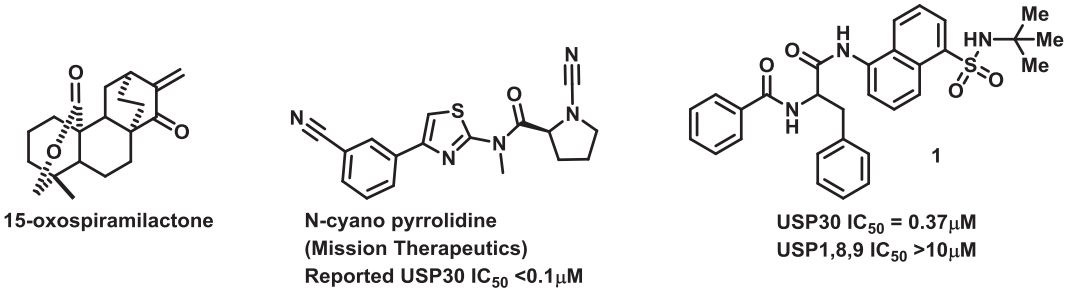
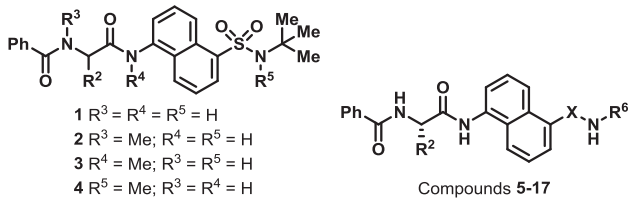


Fig. 1. Inhibitors of USP30.

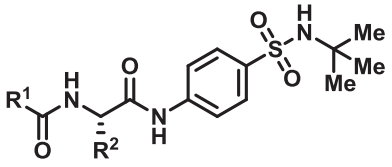
Table 1
SAR.



Cmpd.	R^2	X	R^6	USP30 IC_{50} (μM)*
(***R,S***)**-1**	Bn	SO_2	*t*-Bu	0.37
(***S***)**-1**	Bn	SO_2	*t*-Bu	0.27 ± 0.028
(***R***)**-1**	Bn	SO_2	*t*-Bu	0.25
(***R,S***)**-2**	Bn	SO_2	*t*-Bu	>10
(***R,S***)**-3**	Bn	SO_2	*t*-Bu	>10
(***R,S***)**-4**	Bn	SO_2	*t*-Bu	>10
(***R,S***)**-5**	Bn	CH_2SO_2	*t*-Bu	>10
(***R,S***)**-6**	Bn	SO_2	Et	>10
(***R,S***)**-7**	Bn	SO_2	*i*-Pr	0.51
(***S***)**-8**	Bn	CO	*t*-Bu	>10
(***S***)**-9**	4-Hydroxybenzyl	SO_2	*t*-Bu	0.47 ± 0.21
(***S***)**-10**	4-Methoxybenzyl	SO_2	*t*-Bu	0.09
(***S***)**-11**	4-Methylbenzyl	SO_2	*t*-Bu	>10
(***S***)**-12**	H	SO_2	*t*-Bu	>10
(***S***)**-13**	Me	SO_2	*t*-Bu	>10
(***S***)**-14**	^{i}Pr	SO_2	*t*-Bu	>10
(***S***)**-15**	Ph	SO_2	*t*-Bu	>10
(***S***)**-16**	Phenethyl	SO_2	*t*-Bu	2.27
(***S***)**-17**	Cyclohexylmethyl	SO_2	*t*-Bu	0.67

* The assay was performed in duplicates and the values were typically within 10% of each other. For select compounds, two or more experiments were performed independently. In those instances, the data is reported as Mean ± SD.

Table 3
SAR of benzenesulfonamide series for inhibition of USP30.

