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Abstract

The underlying hypothesis of this proposal is that a breast tissue/cancer proteolytic activity can be identified by screening the extracellular fluid from human breast cancers with a fluorescence quenched random peptide library. The peptide substrate(s) identified from this screening could be used to produce prodrugs that are targeted for specific activation by proteolytic activity present in extracellular fluid of breast cancers while avoiding systemic toxicity. In the first year we developed methods to synthesize large fluorescently quenched peptide libraries as outlined in task 1 and screened for hydrolysis by human glandular kallikrein 2 shown to be present in ~ 75% of breast cancers. A peptide substrate was identified and couple to the thapsigargin analog, L12ADT to produce a prodrug that was readily hydrolyzed by hK2, stable in human plasma in vitro and mouse plasma both in vitro and in vivo, and was selectively cytotoxic to cancer cells in the presence of enzymatically active hK2. These studies demonstrated the feasibility of the approach to identification of protease substrates outlined in tasks 1-3 of the proposal. However, incubation of breast cancer homogenates or concentrated media from breast cancer cell lines did not yield any hydrolyzed peptides. This lack of hydrolysis is most likely is due to a combination of ng/ml concentrations of protease in the extracellular fluid and the need to dilute samples to cover entire bead library (i.e. 10-40 mls). New approaches are needed to identify breast cancer proteases that can concentrate proteases within breast cancer extracellular fluid or which can screen large libraries in much smaller volume. Two such methods, in vitro compartmentalization and macroglobulin complexation are being evaluated in the laboratory.

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INTRODUCTION:

From a very early stage in the disease process, breast cancers are composed of a heterogeneous collection of cells. These breast cancer cells within a given patient have varying sensitivities to commonly used antiproliferative agents. Metastatic breast cancers are often initially responsive to the commonly used chemotherapeutic agents such as doxorubicin and the taxanes. Unfortunately, no patient is cured by these therapies and thus, metastatic breast cancer is uniformly fatal. New effective therapies for breast cancer are therefore urgently needed.

Currently used chemotherapies are for the most part antiproliferative agents and general cytotoxins that take advantage of the differential rate of growth of cancerous versus normal tissue. These therapies, therefore, are not breast cancer cell specific in their cytotoxicity and their use is often associated with significant dose limiting toxicities. New strategies are needed to inhibit breast cancer specific targets while at the same time avoiding toxicity to normal host tissues. The approach outlined in this proposal is to inhibit a ubiquitous intracellular protein whose function is mandatory for survival of all cell types. This approach would overcome the problem of heterogeneity and "resistance" as all cancer cells within a tumor could be killed via this approach. The obvious shortcoming of this approach is that the cytotoxicity would not be cell-type specific and administration of such a general toxin would be associated with significant systemic toxicity.

Previously we and others have identified such a ubiquitous intracellular protein, the Sarcoplasmic/ Endoplasmic Reticulum Ca^{2+} ATPase (SERCA) pump (1-4). We documented that inhibition of this SERCA pump by the natural plant product thapsigargin (TG) could induce apoptosis of all cell types including breast and prostate cancer cell lines (3-5). Because TG does not possess the primary amine needed for coupling to the C-terminal carboxyl of a peptide carrier, primary amine analogs of TG were made (6,7). Based on a model of the TG binding site within the SERCA pump it was determined that modifications of the TG molecule could possibly be made in the side chain in the 8-position without adversely effecting SERCA pump inhibitory activity (6,7). Using this rationale a series of TG analogs (i.e. ~50) modified in the 8-position with primary amine containing side chains were synthesized by my long time collaborator and discoverer of TG, Dr. Soeren Christensen from the Royal Danish School of Pharmacy in Copenhagen (6,7). These analogs were characterized for their ability to inhibit the SERCA pump and elevate intracellular calcium (6,7). In addition, these analogs were assayed for cytotoxic activity against androgen independent human prostate cancer cells in vitro (6,7). The best of these analogs contained a 12-amino dodecanoate side chain (12ADT) coupled to the amino acid leucine (L12ADT) and was found to have an LD_{50} value against prostate cancer cells of ~ 30 nM. This LD_{50} is identical to that reported for TG (25). In addition, we developed a strategy to target this potent TG analog specifically to sites of prostate cancer to avoid systemic toxicity (8). This approach targets the proteolytic activity of the serine protease prostate-specific antigen (PSA) (8). We identified a PSA specific peptide substrate (9) that is can be coupled to the L12ADT analog of TG to produce an inactive prodrug that can only be proteolytically activated by enzymatically active PSA present within the extracellular fluid prostate cancer (9-11).

