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Identification of inhibitors using a cell based assay for monitoring golgi-resident protease activity

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Abstract

Non-invasive real time quantification of cellular protease activity allows monitoring of enzymatic activity and identification of activity modulators within the protease's natural milieu. We developed a protease-activity assay based on differential localization of a recombinant reporter consisting of a Golgi retention signal and a protease cleavage sequence fused to alkaline phosphatase (AP). When expressed in mammalian cells, this protein localizes to Golgi bodies and, upon protease mediated cleavage, AP translocates to the extracellular medium where its activity is measured. We used this system to monitor the Golgi-associated protease furin, a pluripotent enzyme with a key role in tumorigenesis, viral propagation of avian influenza, ebola, and HIV, and in activation of anthrax, pseudomonas, and diphtheria toxins. This technology was adapted for high throughput screening of 30,000 compound small molecule libraries, leading to identification of furin inhibitors. Further, this strategy was utilized to identify inhibitors of another Golgi protease, the β -site APP-cleaving enzyme (BACE). BACE cleavage of the amyloid precursor protein leads to formation of the A β peptide, a key event that leads to Alzheimer's disease. In conclusion, we describe a customizable, non-invasive technology for real time assessment of Golgi protease activity used to identify inhibitors of furin and BACE.

Keywords

Furin; BACE; TGN; Prohormone Convertase; Alzheimers; SEAP; Alkaline Phosphatase; NSAIDs

Introduction

Cellular proteases perform diverse critical functions crucial to proper execution of physiological processes including development, hormone maturation, immunity, blood clotting, pathogenesis of viral and bacterial diseases, and programmed cell death[1;2;3;4;5;6;7;8;9;10]. Proteolytic processing is modulated both temporally and positionally and contributes to protein activation and sub-cellular localization[6]. Proteases involved in maturation of

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secretory proteins typically reside in the Trans-Golgi Network (TGN) where they proteolytically process newly formed proteins from the endoplasmic reticulum before packaging into secretory vesicles. This process of pro-protein maturation involves recognition and cleavage of unique sequences in target protein prodomains by specific proteases[11]. TGN resident proteases include carboxypeptidases[12], prohormone convertase (PC) family members[10;11], and β -site amyloid precursor protein (BACE) family members[13].

PCs, a family of calcium dependant subtilisin-like proteases with homology to the yeast kexin, include furin, PC1/3, PC2, PC4, PACE4, PC5/6, and PC7/8. Furin, an extensively characterized TGN PC, has been shown to process a myriad of proteins containing the RXK/RR cleavage domain found in serum proteins (proalbumin), coagulation factors (pro-vonWillebrand factor), growth factors and hormones (pro- β -nerve growth factor and bone morphogenic factor-4), cell surface receptors (insulin pro-receptor) and matrix metalloproteases (stromolysin-3, MT1-MMP)[14;15]. Processing by furin, and other PC family members, contributes to development of several diseases such as Alzheimer's disease (AD) and arthritis, and enhances invasion and proliferation of cancerous tumors[16;17]. Additionally, furin activity is necessary for propagation of viruses such HIV-1, ebola, and avian influenza, and activation of virulent bacterial pathogens such as anthrax, pseudomonas, and diphtheria[3;4;15;16;18;19;20]. The remarkably crucial role of furin and family members in viral infections is emphasized by the discovery that H5N1 avian influence virus pathogenicity is attributed to the acquisition of a polybasic tract in hemagglutinin glycoprotein, which is effectively cleaved by furin and other PCs. Acquisition of this furin cleavage domain may have extended the virus' ability to infect humans. H5N1 is extremely lethal as ubiquitously expressed furin allows systemic infection of all organs[15]. Additionally, furin activation of anthrax toxin is necessary for infection. The employment of anthrax toxin as a biological weapon by terrorists or rogue nations necessitates the development of sensitive assays that lead to identification of novel furin inhibitors that have potential to treat avian influenza, anthrax, and other furin related diseases.