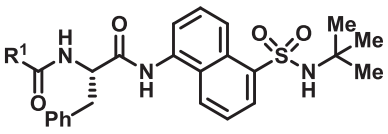


Cmpd.	R^1	R^2	USP30 IC_{50} (μM)*
35	Phenyl	Benzyl	>10
36	Phenyl	Cyclohexylmethyl	0.23
37	Cyclohexyl	Benzyl	0.06 ± 0.012 (n = 3)
38	Cyclohexyl	Cyclohexylmethyl	0.08
39	4-Fluorophenyl	Benzyl	0.02
40	4-Fluorophenyl	Cyclohexylmethyl	0.02
41	2-Fluorophenyl	Cyclohexylmethyl	0.46
42	3-Fluorophenyl	Cyclohexylmethyl	0.06
43	3,4-Difluorophenyl	Cyclohexylmethyl	0.03
44	4-Fluorophenyl	2-Thienylmethyl	0.02
45	Cyclohexyl	2-Thienylmethyl	0.03
46	4-Fluorophenyl	4-Pyridylmethyl	0.02
47	2-Pyridyl	Benzyl	0.25
48	2-Pyridyl	Cyclohexylmethyl	0.12

* The assay was performed in duplicates and the values were typically within 10% of each other. For select compounds, two or more experiments were performed independently. In those instances, the data is reported as Mean ± SD.

modifications for compounds **2**–**4**) shown in Scheme 1. The compounds were tested for USP30 inhibition using a biochemical assay that measures the effect of an inhibitor on USP30 cleavage of the substrate ubiquitin-rhodamine-110-glycine to give a fluorescent

Table 2
SAR for *N*-acyl substituent.



Cmpd.	R^1	USP30 IC_{50} (μM)	Cmpd.	R^1	USP30 IC_{50} (μM)*
1	Phenyl	0.27 ± 0.028	**26**	Benzyl	>10
18	4-Chlorophenyl	0.83	**27**	2-Phenylethyl	>10
19	4-Fluorophenyl	0.049	**28**	Benzyloxy	>10
20	4-Methylphenyl	2.5	**29**	Cyclopropyl	>10
21	4-Methoxyphenyl	9.0	**30**	Cyclopentyl	0.37
22	4-Cyanophenyl	0.27	**31**	Cyclohexyl	0.12 ± 0.0.026
23	4-Hydroxyphenyl	>10	**32**	4,4-Difluorocyclohexyl	0.10
24	2-Pyridyl	0.16	**33**	Cycloheptyl	4.3
25	2-Thiazolyl	0.18	**34**	Adamantyl	>10

* The assay was performed in duplicates and the values were typically within 10% of each other. For select compounds, two or more experiments were performed independently. In those instances, the data is reported as Mean ± SD.

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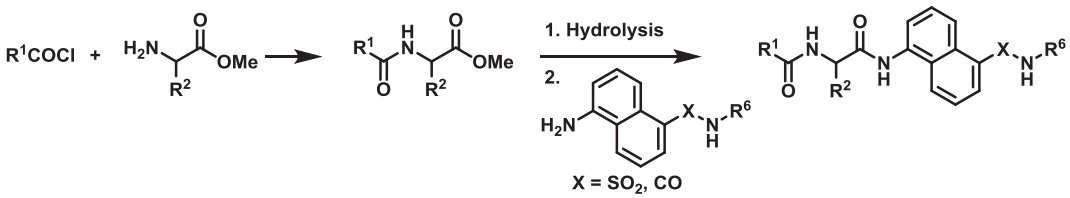


Scheme 1. General method for synthesis of analogs of **1**.

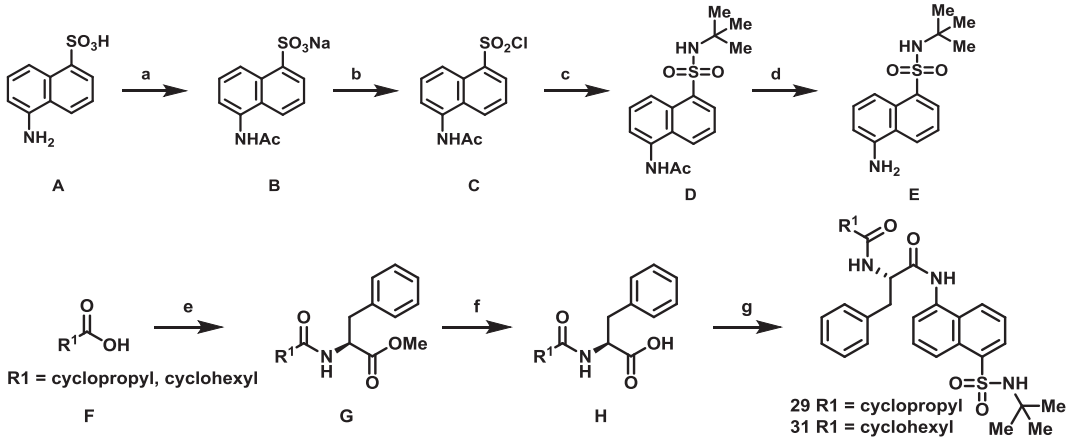
product: rhodamine-110.[21] Compounds with IC_{50} < 1 μM were screened for inhibition of USP's 1, 8 and 9 at 10 μM. The data on selected compounds is summarized in Table 1.