Breast cancer cells, like prostate cancer, secrete a variety of proteases. While a PSA-like protease that is secreted in large amounts in a tissue restricted manner has yet to be identified for breast tissue, there are a number of proteases whose expression has been demonstrated to be relatively increased in breast cancer tissue. Examples of such proteases include cathepsins, kallikreins and members of the matrix metalloproteinase family (12-22). Although newer techniques like differential display and SAGE analysis are powerful tools that are yielding large numbers of putative new therapeutic targets, these techniques do not always provide information about functional activity of identified expression products. This functional information is particularly critical when evaluating protease expression because the activity of these proteins is tightly regulated at a number of levels (e.g. expression levels, processing to active protease, binding to inhibitors, auto-degradation). Therefore, additional methods are needed to that will help define not only the presence but also the functional activity of these proteases. The significance of this proposal is that it proposes a strategy to identify specific peptide substrates for breast cancer/tissue specific proteolytic activities. These studies may help to identify new diagnostic and/or therapeutic targets in breast cancer. In addition, the specific peptide substrates will be

incorporated into prodrugs to yield novel targeted therapies for breast cancer while avoiding toxicity to normal tissue.

BODY:

Hypothesis: The underlying hypothesis of this proposal is that a breast tissue/cancer proteolytic activity can be identified by screening the extracellular fluid from human breast cancers with a fluorescence quenched random peptide library. The peptide substrate(s) identified from this screening could be used to produce prodrugs that are targeted for specific activation by proteolytic activity present in extracellular fluid of breast cancers while avoiding systemic toxicity. The peptide substrate(s) will also be used to isolate and characterize specific protease responsible for specific substrate hydrolysis.

To accomplish the goal of the proposal, we outlined 4 tasks that would be completed over the three year funding period. The objective of **Task 1** (months 0-12) was to synthesize fluorescence quenched random peptide libraries to identify proteolytic activities present in the extracellular fluid of human breast cancer cell suspensions. The objective of **Task 2** (months 3-12) is to synthesize soluble fluorescent peptide substrates to characterize specificity and efficiency of hydrolysis. The objective of **Task 3** (months 9-24) is to synthesize thapsigargin prodrugs by coupling thapsigargin analog to lead peptide substrates identified in tasks 1-3. The objective of Task 4 (months 24-36) is to identify specific protease(s) responsible for proteolytic activity using peptide substrates identified in tasks 1-2.

As an initial step toward accomplishing we needed to develop expertise in the synthesis and characterization of large fluorescence quenched random peptide libraries of ~ 1.5- 2 million peptide sequences containing 6 random amino acids. To produce these libraries we used the "one bead-one peptide" splitting and mixing technique (23-24). This approach produces peptides bound to PEGA grafted "beads" in such a way that each individual bead contains many copies of one unique peptide sequence (25). The peptides are synthesized using a previously described approach with a fluorescent molecule [aminobenzoic acid (ABZ)] at the carboxy terminus and a quencher molecule (nitrotyrosine) at the amino terminus (26-29). This design produces a fluorescence quenched library because the emission spectrum of ABZ overlaps exactly with the absorbance spectrum of nitrotyrosine. Cleavage of the peptide sequence by a protease liberates the quencher moiety resulting in a fluorescent bead that is easily visible and easily removed for peptide sequencing, figure 1.

To accomplish task 1 we proposed to incubate these libraries with extracellular fluid from breast cancers. However, prior to exposing fluid from breast cancers that most likely would contain a mixture of proteases, we decided to work out the methodologies for synthesizing and screening a large random library using a more simplified system. Therefore, prior to screening the breast cancer fluid we chose to use the method to identify a substrate for a single purified protease. For these preliminary studies we selected the serine protease human glandular kallikrein 2 (hK2). HK2 has been well-characterized in prostate tissue but is also known to be selectively produced by ~ 75% of breast cancers (Black MH, et al. Expression of a prostate-associated protein, human glandular kallikrein (hK2), in breast tumours and in normal breast secretions. Br J Cancer. 2000;82:361-7).