Another important TGN resident protein family is the BACE family. Members such as BACE1 and BACE2 are membrane-bound aspartyl proteases that cleave the β -amyloid precursor protein (β APP), resulting in the 39-43 amino acid protein A β . Congregation of this protein contributes to formation of amyloid plaques, leading to AD[21]. Although BACE1 is the primary family member responsible for cleavage of β APP, cleavage by BACE2 plays a role in genetic disorders leading to early onset AD as seen in Down's syndrome and Flemish familial AD[22]. Additionally, a mutation in APP discovered in a Swedish family increases the efficiency of APP cleavage by BACE1 and segregates with family members that develop AD [23]. Pharmaceutical treatment of AD by inhibition of BACE is an active area of investigation [13;24].

Although there have been extensive efforts to study TGN protease biology, a versatile and sensitive assay system to non-invasively monitor their activity in real time has been lacking. Studying TGN enzymes using non-invasive assays allows preservation of the unique intracellular TGN environment (low pH, high Ca^{2+}), which is readily perturbed by invasive assays. Further, strategies to directly monitor TGN enzyme activity in its native physiological environment facilitates discovery of novel pharmaceutical agents that can traverse plasma and Golgi membranes and retain inhibitory activity within microenvironment of TGN. Identification of such inhibitors may have profound influence on the treatment of diseases like Alzheimer's and cancer and may help combat lethal viral and bacterial infections.

We have previously reported development of non-invasive assays for imaging of cytosolic proteases[25]. We describe a non-invasive, real-time cellular assay to monitor the activity of TGN resident proteases employing a chimeric alkaline phosphatase fused to a TGN protease recognition domain and a golgi retention signal. When expressed, this chimeric protein

localizes in the TGN until the protease cleaves its recognition sequence, after which alkaline phosphatase is secreted. Thus, secreted alkaline phosphatase (SEAP) levels present in the media are indicative of protease activity, which is measured using a highly sensitive assay [26]. Decreases in SEAP levels signify a loss of protease activity and allow positive identification of protease inhibitors. Using this technology, we are able to monitor protease activity and identify novel inhibitors for two TGN resident proteins: furin and BACE. Additionally, we have adapted the assay system to high-throughput screening (HTS) and discovered inhibitory compounds for furin by screening three small molecule compound libraries.

Materials and Methods

Reagents

Restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Aspirin (Fisher, Hampton, NH), Ibuprofen (Fisher, Hampton, NH), Celebrex (Pfizer, New York, NY), indomethacin (Calbiochem, San Diego, CA), sulindac sulfide (Calbiochem, San Diego, CA), Z-VLL-CHO (Calbiochem, San Diego, CA), and decRVKR-Chloromethylketone (Bachem, King of Prussia, PA) were purchased. Aspirin, celebrex, sulindac sulfide, β -Secretase Inhibitor II, and decRVKR-CMK were dissolved in DMSO and indomethacin and ibuprofen were dissolved in ethanol. Drugs, except decRVKR-CMK, were prepared fresh on the day of treatment.

Cell Culture and Transfections

COS and LOVO cells were maintained in DMEM containing 10% FBS, 1% ℓ -glutamine, 100ug/mL penicillin, 100ug/mL streptomycin (P/S/G) (Gibco, Carlsbad, CA). Chinese Hamster Ovary (CHO) and furin-deficient CHO (FD11) cells were maintained in Ham's F12 media with 10% FBS, 1uM MEM non-essential amino acids (Gibco, Carlsbad, CA), and P/S/G. Mouse Neuroblastoma (N2a) cells were maintained in 1:1 DMEM:OptiMEM mixture containing 5% FBS and P/S/G. All cells were transfected with plasmid using Eugene 6 (Roche, Indianapolis, IN) according to the manufactures protocol and stable clones were selected with 500ug/mL (N2a) or 800ug/mL G418 (CHO/FD11) (Invitrogen, Carlsbad, CA). Cell lines were incubated at 37°C with 5% CO₂.

Plasmid Construction

pEF-SEAP was generated by PCR and inserted as a 1.6kb Bgl II-EcoRI fragment into the vector pEF. GRAP plasmids were constructed by PCR overlap of the last 192bp of BACE with the cleavage site for GRAPbace using 5'-GGTTAAGATGGACGCAGAGTTCCGC-3' and GRAPbacesw using 5'-TTAACCTAGACGCAGACGTTCC-3' connecting SEAP to the BACE tail. The GRAPfurin cleavage domain was constructed by PCR overlap using primers 5'-GGGCTGAGTGCCCGCAACCGACAGAAGCGCGCGTTGTCCACGCGT-3' and GRAPfurin mutant was constructed using primers 5'-GCTGGACAACGCGGCCGCTGTGCGTTGCGGGCACTCAGC-3'. The PCR was accomplished using Hi Fidelity Taq Polymerase (Invitrogen, Carlsbad, CA). GRAPfurin and GRAPfurinmut were digested with SalI and XbaI on 5' and 3' end, respectively. Ligations were performed using Quick Ligation kit (Roche, Indianapolis, IN).

