Proteasome Activation is a Mechanism for Pyrazalone Small Molecules Displaying Therapeutic Potential in Amyotrophic Lateral Sclerosis

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General Experimental Procedures

All reactions were carried out in oven- or flame-dried glassware open to the air unless otherwise noted. All reactions were monitored by analytical thin layer chromatography using Whatman precoated silica gel glass plates. Visualization was accomplished by UV light (256 nm) or by potassium permanganate and/or phosphomolybdic acid solution as indicator. Flash column chromatography was performed using silica gel 60 (mesh 230-400) supplied by E. Merck. Commercial grade solvents and reagents were obtained from Sigma Aldrich, Pierce Biotechnology, or Alfa Aesar and used without further purification except as indicated. Tetrahydrofuran was distilled from sodium benzophenone ketyl under an atmosphere of dry argon.

^{1}H, ^{13}C, COSY, HMQC and DEPT NMR spectra were recorded on a Bruker Advance III (500 MHz ^{1}H, 125.77 MHz ^{13}C) equipped with a DCH Cryo-Probe. Multiplicities are indicated by s (single), d (doublet), dd (doublet of doublets) t (triplet), q (quartet), m (multiplet), br (broad). Chemical shifts are reported in parts per million (ppm, δ) using $CDCl_3$ (^{1}H 7.26 ppm, ^{13}C 77.36 ppm) and MeOH-d_4 (^{1}H 3.31 ppm, ^{13}C 49.00 ppm) as internal standards. N.O.e experiments were performed on a Varian Inova (500 MHz) or Varian Inova (400 MHz) spectrometer. High resolution mass spectrometry was performed on a VG70-250SE mass spectrometer. High pressure liquid chromatography was performed on a Beckman System Gold chromatograph (Model 125P solvent module and Model 166 detector) with a Vydac (C18, 5μm, 90Å, 4.6mm x 250mm) column (Grace, Deerfield, IL), all samples were assessed to possess >95% purity.

N-(15-(3-((3,5-dichlorophenoxy)methyl)-5-oxo-2,5-dihydro-1H-pyrazol-1-yl)-15-oxo-3,6,9,12-tetraoxapentadecyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide **(BP)**[1]**:** To a solution of 4-(3,5-dichlorophenoxy)-3-oxobutanoate[1] (26 mg, 0.10 mmol) in DMSO-d_6 (4 mL) was added biotin-PEG4-hydrazide (50 mg, 0.10 mmol), and the solution was stirred at room temperature for 7 days. The orange solution was concentrated to provide **BP**, which was analyzed immediately.

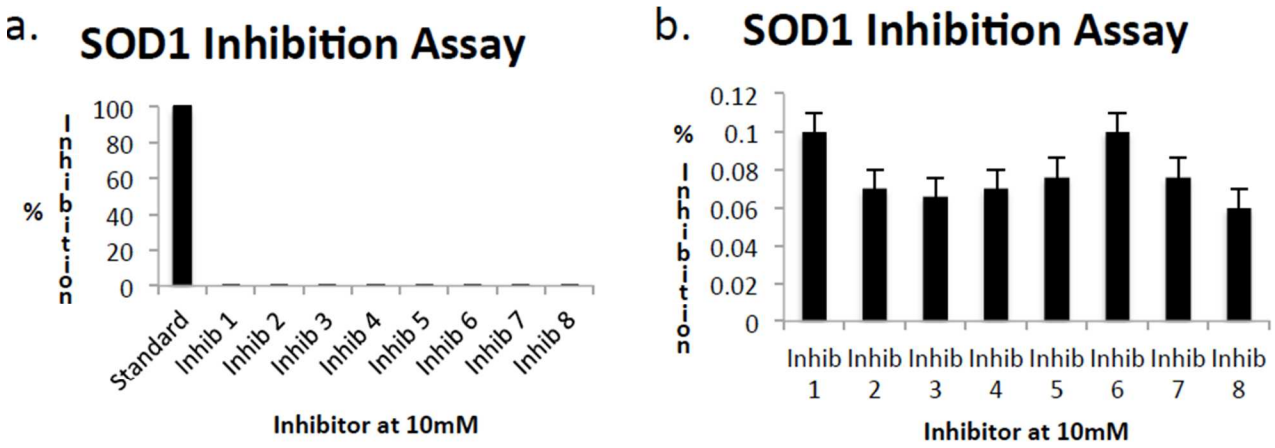
^{1}H NMR (500 MHz; DMSO-d_6): δ_H: 1.30 (2H, m, C*H*$_2$), 1.49 (2H, m, CH_2), 1.60 (2H, m, CH_2), 2.07 (2H, t, *J* = 7.48Hz, CH_2C(O)), 2.25 (2H, t, *J* = 6.52Hz CH_2C(O)), 2.82 (1H, dd, *J* = 5.00 and 7.67Hz, SCH*H'*), 3.09 (1H, m, SC*H*H'), 3.18 (2H, q, *J* = 6.33 and 12.00Hz, C*H*$_2$NH), 3.36 (1H, m, SCH), 3.42 (14H, s, OC*H*$_2$C*H*$_2$O), 3.49 (2H, m, OC*H*$_2$CH$_2$O), 3.59 (2H, t, *J* = 6.39Hz, OC*H*$_2$CH$_2$NH), 4.13 (1H, m, $CH_{bridging}$), 4.19 (2H, s, $CH_{2(pyrazolone)}$), 4.31 (1H, t, *J* = 7.25Hz, $CH_{bridging}$), 5.05 (1H, s, $CH_{pyrazolone}$), 6.40 (2H, d, *J* = 32.10Hz, NH_{biotin}), 7.06 (2H, s, ArCH), 7.19 (1H, s, ArCH), 7.87 (1H, t, *J* = 5.48Hz, CH_2N*H*C(O)), 9.00 (1H, br. s, $NH_{pyrazolone}$).