Through a collaboration with Dr. Hans Lilja at Memorial Sloan Kettering, we have access to large amounts of purified enzymatically active hK2. Therefore, in preliminary studies we generated a fluorescence quenched, combinatorial peptide library and incubated it with hK2. Following incubation, a series of putative peptide substrates were identified and resynthesized as soluble peptide substrates. These soluble substrates were screened for hK2 activity and plasma stability. Finally, the best of these substrates with the sequence Gly-Lys-Ala-Phe-Arg-Arg (GKAFRR) was coupled to a potent analog of thapsigargin to generate the prodrug GKAFRR-L12ADT. This prodrug was then characterized for hydrolysis by hK2, stability in human plasma and selective cytotoxicity against hK2 producing and non-producing cancer cell lines. The rationale, methods and results of these studies using hK2 are described in detail in the attached appendix (appendix 1) which is a manuscript that has been recently submitted for publication.

These studies using purified hK2 allowed our laboratory to develop expertise and techniques required to accomplish goals outlined in tasks 1-3 of the proposal. The results generated using the combinatorial library

approach to identify hK2 peptide substrates suggested that a similar approach, as outlined in the proposal, could successfully yield breast cancer specific substrates. Therefore, a large combinatorial library was synthesized containing ~ 1.5 million random sequences of 6 amino acids in length. The library was screened and a peptide with the sequence Gly-Lys-Ala-Phe-Arg-Arg (GKAFRR) was idenitified that was very efficiently hydrolyzed by hK2 with a Km of 26.5 μM, the k_{cat} at 1.09 sec^{-1} and the k_{cat}/K_m ratio was 41,132 sec^{-1} M^{-1}. This substrate was subsequently attached to a previously identified analog of the potent cytotoxin thapsigargin. The analog, termed L12ADT, is as potent a cytotoxin as thapsigargin but contains an amino acid linker, leucine, which allows for attachment to peptides. On this basis, the prodrug GKAFRR-L12ADT was synthesized. This prodrug is stable to hydrolysis in human plasma. It is more toxic to hK2 producing cell lines than non-hK2 producing cell lines in vitro. Subsequently, the maximally tolerated dose was determined in mice and found to be 6 mg/kg. At this dose, the half life of the drug in mouse plasma is 40 minutes. The prodrug demonstrated significant antitumor effect in vivo while it was being administered, but prolonged intravenous administration was not possible due to local toxicity to tail veins.

On the basis of these results we will screen the prodrug against wild type breast cancer cell lines that produce varying levels of hK2. We will also construct breast cancer cell lines that overexpress hK2 to generate models that can be used for in vivo testing.

Studies with Human Breast Cancer extracts

Our original intention was to incubate this library with extracellular fluid obtained from fresh breast cancer specimens obtained directly from the operating room. Unfortunately, over the course of this first funding year, we have been unable to obtain such fresh samples from the Johns Hopkins Department of Pathology as anticipated. Instead, we have had to rely on frozen specimens to complete these screening assays. On this basis, the newly synthesized combinatorial library was initially incubated with homogenate from 2 breast cancer specimens (total of ~ 200 mg of cancer tissue). This homogenate was incubated with 25% of the peptide library, initially for a period of 48 hrs. At this point in time, no beads had become fluorescent, indicating that no hydrolysis had occurred. The library was incubated for a total of 5 days, again with no positive fluorescent beads.

One possible reason for lack of hydrolysis of peptide containing beads in this assay may be due to small amount of frozen cancer tissue that was available for the assay which, concomitantly, would contain low amounts of proteases. For this assay we obtained homogenized tissue in 1 ml of buffer. The peptide library of ~1 million beads requires ~ 40 ml of solution to wet all of the beads. Thus, the breast cancer homogenate had to be diluted ~ 10-fold more just to cover the beads in 25% of the library. Thus, low levels of protease present in the extracellular fluid would be diluted to levels that may lie below sensitivity of assay, even after 5 days incubation. For example, hK2 is present in media of prostate cancer cells at levels of ~ 10-50 ng/ml. Dilution of this media ~ 10-fold would yield a concentration of hK2 of ~ 1- 5 ng/ml. In the combinatorial library screen a level of 4 μg/ml hK2 was required to identify hydrolyzed peptides over 48 hr incubation period. In the original proposal our plan was to use media from single cell suspension of human breast cancers that had been conditioned for multiple days as the source of extracellular protease. Due to the difficulty in obtaining such fresh specimens we opted to use a human breast cancer cell line, MCF-7, to determine if this combinatorial peptide methodology could be used to identify proteolytic activity. For these assays, MCF-7 cells were grown in standard serum containing media until cells were ~60% confluent. Cells were then transferred into DMEM media without any additional serum to obtain conditioned media that did not have any serum protease inhibitors present. Media was conditioned for 4 days and then media from 5 T-75 flasks (i.e. ~ 5 x 10^7 cells) was collected and concentrated ~8-fold to a volume of 10 ml. Again, this fluid was incubated with beads representing ~ 25% of the library (i.e. ~ 250,000 peptide sequences). After 5 days incubation, 3 fluorescent beads were observed, but Edman sequencing revealed that these beads represented false positives that had not been correctly synthesized.