A series of positional modifications of **1** established several key structural requirements for USP30 inhibitory activity. The racemate and the enantiomers of **1** have similar IC_{50}s suggesting that the chirality of the phenylalanine moiety does not contribute significantly to the binding affinity to the protein. The three exchangeable hydrogens (two amides and one sulfonamide) are essential, since replacement of any one by a methyl group (compounds **2**–**4**) results in drastic drop in USP30 inhibition. The SAR in the sulfonamide region seemed to be fairly restrictive in terms tolerated substitutions. Compound **5** with an additional methylene group between the sulfonamide and the phenyl ring results in complete loss of USP30 inhibition. The ethylsulfonamide analog **6** does not inhibit USP30, whereas compounds containing *t*-butylsulfonamide (**1**) and isopropylsulfonamide (**7**) inhibit USP30 with IC_{50}s around 0.5 μM. When the sulfonamide group is replaced with an amide group, the resulting compound **(*S*)-8** does not inhibit USP30. The complete loss of inhibition seen with amide (***S***)-**8** indicates a critical role the SO_2 moiety through either direct binding to USP30 or through its tetrahedral geometry at the binding site. Based on these results, the two primary amides and the *t*-Bu$NHSO_2$ group were kept constant during exploration of SAR in the other regions.

The structural correlation between potency with the size of the substituent at the α-chiral center is shown in Table 1. Benzyl (**1**) as well as its 4-hydroxy (**9**) and 4-methoxy (**10**) analogs, and cyclohexylmethyl (**17**) all show good inhibition; however, the 4-methyl substitution (**11**) is not tolerated. Analogs in which the L-phenylalanine moiety is replaced with glycine, alanine, valine and phenylglycine (R^1 = H, Me, *i*-Pr and Ph respectively) (**12**–**15** in Table 1) moieties do not inhibit USP30 up to 10 μM concentration. Compound **16**, where R_1 = phenethyl, weakly inhibits USP30 (2.2 μM). Further SAR was then explored by keeping the (*S*)-phenylalanine and the naphthyl-*t*-butylsulfonamide moieties unchanged.

The data summarized in Table 2 demonstrate a significant dependence of inhibition on the structure of R_1. Substitutions at the 4-position of the phenyl ring affect USP30 inhibition, e.g. the 4-fluorophenyl analog (**19**) has an IC_{50} = 0.05 μM and compound **23** (R^1 = 4-hydroxyphenyl) showed no inhibition (at 10 μM). Other compounds such as **18**, **20** and **22** with R_1 = 4-chlorophenyl, 4-methylphenyl and 4-cyanophenyl, respectively, inhibit USP30 with IC_{50}s between 2.5 μM and 0.27 μM. Electron withdrawing substituents (e.g. F, CN) on the phenyl ring are preferred over the electron donating groups (e.g., OMe, OH). Electron deficient heteroaromatic rings are preferred in this region as shown by 2-pyridyl (**24**) and 2-thiazolyl (**25**) with IC_{50}s of 0.16 μM and 0.18 μM, respectively. For further exploration of the lipophilic pocket, the phenyl group was replaced with benzyl (**26**), phenylethyl (**27**) and benzyloxy groups (**28**). None of these compounds inhibit USP30 (at 10 μM). Non-aromatic cycloalkyl groups were tolerated in this region as evidenced by the USP30 inhibition observed with cyclopentyl (**30**), cyclohexyl (**31**) and 4,4-difluorocyclohexyl (**32**) groups. Smaller rings such as cyclopropyl (**29**) or larger rings such as adamantyl (**34**) resulted in complete loss of USP30 inhibition while the cycloheptyl analog (**33**) showed an order of magnitude decrease in USP30 inhibition.