Drug Treatment Assays

For Furin secreted alkaline phosphatase (SEAP) assays, cells were plated at 80,000 cells/well in 6 well dishes and allowed to incubate for 36h. Media was then changed to OptiMEM with drug or vehicle, and cells were allowed to incubate overnight, after which media was collected for SEAP assays or WB, and cells were lysed for WB. For furin substrate assays using transient

transfections (CPA95, vWF), cells were plated as above, 36h post transfection, the media was replaced with optiMEM containing drug or vehicle and allowed to incubate for 2h. Then media was replaced with optiMEM containing drug or vehicle and allowed to incubate overnight. Media was then collected and analyzed by WB. For BACE assays, cells were plated at 7.5×10^5 cells/10cm plate. After incubation overnight, drugs or vehicle were added to the media. Media was replaced with fresh media containing drug or vehicle after 24h. After 36h, media was replaced with reduced serum media containing drug or vehicle. Media was collected and cells were harvested for activity assays and western blot 48h post drug addition.

SEAP activity assays

Cells were placed in low serum (<4% FBS) media or OptiMEM containing indicated concentrations of drug or vehicle during last 12-16h or overnight. The media was collected, filtered (N2a), and tested for SEAP activity using Great Escape SEAP chemiluminescent detection kit (Clontech, San Diego, CA) according to the manufactures protocol for 96 well plates. For BACE assays, the chemiluminescent signal was measured using a Hamamatsu imaging system (Bridgewater, NJ). The collected photon counts were converted to counts/million cells for comparison between samples. For Furin assays, the chemiluminescent signal was measured using a Fluorostar optima plate reader (BMG Labtech, Chicago, IL). Assays were performed in duplicate or triplicate for statistical analysis. All errors are reported as standard deviation from the mean (s.d.).

Western Blotting

Western blotting was performed as described[25]. Briefly, cells were washed in PBS and lysed with a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP40, supplemented with "cComplete™" protease inhibitors cocktail (Roche Diagnostics Corporation, Indianapolis, IN). Protein was estimated by detergent compatible protein assay kit from Bio Rad (Hercules, CA). Media and lysates were separated by SDS polyacrylamide gel electrophoresis, and protein expression was detected by western blot analysis using antibodies. SEAP was detected using a rabbit polyclonal antibody to alkaline phosphatase (Biomedica, Baesweiler, Germany). BACE expression was detected using a rabbit polyclonal antibody to BACE (AB-2) (Calbiochem, San Diego, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected using a mouse monoclonal antibody (Abcam, Cambridge, MA). Carboxypeptidase A was detected using a rabbit polyclonal antibody AB1213 (Chemicon, Temecula, CA). vWF was detected using a rabbit polyclonal antibody (Dakocytomation, Carpinteria, CA). HRP-conjugated secondary antibody followed by detection by chemiluminescent HRP substrate (Pierce, Rockford, IL). Nitrocellulose blots were stained using Aurodye Forte (Amersham Biosciences, Pittsburgh, PA) according the manufactures protocol.

HTS

CHO-GRAPfurin cells were plated in 384 well plates at 6000 cells/well in Ham's media and allowed to incubate (37°C, 5% CO₂) overnight. The media was then removed by inversion and replaced with 30uL optiMEM using a multidrop dispenser (Thermo Labsystems, Waltham, MA). Compounds (or controls) were added using the HDR "pin tool" (Biomek FX, Beckman, Fullerton, CA) in a volume of 0.2μl. Controls were DMSO (1% final) and inhibitor, decRVKR-CMK. 8-10h post compound addition, pNPP yellow substrate (Sigma, St. Louis, MO) was added and colorimetric analysis was performed at ABS 405nm using the PHERAstar microplate reader (BMGLabtech, Chicago, IL). All plates were barcoded for identification and linked to compounds stock plates (Chembridge, San Diego, CA and ChemDiv, San Diego, CA). Final compound concentrations were 4.2-15μM. Assay quality was determined by calculating mean, standard deviation and coefficient of variation (CV, SD/mean) for control

wells on each plate (n=16-32). $Z' > 0.5$ for all plates and for assay[27]. Plates with $Z' < 0.5$ were repeated.