On the basis of the preliminary studies with hK2 and these two experiments with frozen breast cancer tissue and concentrated conditioned media from a human breast cancer cell line, we have concluded that the

combinatorial bead strategy for identifying breast cancer proteases has a number of limitations and will need to be modified in future studies. The major limitations of this approach are:

1. The beads used for solid phase synthesis are ~ 300 micrometers in diameter and therefore, one million beads takes up ~ 40 ml volume. This requires either a large amount of extracellular fluid (ECF) or dilution of ECF multiple-fold to cover all of the beads.

2. The proteases in the ECF of breast cancer cells most likely are in concentrations in the ng/ml range and this bead methodology appears to work best with proteases in the μg/ml range.

3. Proteases greater than ~ 35,000 MW do not easily access the central portions of the beads, resulting in limited hydrolysis and poor sensitivity.

4. Steric constraints imposed by the solid phase surface limit peptide hydrolysis compared to soluble peptide substrates.

Planned Studies for 2005-2006

On the basis of our prior studies it is clear that future success in the project will require development of methods to (1) decrease the physical size of the library; (2)to screen soluble peptides in smaller volume of fluid that can be obtained from ECF of human breast cancers; (3) or to develop alternative method to enrich for breast cancer specific proteases. To accomplish the first task we intend to modify the combinatorial strategy originally outlined in task 1 and adopt the combinatorial screening method recently described by Sepp, et al. (see appendix 2) depicted in Figure 1 (30). In this approach, an oligonucleotide is generated that encodes a random peptide flanked by peptide tags recognized by commercially available antibodies. This oligonucleotide is coupled to biotin and then attached to a streptavidin coated microbead (i.e. one micron diameter). Beads are then compartmentalized in a water-oil emulsion to give on average ~ 1 bead per compartment and are transcribed and translated in the compartment. For our application the gene will encode for a peptide with the general sequence FLAG Tag- Random 8 amino acid Peptide- HA Tag. Peptides will be bound to bead by binding to commercially available biotinylated antibody to FLAG attached to streptavidin surface. Beads are then incubated with protease containing ECF from breast cancers. Non-hydrolyzed beads will bind to commercially available HRP-linked HA antibody and will become fluorescent. Beads containing peptides that have been hydrolyzed by proteases will not bind to HRP-linked HA antibody and will not be fluorescent. These beads can then be rapidly sorted on flow cytometer. Non-fluorescent beads can be captured and nucleotide sequence determined to then determine random peptide sequence. These peptides can then be resynthesized as soluble peptides and rescreened as outlined in task 2. Further modifications to peptide sequence can be made suing this approach to identify an optimal peptide for protease activity present in ECF.

There are many advantages to the In Vitro Compartmentalisation approach for this application. First, the physical volume of the library is greatly reduced by decreasing the size of the bead particles from 300 to 1 micron in diameter. A library of ~ one million sequences will only take up ~50-100 μl volume in this approach (31). In addition, the use of an oligonucleotide to generate the peptide will allow for rapid modification and optimization of peptide sequences. The time to determine peptide sequence and costs of sequencing will be greatly reduced by this approach. Finally, bead screening and sorting can be performed using standard flow cytometer available as a core facility within the Oncology Department. This method, therefore, allows for rapid screening of large numbers of beads in a short period of time.