^{13}C NMR (125.77 MHz; DMSO-d_6): δ_C: 13.95 (CH_2), 25.23 (CH_2), 27.99 (CH_2), 28.17 (CH_2), 34.21 (CH_2C(O)), 35.04 (CH_2C(O)), 38.39 (CH_2NH), 45.19 (CH_2S), 55.40 (CHS), 59.14 $CH_{bridging}$), 60.80 ($CH_{bridging}$), 66.60 (CH_2*C*H_2O), 69.11, 69.46, 69.52, 69.62, 69.66, 69.73 (CH_2O), 72.32 (CH_2OAr), 94.40 ($CH_{pyrazolone}$), 113.88 (ArCH), 120.21 (ArCH), 134.50 (ArC-Cl), 162.69 (C(O)$_{pyrazolone}$), 167.00 (ArC-O), 169.48 (C(O)$_{biotin}$), 172.14 (CHC(O)).

***m/z* (ESI)**: 800.34 [M+EtOH+Na] (47%), 801.4 (16), 804.28 (5).

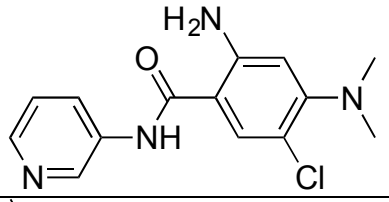
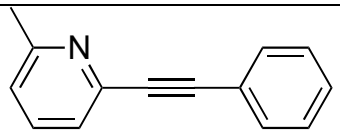
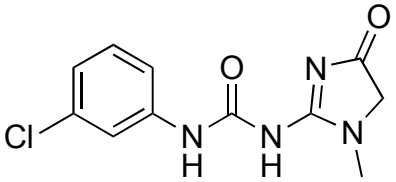
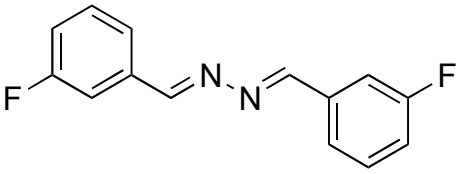
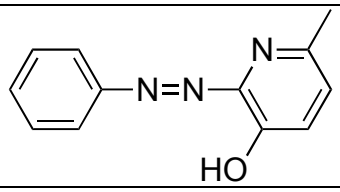
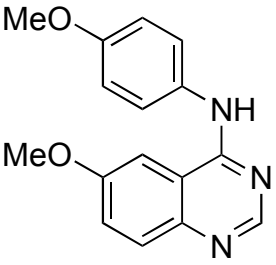
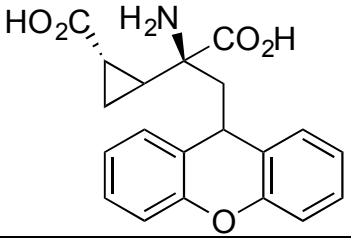
HRMS (ESI): $C_{33}H_{50}Cl_2N_5O_{10}S$ [M+H+EtOH] requires 778.265, Found 778.2659.