Dose response assay

Compounds were transferred from stock plates (3-20mM in DMSO) into plates containing media. Assay plates were prepared by removing all media from CHO-GRAPfurin cells and replacing with optiMEM media. Media with controls or compounds was transferred to assay plates containing cells and serial dilution was performed with final concentration of 33uM to 33pM in 30ul (final DMSO concentration < 2%). After 7 hr incubation, pNPP reagent (30ul) was added and ABS 405nm measured by Pherastar. Analysis of dose response was performed using PRISM software (GraphPad, San Diego, CA) with negative control (DMSO treated cells) compared to compound treated. Positive controls were assayed using 20uM decRVKR-CMK. CHO-SEAP cells were also treated with compounds to identify non-specific inhibitors.

Furin activity assay

Recombinant purified furin (Alexis Biochemicals, San Diego, CA) and inhibitory compound was added to NaOAC buffer (50mM sodium acetate (pH 7.0) and 1mM CaCl_2) and allowed to incubate at RT for 30 minutes. After pyr-RTKR-MCA substrate (Peptide Institute, Osaka, Japan) was added, AMC release was monitored using a Fluorostar optima platereader at an excitation wavelength of 380nm and emission wavelength of 460nm. Data was plotted and kinetic parameters were determined using PRISM software.

BACE activity assay

BACE activity was measured using a Beta-secretase activity kit (R&D systems, Minneapolis, MN) using a fluorometric reaction to measure the proteolytic cleavage of APP_{sw} by BACE in cell lysates. Approximately 40×10^6 N2a cells were collected and lysed according to the manufacturer's protocol. The lysate was quantified and aliquoted for various treatment conditions. 100ug of protein was used for each sample reading within the recommended range. Lysates were preincubated with drug at RT before the initial reading (T=0). The initial reading was subtracted from subsequent readings that were taken every 15 min up to 1h. The samples were read on a fluoromax-2 fluorimeter (Instruments SA, Edison, NJ) at excitation 355nm and emission 510nm with a 495nm cutoff.

Results

Strategy for monitoring TGN protease activity

In an effort to develop a cell based assay to report on TGN resident enzyme activity, we constructed a hybrid reporter protein, GRAP (Golgi Retained Alkaline Phosphatase), consisting of three functional domains: (1) secreted alkaline phosphatase (SEAP), (2) a Golgi protease specific recognition and cleavage site, and (3) the cytoplasmic and transmembrane domains from BACE that retains the reporter within the TGN (Fig. 1a). This fusion protein, when expressed in cells, localizes to TGN until it is cleaved by a specific TGN protease, after which SEAP is secreted into the extracellular media (Fig. 1b). SEAP levels present in the media are indicative of intracellular TGN protease activity[26].

Construction and validation of furin reporter

We constructed GRAPfurin, which contains a 10 amino acid recognition and cleavage site (GLSARNRQKR ↓) from the furin substrate, ST3[28]. To investigate whether this reporter protein is sensitive to furin activity, we transfected the furin expressing Chinese hamster ovary (CHO) cell line and two furin deficient cell lines, FD11(furin-deficient CHO)[3] and LOVO (human colon adenocarcinoma)[29] with GRAPfurin and either empty vector or the furin

