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Miniperspective

Hunter−Killer Peptide (HKP) for Targeted Therapy

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Introduction

Hunter−killer peptides (HKPs[a]) are short chimeric molecules (∼20 amino acids) consisting of two functional domains: a "targeting" domain (5−10 amino acids) that facilitates receptor-mediated binding and internalization into the cytosol of targeted cells and a proapoptotic domain (∼14 amino acids) designed to be nontoxic outside cells but toxic when internalized into targeted cells by the disruption of mitochondrial membranes. HKPs have shown promise as therapeutic agents in animal models of several human diseases. HKPs such as HKP-1, with the amino acid sequence NH_2-$_c$(CNGRC)-GG-$_D$(KLAKLAK)$_2$-COOH (**1**) (which includes a cyclic disulfide bridge between the cysteine residues), are targeted to the angiogenic vasculature of tumors and have strong anticancer activity in models of breast and prostate cancer, reducing tumor volume and metastasis and prolonging survival.[1] HKPs targeted to normal prostate vasculature reduce the size of the prostate gland and have a strong anticancer effect in a transgenic adenocarcinoma of the mouse prostate model (TRAMP).[2] HKPs targeted to the synovial vasculature have strong anti-inflammatory effects in a mouse model of collagen-induced arthritis.[3] Finally, HKPs targeted to the normal blood vessels that feed fat deposits decrease obesity in a transgenic mouse model of obesity.[4] A new generation of HKPs currently under development holds the promise of bringing this class of therapeutic agents from the bench to the clinic.

Background

The "Hunting" Peptides. In pioneering work from the Ruoslahti laboratory, Pasqualini and colleagues used a novel targeting system to identify receptors that are uniquely expressed on the surfaces of the endothelial cells that form the vasculature of specific organs.[5−9] This targeting system uses peptides expressed on the surface of bacteriophage to study organ-specific targeting. In the in vivo procedure, peptides capable of homing to certain organs or tissues are identified by the intravenous injection of the phage followed by recovery from the individual target tissue, in vitro amplification, and reinjection to obtain enrichment. Beyond just binding to the endothelial cell surface, the phage expressing certain peptide sequences are internalized and released into the cytosol of the endothelial cells. The mechanism of internalization does not involve coated pit endocytosis but rather is thought to be similar to the binding and internalization of the pathogenic bacteria Yersinia via its surface protein invasion.[10,11]

Since these original targeting peptide discoveries, as discussed below, this and related targeting technology have been applied to help design novel therapeutics for cancer,[1] arthritis,[3] prostatic hypertrophy,[2] and obesity.[4]

The "Killing" Peptides. Our laboratory established a cell-free system to study mechanisms involved in cell death.[12] In one version, normal (i.e., not derived from apoptotic or necrotic cells) mitochondria were suspended in normal cytosolic extract. Various peptides were then added to the system to determine if they disrupted the mitochondrial membranes sufficiently to induce the release of apoptosis initiators, such as cytochrome

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[a] Abbreviations: HKP, Hunter-Killer Peptides; TRAMP, Transgenic Adenocarcinoma of the Mouse Prostate Model; SGP, Small Globular Protein; APN, Aminopeptidase N; RA, Rheumatoid arthritis; c, cylic

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c, from the intermembrane space. The membrane-disrupting, apoptosis-inducing properties of these peptides were also investigated on mammalian cells in culture, as well as isolated mitochondria.

From these studies, two classes of membrane-disrupting peptides emerged. The peptide venoms, such as melittin, swelled mitochondria and killed mammalian cells at about the same concentration.[1,12−14] However, the peptide antibiotics, such as magainin, swelled mitochondria at concentrations 80- to 100-fold lower than those required to kill mammalian cells in culture.[1,15−17]