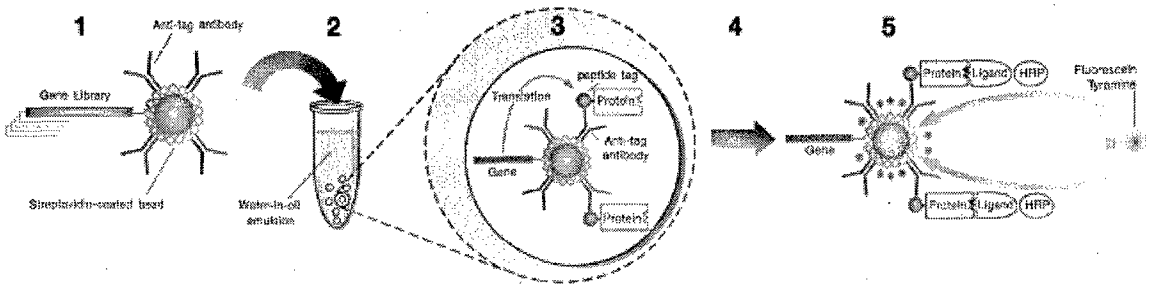

Figure 1. Creation of microbead display libraries by In Vitro Compartmentalisation and selection for binding using flow cytometry. A repertoire of genes encoding protein variants, each with a common N- or C-terminal epitope tag, are linked to streptavidin-coated beads carrying antibodies that bind the epitope tag at, on average, ≤1 gene per bead (1). The beads are compartmentalised in a water-in-oil emulsion to give, on average, <1 bead per compartment (2), and transcribed and translated in vitro in the compartments. Consequently, in each compartment, multiple copies of the translated protein become attached to the gene that encodes it via the bead (3). The emulsion is broken (4), and the microbeads carrying the display library isolated. The beads are incubated with ligand coupled to horseradish peroxidase (HRP), washed to remove unbound ligand and incubated with hydrogen peroxide and fluorescein tyramide (5). Immobilised HRP converts the fluorescein tyramide into a short-lived, free-radical intermediate which reacts with adjacent proteins. Hence, beads displaying proteins that bind ligand become labeled with multiple fluorescein molecules. These beads can then be enriched (together with the genes attached to them) by flow cytometry. [Figure and legend from Sepp et al. FEBS Letters 532:455-458, 2002 (ref 30 and Appendix 2)].

Over the past year we have begun to create components of the In Vitro Compartmentalisation system. These include the construction of the gene library encoding for peptide that would include the appropriate binding tags (i.e. FLAG, etc) and development of methods to generate water-in-oil emulsion system to capture individual gene sequences. We will begin testing to determine if we can achieve adequate translation to produce enough peptide in the individual microsomes for detection by fluorescence based cell sorting. Once we have optimized the system we will begin to analyze extracts from breast tissues and conditioned media from breast cancer cell lines to determine if this In Vitro Compartmentalisation system is applicable to the goal of identifying breast cancer specific peptide substrates.

While work is ongoing to produce small scale libraries that can be used to identify breast cancer proteolytic activity in small volume of fluid we have also initiated studies to capture and characterize specific proteases present in conditioned media and extracellular fluid of breast cancer samples. To accomplish this goal we will take advantage of the fact that protease compartmentalization is abnormal in within cancer sites. Normally, protease activity is tightly regulated within tissues and within the serum. Proteases are frequently produced as inactive zymogens that are secreted into discrete compartments within an organ (e.g. prostatic acinus) and only become activated under specific physiologic conditions and within specific tissue compartments. Dysregulation of protease activation and compartmentalization occurs within cancer tissue. Proteases are released and activated abnormally within the extracellular fluid. These abnormally compartmentalized and activated proteases enter the serum from the extracellular fluid of tumors and bind to serum inhibitors that also target the proteins for clearance and degradation. Examples of protease inhibitors found in the blood include antichymotrypsin and its broad specificity counterpart, alpha-2-macroglobulin.

Alpha-2-macrglobulin (A2M) is a 720kDa tetrameric glycoprotein found in the circulating serum of both vertebrates and invertebrates. In human plasma, it is found at concentrations ranging from 2 to 4 mg/ml, making it one of the most abundant proteins in the circulation. A2M functions principally as a protease inhibitor, employing a unique process to capture a broad spectrum of active proteases, including those with trypsin-like, and chymotrypsin-like activity. Notable examples of proteases captured by A2M include the matrix metalloproteinases (MMPs) and members of the kallikrein family, including human kallikrien-2 (hk2) and

prostate specific antigen (PSA): markers of prostate cancer progression. Once captured, these A2M-protease are removed from the circulation via the liver. The mechanism of protease capture begins with the presentation of a peptide sequence, termed the bait region, which, when cleaved by the target enzyme, causing a large conformational change in A2M. This change leads to the enzyme's twofold capture: 1) it is spatially enveloped by A2M, and; 2) it becomes covalently bound to A2M through exposure to an internal reactive thiol ester, figure 2. The internal β-cysteinyl-γ-glutamyl thiol ester, between cysteine 949 (Cys^{949}) and glutamate 952 (Glu^{952}), interacts with any available lysines on the protease to form ester linkages and releasing a reduced cysteine, figure 2. Upon activation, A2M undergoes a conformational change such that the A2M-protease complex is now able to bind to A2M receptors. Abundant A2M receptor is expressed by the liver and binding results in internalization of the protease/inhibitor complex.