expression plasmid. Co-expression of furin increased processing of GRAPfurin in CHO and LOVO cells leading to a decrease in unprocessed protein when lysates were immunoblotted with BACE antibody to detect both unprocessed (64kDa) and processed (7kDa) fragments. In FD11 cells, an increase in processed protein was observed. When a western blot was performed using the extracellular media and SEAP antibody, co-expression of GRAPfurin and furin lead to an increase in SEAP levels in the extracellular media of CHO, FD11, and LOVO cells (Fig. 1c). This was further confirmed by detection of SEAP activity in the extracellular media. Co-transfection of GRAPfurin and furin resulted in increased SEAP activity in the extracellular media of CHO, FD11, and LOVO cells (Fig. 1d). To further investigate the specificity of this system, we constructed GRAPfurinmut, wherein the furin target recognition and cleavage sequence was mutated to GLSAANAQAA ↓ rendering this reporter non-responsive to furin proteolytic activity. Stable CHO cell lines expressing either GRAPfurin or GRAPfurinmut were transfected with furin expression plasmid and furin activity was monitored by western blotting using BACE and SEAP antibodies and by a SEAP activity assay. Mutation of the furin recognition and cleavage sequence lead to reduction in processed protein in both the lysate and extracellular media (Fig. 1e) and a concomitant decrease in SEAP activity (Fig. 1f). Further, a notable decrease in SEAP activity was observed when CHO-GRAPfurin cells were treated with 25μM of the furin inhibitor, deconyl-RVKR-chloromethylketone (decRVKR-CMK) (Fig. 1g), while similar treatment with decRVKR-CMK caused no decrease in SEAP activity on the control CHO cell line constitutively expressing SEAP (data not shown).

High throughput screening with small molecule libraries using furin reporter

Having established the specificity and sensitivity of the furin reporter system, we adapted this technology for High Throughput Screening (HTS) of several small molecule libraries (ChemDiv, Chembridge, and a collection of natural product extracts) and screened over 30,000 compounds in a 384-well plate format using CHO-GRAPfurin cells and 20uM decRVKR-CMK as a positive control. A representative plate from the HTS is shown in Fig. 2a. Positive hits were defined as compounds that decreased SEAP activity more than 3 s.d. from the mean of untreated cells. From the 30,000 compounds screened, we narrowed the hits down to five compounds using secondary assays that eliminated compounds that inhibited SEAP directly, were cytotoxic, and those whose intrinsic fluorescence interfered with the AP substrate readout. Compound CCG 8294 was selected from the five and inhibition was shown to occur in a dose dependent manner in CHO-GRAPfurin. Treatment of CHO-GRAPfurin with CCG 8294 had no effect on furin activity until ~100nM where a decrease in SEAP activity was first observed. Furin activity decreased in a dose dependant manner from 100nM-100μM in this experiment, resulting in a pIC50 of 5.26. When repeated, an average pIC50 of 4.98 (Fig. 2b) or ~10⁻⁵M was obtained.

Validation of CCG 8295 as furin inhibitor

We then tested whether CCG 8295 inhibited furin mediated processing of two well studied furin substrates, vonWillebrand Factor (vWF) and the engineered substrate CPA95[30]. vWF is a secretory protein that is processed by furin and other PC family members[31]. When transfected into CHO cells, vWF secreted into the extracellular media was almost entirely processed. Addition of 20μM CCG 8294 inhibited furin processing of vWF and an increase in the unprocessed form of the protein was observed by western blotting with vWF antibody (Fig. 3a). Addition of 20μM decRVKR-CMK also inhibited processing of vWF, resulting in an increase in the unprocessed form. CPA95 is a soluble furin substrate that has been engineered by addition of a furin cleavage domain to carboxypeptidase A. When CPA95 was transfected into CHO cells, furin mediated processing occurred, resulting in the appearance of both unprocessed (43kDa) and processed (32kDa) forms of the protein in the extracellular media. Addition of both decRVKR-CMK and CCG 8294 resulted in a decrease in the processed protein and increase in the unprocessed protein as observed by western blot analysis with CPA

antibody. Further, CPA95 processing was inhibited by increasing doses of CCG 8294 (Fig. 3c). To further validate CCG 8294 as a furin inhibitor, an *in vitro* activity assay was performed using purified recombinant furin and the furin substrate pyr-RTKR-MCA. Addition of increasing concentrations of CCG 8294 resulted in a dose dependant decrease in furin activity as measured by processing of the fluorescent substrate with an average IC₅₀ of ~22uM (Fig. 3d).