Both classes of peptides are linear and α-helical. A reason for the specificity of these membrane disrupting of antimicrobial peptides for mitochondrial membranes can be due to their positively charged structure. In addition to being linear and α-helical, these antibacterial peptides are also amphipathic, with hydrophobic residues distributed on one side of the helical axis and cationic residues on the other.[13] Since their cationic amino acids are attracted to the head groups of anionic phospholipids, these peptides preferentially disrupt negatively charged membranes. Once electrostatically bound, their amphipathic helices distort the lipid matrix, resulting in the loss of membrane barrier function.[13,18] The mechanism of membrane disruption is physical, not receptor-mediated, and is thought to be due to the formation of physical pores or to a gradual increase in the "carpeting" or covering of the membrane surface, which eventually leads to the loss of membrane integrity.[1,13,18] Both prokaryotic cytoplasmic membranes and eukaryotic mitochondrial membranes maintain large transmembrane potentials and have a high content of anionic phospholipids, presumably because bacteria and mitochondria share a common ancient heritage.[18−20] In contrast, eukaryotic plasma membranes (outer leaflet) generally have low, or no, membrane potential (with the exception of neurons) and are almost exclusively composed of zwitterionic phospholipids.[21,22] Such antibacterial peptides, therefore, preferentially disrupt prokaryotic membranes and eukaryotic mitochondrial membranes over eukaryotic plasma membranes (outer leaflet).

In contrast, peptide venoms, while they can be amphipathic, are generally much more hydrophobic than the antibacterial peptides, with just enough charged amino acids to permit solubility in aqueous solution. This generally higher hydrophobicity facilitates the peptide venoms in their insertion into and disruption of any membrane, regardless of its charge.[17,23]

There are well over 100 naturally occurring antibacterial peptides, and in addition, their de novo design has received much attention.[17,23] Properties like the individual residue hydrophobicity, the peptide average hydrophobicity, the peptide hydrophobic moment, the peptide length, and the angle subtended by the charged residues all contribute to the specificity and efficacy of a given peptide to disrupt negatively charged membrane surfaces.[24] By use of these principles, antibiotic peptides have been designed to kill prokaryotes at concentrations 100 times less than those required to kill eukaryotic cells.[17] Indeed, one of these antibacterial/antimitochondrial peptides with the amino acid sequence NH_2-KLAKLAKKLAKLAK-COOH (**2**), composed of lysine (K), leucine (L), and alanine (A) residues, became the first generation "killing" domain of HKPs.[1]

Design, Mechanism, and Results

Design of the First HKP. Just as there are unique vasculature markers that vary from tissue to tissue, the endothelial cells that form a tumor's vasculature express a number of cell-surface proteins that are not detectable in normal quiescent blood vessels.[25,26] These include some of the receptors for angiogenic growth factors[27] and the αV integrins.[28] On the basis of this, Pasqualini and co-workers used phage-displayed peptides to target tumors.[29,30] Among several successful moieties, they found that the cyclic peptide NH_2-$_c$(CNGRC)-COOH (**3**) structure, composed of cysteine (C), asparagine (N), glycine (G), and arginine (R) residues, yielded the highest tumor staining in comparison to the control organ background among all the tumor vasculature homing peptides they tested, with ratios exceeding 2 orders of magnitude.

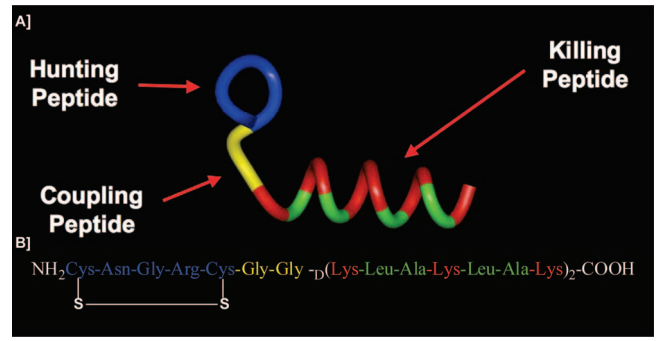

Figure 1. (A) Computer-generated model of the prototype hunter−killer peptide, HKP-1 (**1**), which is composed of a "hunter" domain (blue) designed to selectively guide the peptide to the angiogenic endothelial cells of tumor vasculature, but not to the endothelial cells of normal vasculature, and a "killer" domain (red hydrophilic and green hydrophobic residues) designed to selectively disrupt the negatively charged membranes of mitochondria, but not the outer leaflet of mammalian plasma membranes, joined by a coupling domain (yellow). (B) Amino acid sequence of HKP-1. The "killer" domain is composed of all D-amino acids, which were incorporated to prevent peptide proteolysis.