As a strategy to reduce the molecular weight and the number of aromatic rings in the compounds, 5-amino-naphthalene sulfonamide at the C-terminus of the amino acid core was replaced with 4-aminobenzene sulfonamide moiety (Table 3). The *N*-acyl group and the L-phenylanine moiety were simultaneously modified using the insights gained from the SAR in the naphthylsulfonamide series. Therefore, optimum substituents from Tables 1 and 2 (R^1 = phenyl, cyclohexyl and 2-pyridyl) were matched with the optimum R^2 substituents (benzyl, 4-fluorobenzyl, and cyclohexylmethyl). As anticipated, several compounds inhibit USP30 with IC_{50}s < 100 nM as shown in Table 3. All the compounds with



Scheme 2. Synthesis of compounds **29** and **31**. Reagents and conditions: a) Acetic anhydride, aq. NaOH (2 N), toluene, 100 °C, 2 h; b) Chlorosulfuric acid, rt, 2.5 h; c) 2-Methylpropan-2-amine, Et_3N, dry THF, rt, 16 h; d) aq. NaOH (5 N), MeOH, 80 °C, 16 h. e) Methyl-L-phenylalaninate hydrochloride, EDC-HCl, HOBT, DIPEA, DMF, rt, 16 h; f) LiOH-$2H_2O$, THF, MeOH, H_2O, rt, 6 h; g) 5-amino-*N*-(*tert*-butyl)naphthalene-1-sulfonamide (E), EDC-HCl, HOBt, DMF, rt, 48 h.

IC_{50}s < 1 μM were screened for USP1, USP8 and USP9 inhibition. None of the compounds inhibit these proteases, which demonstrate the selectivity of this series of compounds.

Since structurally related analogs **31** (MF-094) and **29** (MF-095) have significantly different inhibitory activity for USP30, the compounds were used to probe the biology of USP30. The synthesis of **31** and **29** is shown in Scheme 2. Both compounds have <30% inhibitory activity for a panel of 22 ubiquitin specific protease assays up to 10 μM at Ubiquigent Ltd (Dundee, UK).[22] The compounds were used to evaluate binding to the active site on the surface of isolated mitochondria.[23]

Mitochondria were isolated from C2C12 myoblasts, which are of murine origin, and the intact organelles were exposed to a DUB specific activity based probe, biotin-UbVME (biotin-ubiquitinylated methyl vinyl ether).[24] USP30 enzymatic activity results in covalent modification of the active site cysteine with biotinylated ubiquitin, which was detected after SDS-PAGE and transfer to a membrane as shown in Fig. 2. The upper band in Fig. 2 has been assigned previously as methylcrotonyl CoA carboxylase and propionyl CoA carboxylase;[25] these enzymes were endogenously biotinylated and so appear irrespective of biotin-UbVME. Compound **31** protected USP30 from modification at concentrations from 0.2 to 5 μM whereas **29** did not protect mitochondrial USP30 from modification.

The effects of **31** and **29** were examined in a recently described mitophagy assay that tracks the disappearance of bromodeoxyuridine-labelled mitochondrial DNA in terminally differentiated C2C12 cells (Fig. 3).[26] Inhibition of USP30 is expected to increase the net rate of ubiquitin accumulation on mitochondria by slowing the de-ubiquitination of outer membrane proteins. In turn, faster accumulation of ubiquitin will lead to an increased rate of mitophagy. Compound **31** accelerated the disappearance of BrdU from the mitochondrial DNA after 6 h. In contrast, **29** did not elicit a significant effect.

We have identified a chemical series of highly selective USP30 inhibitors. Two structurally similar compounds **31** (MF-094) and **29** (MF-095), which inhibit USP30 with IC_{50}s that are at least two orders of magnitude different, were used to demonstrate the role of USP30 inhibition in accelerating mitophagy in C2C12 myotubes. We believe that compounds **31** and **29** represent unique tools to probe the underlying biology of USP30 and further work will be reported in due course.