Supplementary Chart S1. Wild-type SOD1 activity inhibition by selected compounds of the ASP, PYT and CHD classes.



A variety of active compounds in the ^{G93A}SOD1 cellular assay do not directly inhibit wild-type SOD1. a) Percentage inhibition of wild-type SOD1 by 10mM of the selected inhibitor. b) Expansion of a. Experimental protocol was followed as described in the kit manufacturers guidelines (Cayman Chemical). Standard is 10mM concentration of KCN. Data are represented as mean ± Standard deviation. All data shown are representative of three independent experiments.

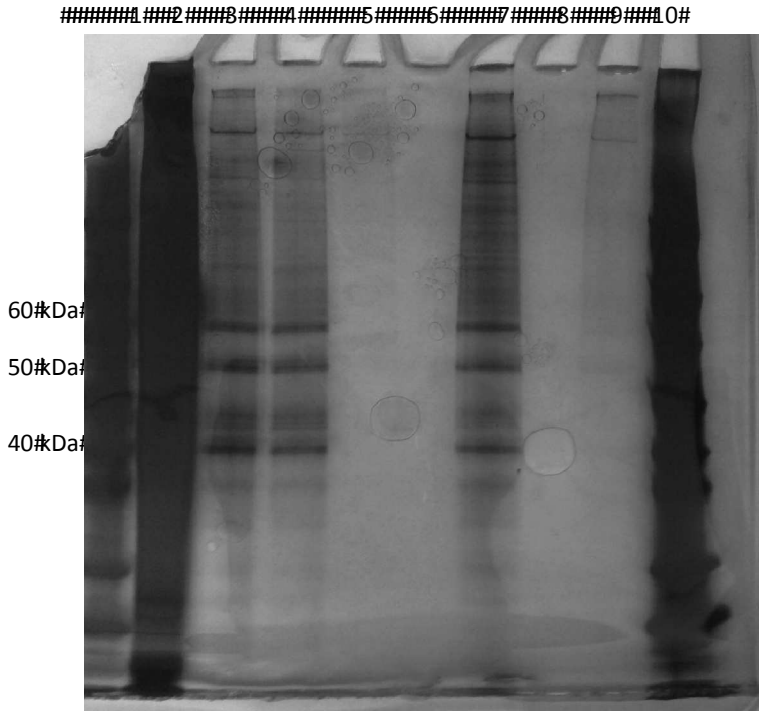
Supplementary Table S1. Activities of known mGluR inhibitors in ALS cell-based assay.

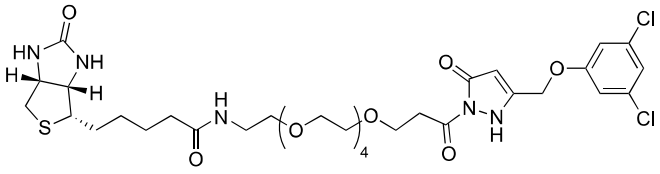
Name / Acronym	Structure	Target Specificity	Known Potency (IC_{50})	Assay (EC_{50})
ACDPP		$mGluR_5$	Ki = 295 nM	> 32 μM
MPEP		$mGluR_5$	36 nM	> 32 μM
Fenobam		$mGluR_5$	87 nM	> 32 μM
DFB		$mGluR_5$	3-5 fold potentiation	> 32 μM
SIB		$mGluR_5$	0.4 μM	> 32 μM
LY 456236		$mGluR_1$	143 nM	> 32 μM
LY 341495		$mGluR_2$ / $mGluR_3$	2.3 nM 1.3 nM	> 32 μM

Cell Culture Conditions.

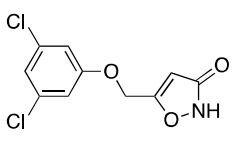
Cultured PC12-^{G93A}SOD1 cell lines were obtained by the published procedure.[2]

Supplementary Figure S1. SDS-PAGE gel of biotinylated probe pull-down using PC12-^{G23A}SOD1. Lanes 1 and 10 are the MW ladder; lane 2 is PC12-^{G93A}SOD1 cell lysate; lane 3 is biotinylated probe **8** post treated with inactive analogue **9**[1]; lane 4 is biotinylated probe **8** pull-down; lane 5 is **4** pretreated lysate incubated with biotinylated probe **8**; lane 6 is blank; lane 7 is biotinylated probe **8** pull-down; lane 8 is blank; lane 9 is biotinylated probe **8** pull-down post treated with **4**.