Using the "hunter" and "killer" peptide domains described above, we designed the first HKP (HKP-1) and applied our new technology to cancer therapy.[1] We used a short coupling peptide (-Gly-Gly-) to link the "hunter" and "killer" peptide domains. HKP-1 is a 21 amino acid peptide that has been synthesized by standard solid-phase peptide synthesis. HKP-1 targets and selectively eliminates the angiogenic endothelial cells of tumor blood vessels.[1] A low energy conformation of HKP-1 and its two functional domains are shown in Figure 1. The first domain was the tumor vasculature targeting sequence, the cyclic peptide CNGRC, designed to guide and internalize HKP-1 into angiogenic endothelial cells. The second domain was the D-amino acid version of the antibacterial/-mitochondrial peptide, **2**, designed to be nontoxic when outside cells but proapoptotic when internalized into targeted cells by the disruption of mitochondrial membranes. We chose D-amino acids for the antibacterial "killer" domain to prevent the proteolysis of HKP-1. The "hunter" domain CNGRC being cyclic (disulfide bridges between the two cysteines) binds to its receptor, aminopeptidase N and was anticipated to be resistant to proteolysis.[31] Aminopeptidase N (APN), also called CD13 (EC 3.4.11.2), is a type II transmembrane protein and an M1 class metalloprotease containing a Zn^{2+} binding motif, HEXXH.[32] APN is expressed in endothelial cells and is involved in angiogenesis including the neovascularization associated with tumor growth. The dysregulated expression of APN is also observed in various cancer cells and affects tumor cell functions such as proliferation, migration, and invasion. The cyclic peptide NH_2-$_c$(CNGRC)-COOH (**3**) binds to APN, which is selectively expressed on the endothelial cells that form tumor vasculature[31] and not on normal endothelial cells or myeloid cells.[33]

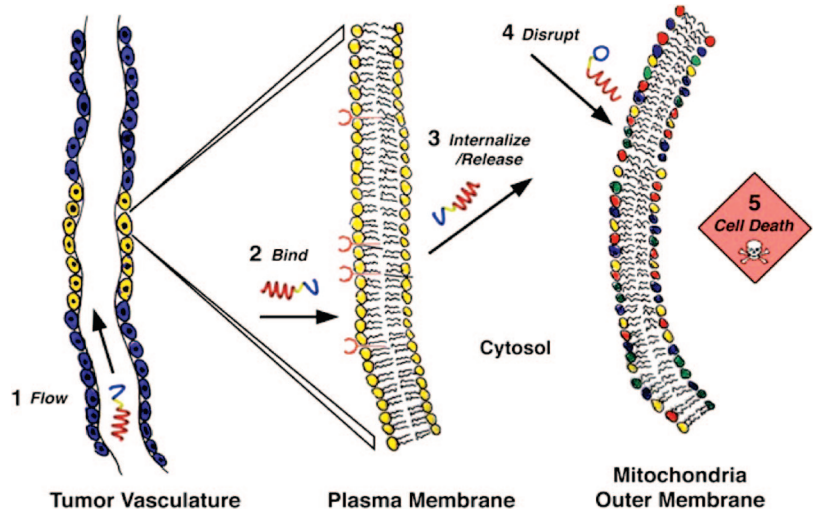

Figure 2. HKP mechanism of action. Diagram shows the putative mechanism of HKPs, using tumor blood vessel targeting and destruction as the example. HKPs (1) remain in circulation without harming the endothelial cells that comprise normal blood vessels (blue endothelial cells) until they enter tumor blood vessels (yellow endothelial cells). They then (2) bind to receptors (red semicircles) on the plasma membrane surface of the tumor blood vessel endothelial cells (receptors not found on normal endothelial cells). They then are (3) internalized into the cytosol of the targeted cells, where they then (4) disrupt the outer membranes of mitochondria, (5) leading to the cell death of the targeted endothelial cells.