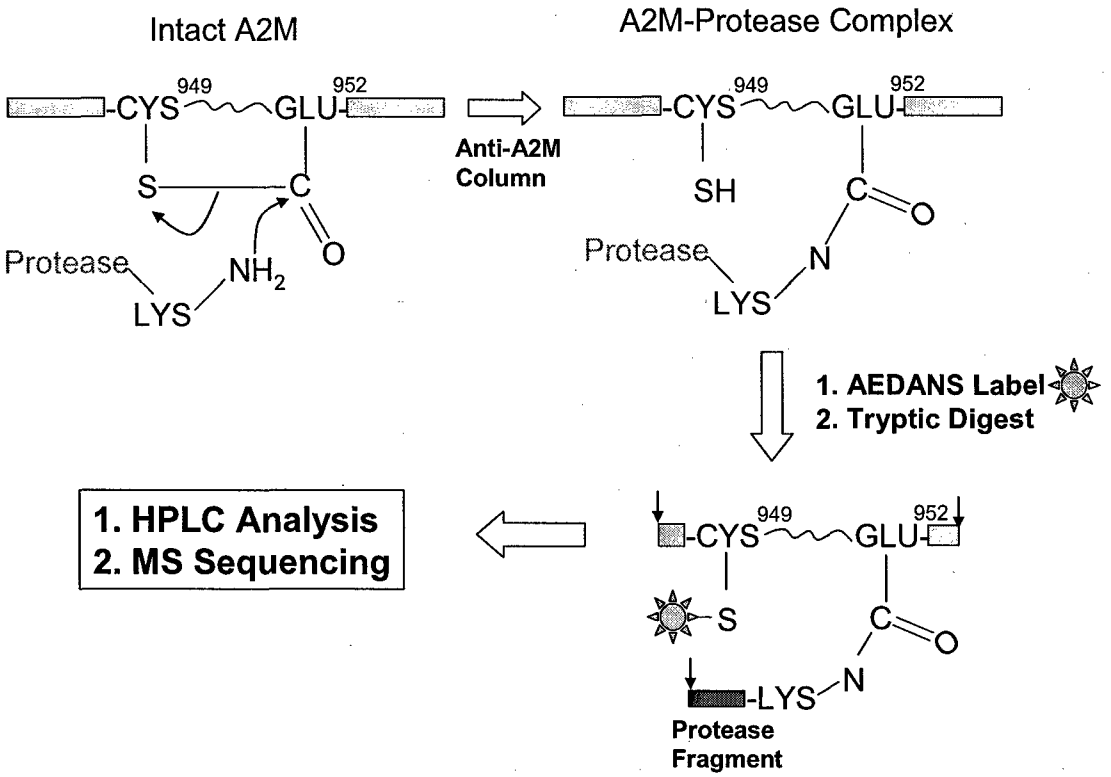

Figure 2. Development of a method for analysis of A2M-bound proteases using breast cancer cell lines. The commonly used breast cancer cell lines MCF-7 and MDA-MB231 will be grown to 80% confluence in standard tissue culture conditions. They will then be washed before conditioning serum-free media overnight. This protease-enriched media is then incubated with commercially available human A2M. The A2M-containing media is then run through an immunoaffinity column containing resin beads conjugated to commercially available antibodies specific to A2M. The column is then washed before the captured A2M is eluted by lowering the pH. The sole free thiol group of the activated A2M is labeled with the commercially available, sulfhydryl reactive fluorophore AEDANS [N-(acetylaminoethyl)-8-naphthylamine-1-sulphonic acid] . Complexes are then subjected to trypsin proteolysis. Trypsin is then removed by a size exclusion column and the filtrate is subjected to profiling by HPLC fitted with a fluorescence detector. This methodology has the advantage that we will only interrogate a very small fraction of the fragments produced by trypsin digestion of the A2M-protease complexes. Fluorescent labeling of the A2M-protease fragment will allow us to both identify as well as quantify the amount of each specific protease within the macronome.

Using biochemical assays with purified proteins, previous investigations have identified a broad range of proteases that complex with A2M. Because A2M completely engulfs the captured protease leaving no exposed epitopes, serum A2M-protease complexes cannot be measured directly using standard ELISA-based assays.