Construction and validation of BACE reporter

We next sought to determine if this TGN protease activity reporter system can be customized to report activity of BACE, another TGN protease. For this, we generated GRAPbace and GRAPbacesw from GRAPfurin by substituting the furin cleavage domain with wild type or Swedish mutant β -secretase recognition and cleavage sequences from β APP (Fig. 1a). Originally found in a Swedish family, the Swedish mutation within the β APP cleavage site increases processing by BACE and predisposes family members with the mutation to Alzheimers disease[13]. Since BACE activity plays a key role in Alzheimers disease, we chose a cell line of neuronal origin (N2a) for these experiments. To monitor BACE activity, we created stable N2a cell lines expressing GRAPbace and GRAPbacesw. N2a-GRAPbace and N2a-GRAPbacesw cells were transfected with empty vector or human BACE, and lysates and extracellular media were immunoblotted with SEAP antibodies. Both processed (64kDa) and unprocessed (56kDa) BACE reporter protein fragments were detected in the lysates of N2a-GRAPbace and N2a-GRAPbacesw cells, and SEAP protein was not detected in the conditioned media. BACE expression resulted in appearance of SEAP protein in the conditioned media of N2a-GRAPbace cells. In N2a-GRAPbacesw cells, BACE expression resulted in increased processing of BACE reporter protein as indicated by a decrease in the unprocessed form present in cell lysates and an increase in SEAP protein detected in the extracellular media (Fig. 4a). Further, SEAP levels were notably higher in the media of GRAPbacesw cells compared to GRAPbace (Fig. 4a). Transfection of the BACE expression plasmid into GRAPbace and GRAPbacesw cells resulted in a five-fold increase in SEAP activity (Fig. 4b). To evaluate the effects of a BACE inhibitor on the reporter system, N2a-GRAPbacesw cells were treated with peptidyl BACE inhibitor, Z-VLL-CHO (BACE Inhibitor II) and SEAP activity in extracellular media was measured. A dose dependent decrease in alkaline phosphatase activity was observed in N2a-GRAPbacesw cells treated with BACE inhibitor II (Fig. 4c).

Screening and validation of BACE inhibition by NSAIDs

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce incidence of AD by 60-80% [32;33]; however, the mechanism by which NSAIDs restrain AD progression is not clear. We tested whether NSAIDs inhibited BACE activity. For this, N2a-GRAPbacesw cells were treated with ibuprofen, sulindac sulfide, celebrex, and aspirin. At physiologically relevant doses, ibuprofen, indomethacin, and the Cox-2 inhibitor, celebrex, had no noticeable effect on SEAP activity in the extracellular media of N2a-GRAPbacesw expressing cells. Treatment of N2a-GRAPbacesw cells with sulindac sulfide and aspirin substantially inhibited BACE activity (Fig. 5a). In contrast, none of these agents influenced SEAP secretion from N2a control cells constitutively expressing SEAP (data not shown). A dose response analysis of the ability of sulindac sulfide and aspirin to inhibit BACE in N2a-GRAPbacesw revealed that sulindac sulfide and aspirin decreased BACE activity in a dose dependant manner (Fig. 5b and 5c). To confirm that sulindac sulfide and aspirin are able to inhibit BACE, we performed an *in vitro* BACE activity assay wherein N2a cell lysates were incubated with sulindac sulfide or aspirin and proteolytic cleavage of a fluorescently labeled APP substrate was monitored. Both sulindac sulfide and aspirin inhibited BACE cleavage of fluorescent APP in a dose dependant manner (Fig. 5d).

Discussion

Many current methods of assessing cellular TGN protease activity are invasive and prohibit direct, real-time monitoring of TGN proteases in their natural environment. Many TGN proteases such as PC family members play a critical role in a number of pathologies; however, there are few potent, non-toxic inhibitors and none have reached clinical trials. This may be due to a scarcity of sensitive and specific cell-based assay systems for monitoring furin/PC activity. The technology described here is highly sensitive, specific, and adaptable to a number of proteases and allows direct monitoring of protease activity in undisturbed cells.

GRAPfurin possesses the tetrabasic cleavage domain, RX(K/R)R. Although this domain is cleaved by furin, furin homologues can also efficiently process substrates containing this site. For example, cleavage of GRAPfurin occurred, to a lesser extent, in the furin deficient cell lines, LOVO and FD11 (Fig. 1c). Though these cell lines exhibit furin deficiency, some furin-like activity occurs due to the activity of furin homologues in the cells[34;35;36].