The use of D-amino acid in the sequence KLAKLAKKLAK-LAK was possible, since the mechanism of membrane destruction by the "killer" domain is physical and not receptor mediated. The peptide structure, which is α-helical, forms pores in the membrane. Changing from L to D-amino acids simply changes the handedness of the α-helix, which inserts into and destroys mitochondrial membranes with equal effectiveness either way.[1,34] The two functional domains were linked by a glycine−glycine bridge so that any possible steric hindrance between the functional domains could be limited. Thus, our peptides combined two novel principles of specificity: homing to targeted cells and selective apoptosis of those targeted cells.

Dual-Purpose Mechanism of HKP Activity. Our original paper on HKPs applied to cancer therapy,[1] lent strong support to the idea that HKPs (a) selectively guide themselves to the endothelial cells of tumor vasculature, where they are then internalized into the cytosol of these targeted cells, and then (b) selectively disrupt negatively charged membranes, including at the very least mitochondrial membranes.[35] This then (c) results in the death of the targeted angiogenic endothelial cells and, consequently, the destruction of the tumor vasculature (Figure 2). This data included demonstration that fluorescently labeled peptides entered the cytosol of targeted but not untargeted cells and that mitochondrial swelling/disruption was detected early in the cell death of targeted cells.[1]

HKPs for Cancer Therapy. The first application of HKPs was as a possible cancer therapy.[1] Nude mice bearing MDA-MB-435 derived human breast carcinoma xenografts were treated with HKP-1. The results showed that tumors in the groups treated with HKP-1 were on average $^1/_{10}$ the size of tumors in the control groups (Figure 3A). The survival of the mice was also enhanced in these groups compared with control groups (Figure 3B). The control was a nontargeted peptide-mimic NH_2-$_c$(CARAC)-GG-$_D$(KLAKLAK)$_2$-COOH (4); the CARAC group of peptide 4 has a charge, size, and general structure similar to that of CNGRC group of peptide 1. Most notably, 60% of the HKP-1 treated mice were still alive after 120 days, 20 days after all of the control mice had already died. Indeed, some of the mice treated with HKP-1 outlived control mice by several months, indicating that both primary tumor growth and metastasis were inhibited by HKP-1. Furthermore, in subsequent experiments, dramatic effects of HKP-1 on metastasis to the lung could be seen by visual inspection (Figure 3C). Histopathological and TUNEL analysis showed cell death in the treated tumors and evidence of apoptosis and necrosis (data not shown). Similar results were also obtained with HKP-2 (a bicyclic RGD-based HKP) with the amino acid sequence NH_2-ACDCRGDCFC-GG-$_D$(KLAKLAK)$_2$-COOH (**5**), which binds selectively to the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, known also to be involved in angiogenesis.[1] The exact disulfide arrangement of HKP-2 (**5**) has not been determined.[36]

Following these studies, we synthesized an HKP called HKP-3 with the amino acid sequence NH_2-SMSIARL-GG-$_D$(KLAK-LAK)$_2$-COOH **(6)** targeted to endothelial cells that comprise the normal prostate vasculature. HKP-3 was evaluated for efficacy in a mouse model of prostate cancer, transgenic adenocarcinoma of the mouse prostate model (TRAMP) mice.[37] The life span extension in treated TRAMP mice was 6−8 weeks beyond that of control treated mice, close to 20% of the life span.