Additionally, since A2M and the protease become covalently linked, these complexes cannot be readily dissociated using under variety of denaturing conditions. Standard proteomic methodologies, therefore, cannot measure these complexes and typically do not assay proteins in this high MW range. As stated, protease production and compartmentalization is dysregulated in cancers. This suggests that a method to analyze proteases present in the A2M fraction, (i.e. the macronome) could identify alterations in levels of already described tissue proteases and also leaves the possibility for the identification of previously uncharacterized proteases, figure 2. Therefore, we have initiated studies to identify the "macronome" within breast cancer tissue using methodology outlined in figure 2. This method will allow us to concentrate active proteases in conditioned media from breast cancer cell lines by addition of purified A2M to serum-free conditioned media. A2M protease complexes can be readily separated following tryptic digest and HPLC/MS sorting and characterization. The presence of the fluorescent tag will also allow for quantification of levels of individual proteases present in the media, figure 3. Once optimized in tissue culture, the method can then be extended to analyze breast cancer samples and/or serum from patients with and without breast cancer.

Trypsin Digest A2M Fragment

ASVSVLGDILGSAMQNTQNLLQMPYGCGEQNMVLFAPNIYVLDYLDYLNETQQLTPEVK

- **46 amino acids, MW – 6161.03**
- **Largest peptide fragment of A2M produced by a tryptic digest**

CNBr Cleavage A2M Fragment

PYGCGEQNM

- **9 amino acids, MW – 977.10**
- **Smallest fragment produced by CNBr**

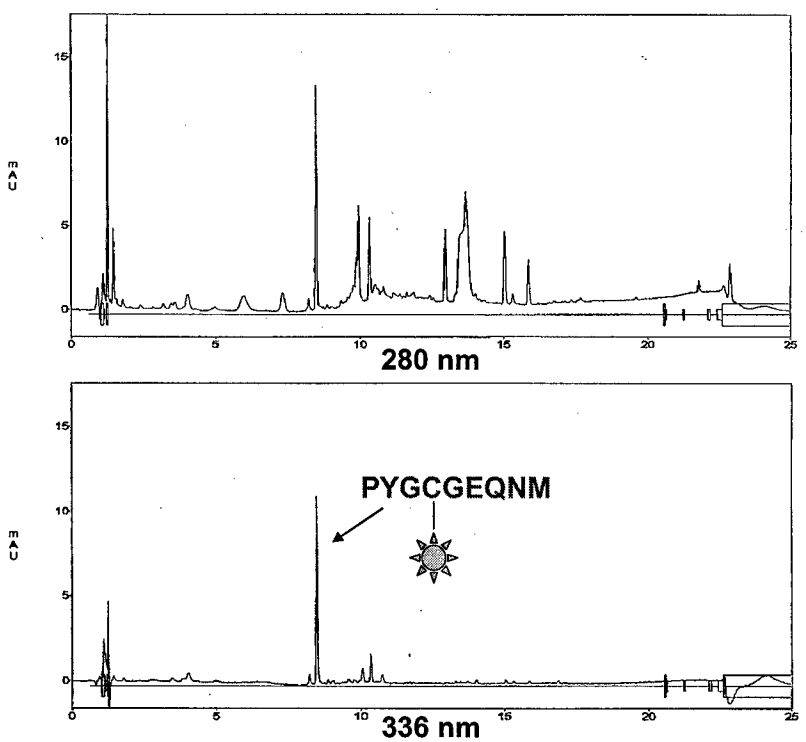

Figure 3. Preliminary HPLC analysis of fluorescently labeled macronome fragment following digestion with CNBr.

KEY RESEARCH ACCOMPLISHMENTS:

1. Synthesized and characterized a large fluorescently quenched combinatorial peptide library and successfully identified a substrate for the serine protease human glandular kallikrein 2 (hK2), which is expressed by human prostate cancers and by ~ 50% of human breast cancers.
2. Characterized hK2 hydrolysis of soluble hK2 substrates and determined plasma stability
3. Synthesized a hK2-activated thapsigargin prodrug that is efficiently hydrolyzed by hK2 and stable in human plasma
4. Demonstrated that this hK2 prodrug is selectively toxic in the presence of enzymatically active hK2 in vitro.
5. Completed prodrug distribution studies in vivo using radiolabeled prodrug.
6. Completed pharmacokinetic studies in vivo using hK2 activtaed prodrug
7. Screened homogenates of frozen human breast tumors and conditioned media from the human breast cancer cell line MCF-7 but did not identify a putative peptide substrate for a breast cancer protease.
8. Designed random gene library to be used in In Vitro Compartmentalization studies
9. Developed methodology to identify breast cancer selective proteases bound to human-alpha-2 macroglobulin

REPORTABLE OUTCOMES:

Presentations:

Janssen S, **Denmeade SR.** Identification of Tumor Associated Protease Substrates Using Combinatorial Chemistry. AACR/EORTC Molecular Targets and Cancer Therapeutics Meeting, Frankfurt, Germany 2002.