Several protein/peptide based furin inhibitors have been reported such as α 1-PDX[37], D6R [38], and D9R[39]. Unfortunately, use of these inhibitors as pharmaceutical agents is hampered by their large size, lack of stability, and/or toxicity. Another inhibitor, decRVKR-CMK is a potent inhibitor of furin with an IC₅₀ in the pM range but is extremely toxic[40]. To date, the only non-protein/peptide inhibitor of furin is a naturally occurring neoandrographolide and its succinoyl ester derivatives[18] with IC₅₀ values ranging from high μ M to low mM. Although copper complexes of terpyridine derivatives[41] show inhibitory concentrations in the μ M range, there is currently no documentation of efficacy against furin related diseases *in vivo*. There is urgent need for new inhibitors and the cell based assay described here will expedite drug discovery as library screening will allow identification of inhibitors with desired characteristics in context of toxicity, solubility, and ability to interact with the protein target in its appropriate subcellular compartment. We identified furin inhibitors from a screen of 30,000 molecules, and one is described in detail here. CCG 8294 has shown great promise as a furin inhibitor with high efficacy in cells and has also demonstrated a dose dependant inhibition of furin mediated processing of polypeptides within the secretory pathway (CPA95). Furin mediated cleavage of vWF was inhibited at 20 μ M (Fig. 3a). Limited toxicity at 20 μ M was evident as dose dependant cleavage of CPA95 showed some reduction in protein expression at 20 μ M. To improve the specificity and potency while reducing toxicity, a number of structural analogs of CCG 8294 will be synthesized and efficacy tested. Additionally, other libraries will be screened for identification of novel inhibitors. Future studies will determine whether CCG 8294, its chemically modified version, or other newly identified molecules will be used clinically to treat diseases or prophylactically to enhance national security.

Our cell based assay can easily be extended to studies of other TGN enzymes by making alterations within the proteolytic cleavage domain. We developed an assay to report on the activity of the TGN protease, BACE. Oh et.al. also utilized a cell based assay for BACE to identify peptide mimetic inhibitors using drosophila cells[42]. BACE1 and its significance to Alzheimers disease development has been extensively studied[43;44]. BACE1 processes β APP, generating A β ₄₂, which accumulates in Alzheimers disease (AD) patients with deleterious impact on mind and memory[43]. Several identified mutations in the β APP sequence segregate with the disease in inherited forms of AD known as early onset familial AD and result in increased A β production[45]. A mechanism of early onset AD occurs in Down's syndrome patients, who have increased expression of β APP and BACE2 due to an additional copy of these genes on chromosome 21[22]. The Swedish mutation increases affinity of BACE for the β APP cleavage domain, resulting in increased processing of APP. Our SEAP activity assay directly supports this, as transfection of human BACE plasmid in N2a-GRAPbacesw produced a five-fold increase in SEAP activity due to increased processing of the BACE reporter within the TGN.

Whether AD develops due to family genetic predisposition or through normal plaque accumulation, discovery of drugs to treat the disease will continue intensely as according to the World Health Organization, over 18 million people are currently suffering with this disease. Since BACE1 processing is the first step in generation of A β ₄₂, BACE1 has become a key target for pharmaceuticals development to treat AD. There are currently several polypeptide based inhibitors of BACE. Unfortunately, their use as pharmaceuticals is limited as peptides do not significantly penetrate the blood-brain barrier. In light of this, it is important to screen libraries for small molecule inhibitors of BACE [43]. As we have shown with furin, a HTS using small molecule libraries can be accomplished with this cell-based assay to identify inhibitors of BACE.

While the search for BACE inhibitors continues, the medical community has observed that chronic users of NSAIDs have decreased AD incidence, slowed progression, delayed onset, and reduced symptomatic severity[46]. Though many theories have been proposed, the mechanism by which NSAIDs achieve their ameliorating effects is presently unclear[47;48; 49;50;51;52]. Many believe NSAIDs anti-inflammatory activity contributes to reduction of AD noting that disease progression is associated with chronic brain inflammation. Our studies using the cell-based BACE reporter and BACE activity assay demonstrate that NSAIDs aspirin and sulindac sulfide act directly to inhibit BACE. Recent *in vitro* studies have shown that NSAIDs reduce A β ₄₂ production by inhibition of γ -secretase activity or inhibition of substrate cleavage[49;53]. Although γ -secretase inhibition may provide relief from A β accumulation, it cleaves many other substrates required for normal brain function, thus inhibition may lead to side effects [24]. In light of this, BACE inhibition may provide an alternate avenue for drug development as BACE knockout mice are phenotypically normal, and a BACE inhibitor should reduce APP cleavage without altering γ -secretase activity [21].