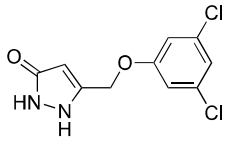




8



9



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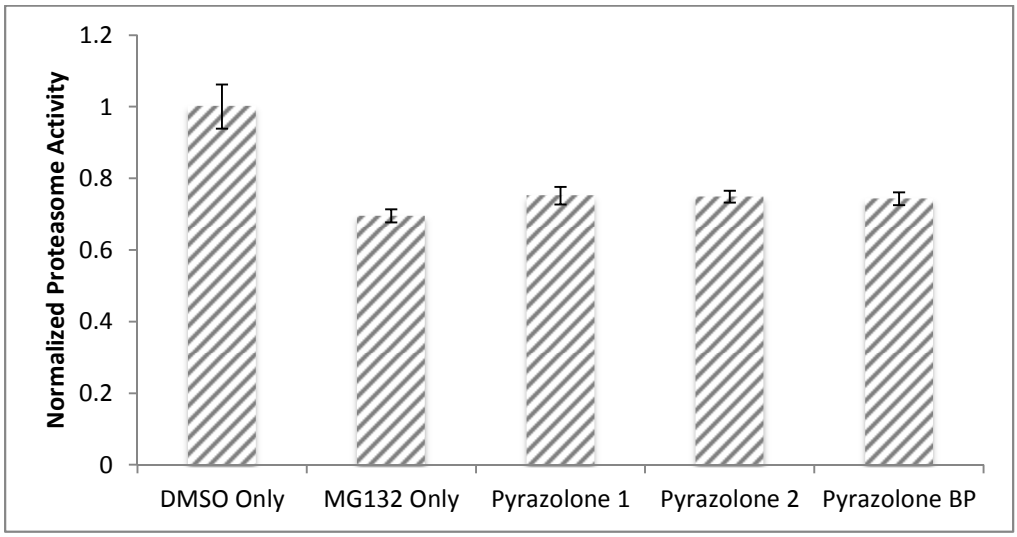
Supplementary Chart S2. Effect of pyrazolones **1**,**2** and **BP** to reverse MG132 proteasome inhibition (measured as rate of degradation of fluorogenic proteasome substrate III). Each pyrazolone was dosed at the respective EC_{50} value (**1**; 700 nM, **2**; 70 nM, **BP**; 670 nM).



The low S.D. demonstrate that the pyrazolones are indeed reversing the effects of MG132 treatment. An unpaired, two sample, one-tailed, t-test was performed between each compound and the MG132 treated control to determine if a MG132 reversing effect is detected. The p-values calculated are:

Compound	**p-value**
Pyrazolone **1**	0.00070025
Pyrazolone **2**	0.00019962
Pyrazolone **BP**	0.000875604

As each value is below 0.005 a statistical significance exists between each compounds proteasome activity versus MG132 proteasome activity.

Trypsin Digestion and LC/MS/MS

In-gel trypsin digestion was done following a published protocol.[3]

50 uL of protein from beads with inhibitor and blocked beads were run on a 4-20% precast biorad mini protean gel. 10 bands from each lane between 5 and 250 kDals were excised and trypsinized. Additionally, in solution digests of both samples without prior protein separation were performed. In both cases, the treated peptides were subsequently separated on a self-packed nanocapillary column (5 μm Jupiter C18, 100 mm _ 75 μm) using a Dionex UltiMate 3000 RSLCnano system (Thermo Scientific). LC solvents included 5% acetonitrile in water with 0.1% formic acid (mobile phase A) and 95% acetonitrile with 0.1% formic acid (mobile phase B). The LC gradient was set as follows: 0 min, 0% B; 55 min, 45% B; 63 min, 80% B; 67 min, 0% B with re-equilibration of 0% B until 90 min. Peptides were eluted into a nanoelectrospray ionization (nESI) source on a Velos Pro Orbitrap mass spectrometer (Thermo Fisher Scientific). Peptides were first detected in an orbitrap cell with resolving power setting of 30000 (at 400 m/z). Intact peptide data were collected in the 400_2000 m/z range, and MS/MS spectra were acquired using data dependent mode where the top ten most abundant peaks from the FTMS full scan were selected for collision induced dissociation (CID) fragmentation followed by mass analysis of the fragment ions in the linear ion trap.