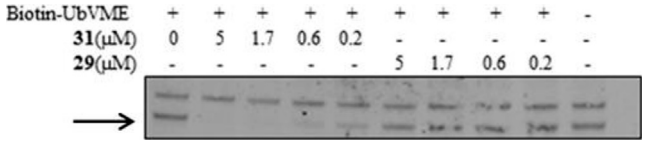


Fig. 2. Compound **31** protects the active site of endogenous USP30 from modification by biotin-UbVME, an activity-based probe. USP30 is indicated by the arrow.

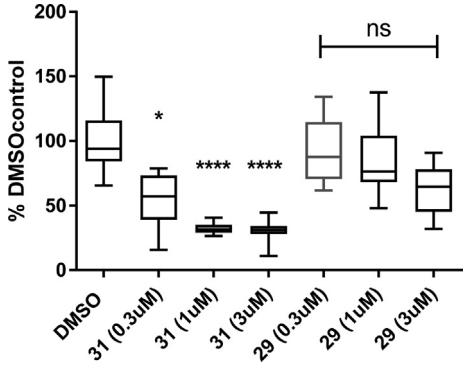


Fig. 3. C2C12 myotubes were incubated with compounds for 24 h and the mitochondrial DNA was labeled with BrdU for 2 h. Six hours after labeling, **31** enhanced mitophagy as measured by loss of BrdU from mtDNA ($p < 0.05$ at all concentrations). Compound **29** did not enhance mitophagy under the same conditions (ANOVA with Kruskal Wallis post test).

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Author contributions

The manuscript was written through contributions of all authors, all authors have given approval to the final version of the manuscript.

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21. The activity of USP30 activity was determined by the increase in fluorescence measured as a result of the enzyme catalyzed cleavage of the fluorogenic substrate Ubiquitin-Rhodamine110-Glycine generating Ubiquitin and Rhodamine110-Glycine. USP30 enzyme assay was performed by pre-incubating 20 nM of USP30 with varying concentration of NCE (the compound) in the assay buffer (PBS (pH 7.4), 1 mM DTT, 0.01% Tween 20, 0.01% BSA, DMSO final conc :1%) for 15 min at room temperature, following

which 100 nM of substrate Ub rhodamine was added and incubated at room temperature for 2 h and the plate was read for fluorescence intensity at Ex/Em 485 nm/535 nm in VICTOR X5 plate reader. The percent inhibition of activity of the enzyme is calculated by comparing counts in the presence and absence of compounds. The compounds were screened at various concentrations and dose response curves were generated for compounds from the data generated at 10–12 tested concentrations using GraphPad Prism software Version 7 (San Diego, California, USA) using non linear regression curve fit for sigmoidal dose response (variable slope).

22. USPs tested were USP1, USP2, USP4, USP5, USP6, USP7, USP8, USP9, USP11, USP14, USP15, USP16, USP19, USP20, USP21, USP25, USP27, USP28, USP35, USP36, USP45, CYLD.
23. Some of the biological results were presented previously. Thmpson JE, Terada Y, Klug AF, et al. FASEB SRC mitochondrial biogenesis and dynamics in health, disease and aging, May 21–26, 2017, West Palm Beach, FL.
24. Mitochondria were isolated from C2C12 myoblasts using the mitochondrial isolation kit for cultured cells (Abcam, Cambridge, Massachusetts). Mitochondria were incubated with compound **29**, compound **31** or DMSO and 1.5 μM biotin-UbVME (UbiQ, Amersterdam, Netherlands) in 6 mM phosphate buffer pH 7.4, 150 mM NaCl, 50 μM tris(2-carboxyethyl) phosphine, 1% DMSO at ambient temperature for 5 min. Reactions were quenched by addition 0.33 volumes of 4× SDS-PAGE sample buffer (Bio-rad, Hercules, California), 14 mM beta-mercaptoethanol and heating at 95 °C for five minutes. After SDS-PAGE and transfer to polyvinyldifluoride the membrane was blocked with 1% fish gelatin blocking buffer (Boston Bioproducts, Ashland, Massachusetts). The membrane was incubated with 100 ng/ml streptavidin dylight (800 nm emission maximum) in 1% fish gelatin buffer with detergent (Boston Bioproducts, Ashland, Massachusetts) for 60 min, rinsed and the image collected using a Licor Odyssey Clx.
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