The long term goal of this study is to utilize these novel assays to better understand the biology of golgi-resident proteases, their regulation, and their role in specific pathologies. The data presented here describes the utility of these assays in identification of novel modulators of protease activity and can be extended to studies of other golgi proteases.

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Figure 1. Strategy for non-invasive monitoring of TGN proteases and validation of furin reporter
 (a) A chimeric reporter consisting of signal peptide, alkaline phosphatase, protease cleavage domain and golgi retention signal was constructed. (b) When expressed in cells, this reporter molecule is retained in the TGN. Proteolytic cleavage mediated by a specific golgi-resident protease cleaves the reporter protein and releases alkaline phosphatase for secretion into extracellular media. (c) A representative western blot of lysates of CHO, FD11, and LOVO cells transfected with GRAPfurin alone or in combination with furin with BACE antibody and the extracellular media from the same cells using SEAP antibodies. (d) SEAP activity detected in extracellular media of CHO, FD11, and LOVO cells transfected with GRAPfurin alone or in combination with furin. (e) A representative western blot of lysates from CHO-GRAPfurin

or CHOGRAPfurinmut cells transfected with either empty vector or furin using BACE antibodies. GAPDH antibody was a loading control. Alkaline phosphatase in the extracellular media from the same cells was detected using SEAP antibody and for loading control the membrane was stained with Aurodye. (f) SEAP activity in the extracellular media of the CHO-GRAPfurin or CHO-GRAPfurinmut transfected with either empty vector or furin (n=3). (g) SEAP activity in the extracellular media of CHO-GRAPfurin in the presence or absence of furin inhibitor decRVKR-CMK (n=3). (Mean +/- SD).

Figure 2. High throughput screen of small molecule libraries for furin inhibitors

(a) Representative results of high throughput screening of inhibitors from one 384 well plate. Blue squares represent negative control (DMSO alone), red squares represent positive control (decRVKR-CMK), and green squares represent compounds screened. (b) Structure of CCG 8294. (c) A representative graph of dose dependent inhibition of furin activity by CCG 8294 resulting in a pIC₅₀ of 5.26 (n=1). CCG 8294 produced an average pIC₅₀ of 4.96 (n=2). (Mean $\pm$ SD).

Figure 3. Validation of CCG 8294 as furin inhibitor *in vitro*

(a) Representative western blot on extracellular media of CHO cells transfected with vWF treated with or without 20 μ M CCG 8294 using vWF antibody. (b) Representative western blot of extracellular media of CHO cells transfected with CPA95 treated with or without 20 μ M CCG 8294 using CPA antibody. (c) Representative western blot of extracellular media of CHO cells transfected with CPA95 treated with increasing concentrations of CCG 8294 (0-20 μ M) using CPA antibody. All experiments utilized decRVKR-CMK as a positive control. (d) *In vitro* furin activity assay using purified recombinant furin, a fluorescent substrate, and CCG 8294 (n=3). (Mean $\pm$ SD).

Figure 4. Validation of BACE reporter

(a) Representative western blot of lysates and extracellular media of N2a-GRAPbace and N2a-GRAPbacesw cells transfected with either empty vector or human BACE using SEAP antibody. Lysates and extracellular media from N2a cells expressing SEAP were used as a control. (b) SEAP activity in the extracellular media of the N2a-GRAPbace or N2a-GRAPbacesw cells transfected with empty vector or human BACE (n=3). (c) SEAP activity in the extracellular media of the N2a-GRAPbacesw cells in the presence or absence of BACE inhibitor II (0-2.5 μM) (n=3). (Mean±SD).

a **b** **c** **d** **e** **Figure 5. Affect of NSAIDs on BACE activity**

(a) SEAP activity in the extracellular media of N2a-GRAPbacesw cells treated with NSAIDs ibuprofen (50μM), sulindac sulfide (2μM), celebrex (1μM), and aspirin (1mM) (n=3). SEAP activity in the extracellular media of the N2a-GRAPbacesw cells in the presence or absence of sulindac sulfide (0-25μM) (b) or aspirin (0-2mM) (c) (n=3). BACE activity assay using N2alysates and fluorescently labeled APP in combination with sulindac sulfide (0-100μM)(d) or aspirin (0-2mM) (e) (n=3). (Mean +/- SD).