LC/MS/MS Data Analysis

LC/MS/MS raw data files were converted to mgf files by Compass 1.0.4.0, assuming precursor charge states of 2+, 3+ and 4+.[4] The mgf files generated were searched against the SwissProt rat database using Macot (www.matrixscience.com or Electrophoresis, 20(18) 3551-67 (1999)). The following parameters were applied during database searching: 12 ppm precursor mass error tolerance, peptide charge states allowed were 2+ to 4+ and 0.5 Da fragment mass error tolerance. Variable modifications included carbamidomethylation for cysteines (+57 Da) and oxidation of methionines (+16 Da); two missed cleavage sites and two variable modification combinations were allowed. Result files were then compiled and compared using Scaffold 3 (http://www.proteomesoftware.com).[5]

List of Protein Hits

ratALS090512

#	Identified Proteins (141)	Accession #	Mw (kDa)	Probability
49	Synaptic vesicle membrane protein VAT-1 homolog OS=Rattus norvegicus GN=Vat1 PE=1 SV=1	Q3MIE4	43	100%
53	ATP synthase subunit beta, mitochondrial OS=Rattus norvegicus GN=Atp5b PE=1 SV=2	P10719	56	100%
66	Aconitate hydratase, mitochondrial OS=Rattus norvegicus GN=Aco2 PE=1 SV=2	Q9ER34	85	100%
72	Fatty acid synthase OS=Rattus norvegicus GN=Fasn PE=1 SV=3	P12785	272	100%
73	Extended synaptotagmin-1 OS=Rattus norvegicus GN=Esyt1 PE=2 SV=1	Q9Z1X1	121	100%
74	ATP synthase subunit alpha, mitochondrial OS=Rattus norvegicus GN=Atp5a1 PE=1 SV=2	P15999	60	100%
75	Profilin-1 OS=Rattus norvegicus GN=Pfn1 PE=1 SV=2	P62963	15	100%
76	Aldose reductase OS=Rattus norvegicus GN=Akr1b1 PE=1 SV=3	P07943	36	100%
83	26S protease regulatory subunit 4 OS=Rattus norvegicus GN=Psmc1 PE=2 SV=1	P62193	49	100%
87	Adenosylhomocysteinase OS=Rattus norvegicus GN=Ahcy PE=1 SV=3	P10760	47	100%
88	Annexin A6 OS=Rattus norvegicus GN=Anxa6 PE=1 SV=2	P48037	75	100%
94	T-complex protein 1 subunit alpha OS=Rattus norvegicus GN=Tcp1 PE=1 SV=1	P28480	60	100%
95	T-complex protein 1 subunit epsilon OS=Rattus norvegicus GN=Cct5 PE=1 SV=1	Q68FQ0	59	100%
96	Syntaxin-binding protein 1 OS=Rattus norvegicus GN=Stxbp1 PE=1 SV=1	P61765	67	100%
97	26S protease regulatory subunit 6B OS=Rattus norvegicus GN=Psmc4 PE=1 SV=1	Q63570	47	100%
98	40S ribosomal protein S6 OS=Rattus norvegicus GN=Rps6 PE=1 SV=1	P62755	29	100%
99	Calpain-2 catalytic subunit OS=Rattus norvegicus GN=Capn2 PE=1 SV=3	Q07009	80	100%
100	Rab GDP dissociation inhibitor alpha OS=Rattus norvegicus GN=Gdi1 PE=1 SV=1	P50398	50	100%
101	Nucleolin OS=Rattus norvegicus GN=Ncl PE=1 SV=3	P13383	77	100%
102	Peroxisomal multifunctional enzyme type 2 OS=Rattus norvegicus GN=Hsd17b4 PE=1 SV=3	P97852	79	100%
108	40S ribosomal protein S3a OS=Rattus norvegicus GN=Rps3a PE=1 SV=2	P49242	30	100%
109	Aspartate--tRNA ligase, cytoplasmic OS=Rattus norvegicus GN=Dars PE=2 SV=1	P15178	57	100%
111	Microtubule-associated protein 4 OS=Rattus norvegicus GN=Map4 PE=1 SV=1	Q5M7W5	110	100%
112	60S ribosomal protein L8 OS=Rattus norvegicus GN=Rpl8 PE=2 SV=2	P62919	28	100%
113	Histidine triad nucleotide-binding protein 1 OS=Rattus norvegicus GN=Hint1 PE=1 SV=5	P62959	14	100%
114	Nischarin OS=Rattus norvegicus GN=Nisch PE=2 SV=2	Q4G017	166	100%

