Identifying Protease Specific Small-Molecule Inhibitors of Ras-converting Enzyme

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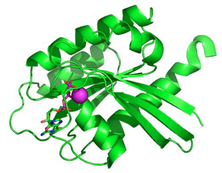


Introduction

Background:
Ras proteins are GTPases that relay extracellular cues via signalling cascades through the cytoplasm. One of the main Ras pathways regulates gene expression and has been involved in tumorigenesis. Ras genes coding for activating mutations of Ras proteins have been implicated in up to 30% of some types of human cancer.

Post-translational modification of Ras is a vital component of the maturation of Ras proteins. Ras-converting enzyme (Rce1p), a protease, catalyzes the cleavage of the CaaX (C = cysteine, a = aliphatic amino acid, X = any amino acids) tetrapolypeptide motif in the C-terminus of the immature Ras peptide. The cleavage of the last three amino acids (aaX) in the CaaX sequence enables the Ras protein to localize to the inner membrane after production in the cytoplasm.

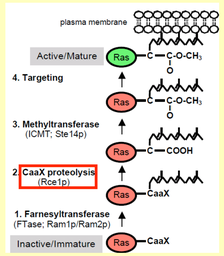


Fig.1. Post-translational modification of Ras protein (Schmidt WK)

Justification:
Ras is difficult to drug directly through competitive inhibition because of its high binding coefficient for GTP and the high concentration of GTP within the cell. Therefore, the enzymes necessary for Ras maturation have been identified as possible targets for drug-induced inhibition. Preliminary studies have shown the ability to identify inhibitors of Rce1p through high-throughput small molecule screening (HTS).

Previous Research:
To this aim, a primary HTS screen was carried out testing over 300,000 compounds from the NIH Molecular Libraries Small Molecule Repository (MLSMR) at single point doses to find inhibitors of Rce1p. From this screen, 714 "hits", or putative inhibitors were identified.

Project Goal:
• The overall goal of the study was to validate the activity of small-molecule inhibitors of Rce1p initially identified in the primary screen

• The specific objectives were to:
a) confirm the results of a primary screen through a retesting at dose, and
b) test compounds against related proteases (trypsin and Ste2) in order to determine protease-specificity

Assay Development

Fluorescence Mechanism:
To determine inhibition of Rce1p, the ability of Rce1p to cleave a recombinant Ras peptide labeled with the fluorochrome ortho-aminobenzoic acid (ABZ) and the fluorogenic quencher lysine ε-dinitrophenyl (Q_L) was measured. **In the case of inhibition**, cleavage of this peptide substrate would be prevented and emitted fluorescence from the ABZ label would be quenched by Q_L. **In the absence of inhibition**, cleavage of the peptide would result in physical separation of the ABZ and Q_L , allowing for emitted fluorescence to be detected.

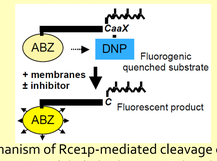


Fig.2. Mechanism of Rce1p-mediated cleavage of Q_L quencher from ABZ-labeled substrate (Schmidt WK)

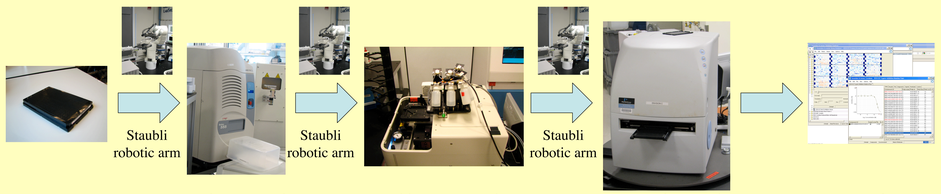
To identify the compounds that were Rce1p-specific, counterscreens were run against trypsin, a broadly active protease, and Ste24, another CaaX protease.

	Rce1p	Trypsin	Ste24
Endogenous Substrate	Ras	Most peptides with lysine or arginine residue	Yeast a-factor mating pheromone
Experimental Substrate (amino acid seq.)	ABZ-KSKTKC(f) Q_LIM	ABZ-KSKTKC(f) Q_LIM	ABZ-KSKTKC(f) VIQ$_L$
Experimental Enzyme Conc.	15µg/ml	25µg/ml	5×10^{-4} % (w/v)
Experimental Substrate Conc.	20µM	20µM	10µM

Titration:
Before screening, the proteases and their substrates had to be titrated to determine concentrations within the dynamic range, in which a change in enzymatic activity will proportionally change the fluorescent signal detected.

Controls:
To account for background fluorescence and identify the range of fluorescence signal within the assay, wells with substrate but no enzyme served as a positive control for inhibition, and wells with enzyme and substrate but no compounds served as a negative control for inhibition in each plate.

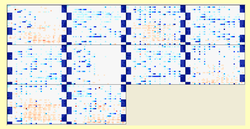
Chemical Screening Process

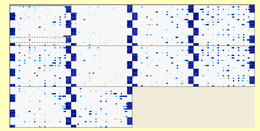


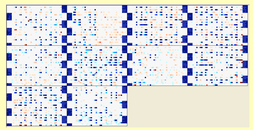
Prepare 1536-well plate | Load compounds acoustically with Echo machine | Add reagents with BioRapTR | Screen plates with EnVision plate reader | Data visualization and analysis with GeneData

Results

Plate Views







	Rce1p Retest	Trypsin Counterscreen	Ste24 Counterscreen
%CV of Negative Control	10.08% to 12.21%	5.257% to 21.38%	7.408% to 11.73%
%CV of Positive Control	1.965% to 4.833%	1.187% to 10.41%	3.818% to 5.396%
Z' (measure of assay robustness)	0.4429 to 0.6276	0.3103 to 0.7974	0.4893 to 0.662

Selective Compound Identification

300K → Primary screen for Rce1p inhibition at single dose → 714 → 50% inhibition of Rce1p in Retest at dose → 183 → 50-fold selectivity for Rce1p over trypsin → 143 → 10-fold selectivity for Rce1p over Ste24 → 118

Structural Activity Relationships With Active Compounds

Compound Nomenclature	Rce1p Retest	Trypsin Counter Screen	Ste24 Counter Screen
BRD-K55	Log AC50 Rce1p = -6.87		
BRD-K81	Log AC50 Rce1p = -6.85		
BRD-K03	Log AC50 Rce1p = -6.31		
BRD-K09	Log AC50 Rce1p = -5.84		
BRD-K65	Log AC50 Rce1p = -5.68		
BRD-K31	Log AC50 Rce1p = -6.59		

Specificity of Identified Compounds (Hit Rates in PubChem Bioassays)

References

Schmidt WK, Discovery of Specific Cell-Permeable Inhibitors of the Ras Converting Enzyme, NIH-R03 MH085698-01

Acknowledgments

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Future Directions

• Retest identified compounds from powder stocks to ensure consistent activity
• Test compounds in cell-based assays to confirm ability to act intracellularly
• Further chemical optimization to improve potency/selectivity