Manuscripts and Abstracts:

1. Janssen S, **Denmeade SR.** Identification of Tumor Associated Protease Substrates Using Combinatorial Chemistry. Eur J Cancer 38 Suppl 7:S97, 2002.
2. **Denmeade, S.R.,** Sokoll, L.J., Dalrymple, S., Rosen, D.M., Gady, A.M., Bruzek, D., Ricklis, R.M., Isaacs, J.T. Dissociation Between Androgen Responsiveness for Malignant Growth vs. Expression of Prostate Specific Differentiation Markers PSA, hK2 and PSMA in Human Prostate Cancer Models. Prostate, 2003;54:249-257.
3. **Denmeade, S.R.,** Jakobsen, C., Janssen, S., Khan, S.R., Lilja, H., Christensen, S.B. and Isaacs, J.T. Prostate-Specific Antigen (PSA) Activated Thapsigargin Prodrug as Targeted Therapy for Prostate Cancer, J Natl Cancer Inst 2003;95:990-1000.
4. Janssen S, Jakobsen CM, Rosen DM, Reineke U, Christensen SB, Lilja H, **Denmeade SR**. Screening a combinatorial peptide library to develop a human glandular kallikrein-2 activated prodrug as targeted therapy for prostate cancer. Mol Cancer Ther. 2004;3:1439-50.
5. Janssen S, Rosen DM, Ricklis RM, Dionne CA, Lilja H, Christensen SB, Isaacs JT, **Denmeade SR**. Pharmacokinetics, Biodistribution and Antitumor Efficacy of a Human Glandular Kallikrein 2 (hK2) - Activated Thapsigargin Prodrug. Prostate, In press, 2005
6. Lebeau A, Janssen S, Denmeade SR. Identification of breast cancer specific proteolytic activities for targeted prodrug activation. Proceeding, Era of Hope Meeting, 2005;P67-4.

Support for Post-Doctoral Fellow, Dr. Samuel Janssen to carry out experiments outlined in this proposal from May 1, 2003 to August 30, 2003. Dr. Janssen subsequently obtained a senior staff scientist position at Amylin, Inc., San Diego, CA.

Salary support for graduate student, Aaron LeBeau, beginning 1/1/04 to complete tasks 1-3 outlined in proposal.

CONCLUSIONS:

At the end of two years of funding we have developed methods to synthesize large fluorescently quenched peptide libraries as outlined in task 1. In preliminary studies, we incubated these libraries with a purified serine protease, human glandular kallikrein 2, and identified a series of putative substrates. These substrates were resynthesized as soluble peptides and characterized for hK2 hydrolysis and plasma stability. Peptides were then identified that were excellent hK2 substrates but unstable to non-specific hydrolysis in human plasma. Coupling these peptides to the thapsigargin analog, L12ADT, however, produced a prodrug that was readily hydrolyzed by hK2, stable in human plasma in vitro and mouse plasma both in vitro and in vivo, and was selectively cytotoxic to cancer cells in the presence of enzymatically active hK2. The hK2 substrate was tested in vivo and showed some antitumor efficacy, however, improved formulations are required to allow for sustained delivery of the compound. These studies demonstrated the feasibility of the approach to identification of protease substrates outlined in tasks 1-3 of the proposal. However, incubation of breast cancer homogenates or concentrated media from breast cancer cell lines did not yield any hydrolyzed peptides. This lack of hydrolysis most likely is due to combination of ng/ml concentrations of protease in extracellular fluid and need to dilute samples to cover entire bead library (i.e. 10-40 mls). A new approach for combinatorial screening is required that would yield libraries of small physical volume to screen small volume of extracellular fluid from breast cancers without requiring substantial dilution. To accomplish this, we are developing methods to adapt and apply the recently described combinatorial technique of in vitro compartmentalization that allows for rapid screening of large libraries in volumes of < 100 μl for this specific application. This method has been used to screen for binding moieties, but has never been used to screen for proteolytic substrates. In addition, we have designed methods to identify proteases in breast cancer fluid through capture by the abundant and ubiquitous pan-protease inhibitor alpha-2-macroglobulin. The "macronome" for conditioned media will be defined using this technique over the current funding period.

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