117	Atlastin-1 OS=Rattus norvegicus GN=Atl1 PE=1 SV=1	Q6PST4	63	100%
118	Hypoxia up-regulated protein 1 OS=Rattus norvegicus GN=Hyou1 PE=1 SV=1	Q63617	111	100%
119	Myosin-10 OS=Rattus norvegicus GN=Myh10 PE=1 SV=1	Q9JLT0	229	100%
120	Arginine--tRNA ligase, cytoplasmic OS=Rattus norvegicus GN=Rars PE=1 SV=2	P40329	76	100%
121	Tryptophan--tRNA ligase, cytoplasmic OS=Rattus norvegicus GN=Wars PE=1 SV=2	Q6P7B0	54	100%
122	Coatomer subunit gamma-1 OS=Rattus norvegicus GN=Copg1 PE=2 SV=1	Q4AEF8	98	100%
123	Peptidyl-prolyl cis-trans isomerase FKBP1A OS=Rattus norvegicus GN=Fkbp1a PE=1 SV=3	Q62658	12	100%
124	Large neutral amino acids transporter small subunit 1 OS=Rattus norvegicus GN=Slc7a5 PE=1 SV=2	Q63016	56	100%
125	Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial OS=Rattus norvegicus GN=Etfdh PE=2 SV=1	Q6UPE1	68	100%
126	Polyadenylate-binding protein 1 OS=Rattus norvegicus GN=Pabpc1 PE=2 SV=1	Q9EPH8	71	100%
128	Coatomer subunit delta OS=Rattus norvegicus GN=Arcn1 PE=2 SV=1	Q66H80	57	100%
129	Septin-11 OS=Rattus norvegicus GN=Sept11 PE=1 SV=1	B3GNI6	50	100%
130	Glutamate dehydrogenase 1, mitochondrial OS=Rattus norvegicus GN=Glud1 PE=1 SV=2	P10860	61	100%
131	40S ribosomal protein S11 OS=Rattus norvegicus GN=Rps11 PE=1 SV=3	P62282	18	100%
132	Transgelin-2 OS=Rattus norvegicus GN=Tagln2 PE=1 SV=1	Q5XFX0	22	100%
133	Profilin-2 OS=Rattus norvegicus GN=Pfn2 PE=1 SV=3	Q9EPC6	15	100%
134	Phosphatidylinositide phosphatase SAC1 OS=Rattus norvegicus GN=Sacm1l PE=1 SV=1	Q9ES21	67	100%
135	Drebrin-like protein OS=Rattus norvegicus GN=Dbnl PE=1 SV=1	Q9JHL4	49	100%
136	Aminopeptidase B OS=Rattus norvegicus GN=Rnpep PE=1 SV=2	O09175	72	100%
137	6-phosphofructokinase, liver type OS=Rattus norvegicus GN=Pfkl PE=2 SV=3	P30835	85	100%
138	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial OS=Rattus norvegicus GN=Acadvl PE=1 SV=1	P45953	71	100%
139	Alanine--tRNA ligase, cytoplasmic OS=Rattus norvegicus GN=Aars PE=1 SV=3	P50475	107	100%
140	Translation initiation factor eIF-2B subunit delta OS=Rattus norvegicus GN=Eif2b4 PE=2 SV=1	Q63186	58	100%
141	Nuclear autoantigenic sperm protein OS=Rattus norvegicus GN=Nasp PE=2 SV=1	Q66HD3	84	100%

References

(1) Trippier, P. C.; Benmohammed, R.; Kirsch, D. R.; Silverman, R. B. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 6647.

(2) Matsumoto, G.; Stojanovic, A.; Holmberg, C. I.; Kim, S.; Morimoto, R. I. *J. Cell. Biol*. **2005**, *171*, 75.

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(4) Wenger, C. D.; Phanstiel, D. H.; Lee, M. V.; Bailey, D. J.; Coon, J. J. *Proteomics* **2011**, *11*, 1064.

(5) Searle, B. C. *Proteomics* **2010**, *10*, 1265.