HKPs for Arthritis Therapy. In rheumatoid arthritis (RA), the synovium is characterized by hyperplasia of the intimal lining and mononuclear infiltration of the sublining, leading to erosion of cartilage and subchondral bone by invasive pannus.[38] Angiogenesis plays a critical role in the formation of pannus and the extensive neovasculature facilitates recruitment of mononuclear cells.[39] $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins are very important in angiogenesis.[25,28,40] $\alpha v\beta 3$ is expressed on synovial blood vessels in rheumatoid arthritis,[41,42] and αv antagonists were injected directly into the joint suppress synovitis in rabbits.[42] Because angiogenesis plays a major role in the perpetuation of inflammatory arthritis, we naturally wondered if HKPs could selectively target and destroy new synovial blood vessels.[3]

Intravenous treatment of mice with established arthritis using peptide **5** (HKP-2) induced apoptosis of endothelial cells in the inflamed synovium, which was associated with decreased severity of arthritis compared with untreated mice or mice treated with a mixture of uncoupled peptides NH_2-ACDCRGD-CFC-COOH (**7**) and NH_2-$_D$(KLAKLAKKLAKLAK)-COOH (**8**) (Figure 4).[3] These data showed that the targeted apoptosis of synovial neovasculature by HKP-2 induced apoptosis and suppressed clinical arthritis. Thus, HKP therapy demonstrated its potential utility in the treatment of inflammatory arthritis.

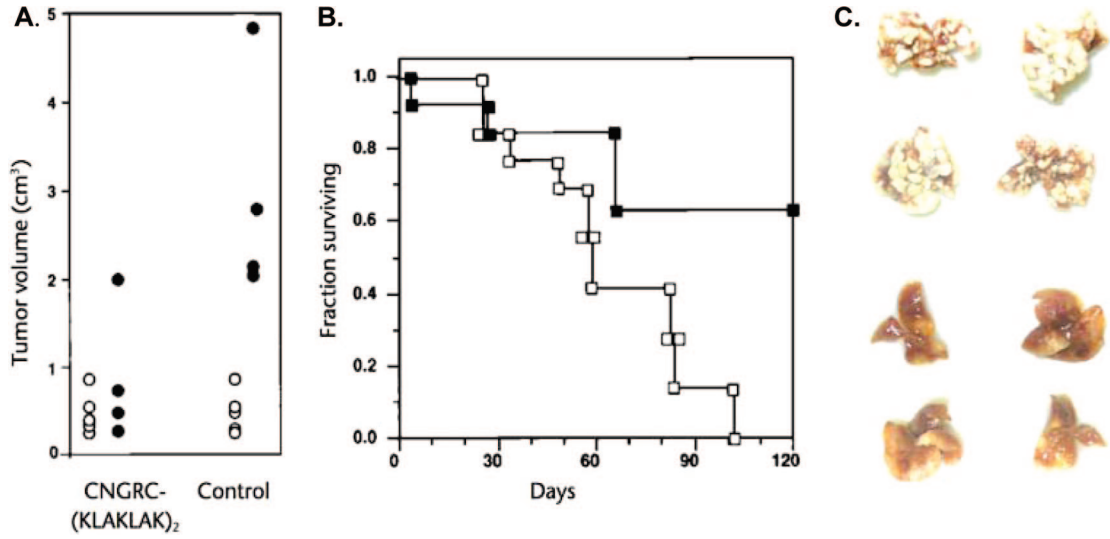

Figure 3. Treatment of nude mice bearing MDA-MB-435-derived human breast carcinoma xenografts with CNGRC-GG-D(KLAKLAK)2 (**1**). (A) Tumors treated with **1** are smaller than control tumors treated with CARAC-GG-D(KLAKLAK)2 (**4**), as shown by differences in tumor volumes between day 1 (○) and day 50 (●). $P = 0.027$, t test. (B) Mice treated with CNGRC-GGD(KLAKLAK)2 (**1**, ■) survived longer than control mice treated with an equimolar mixture of NH_2-D(KLAKLAK)$_2$-COOH (**8**) and NH_2-CNGRC-COOH (**3**) (□), as shown by a Kaplan−Meier survival plot ($n = 13$ animals/group). $P < 0.05$, log-rank test. (**C**) Mice treated with control peptide **4** exhibited extensive lung metastases (upper four lungs), many more than those treated with HKP-1 (lower four lungs).

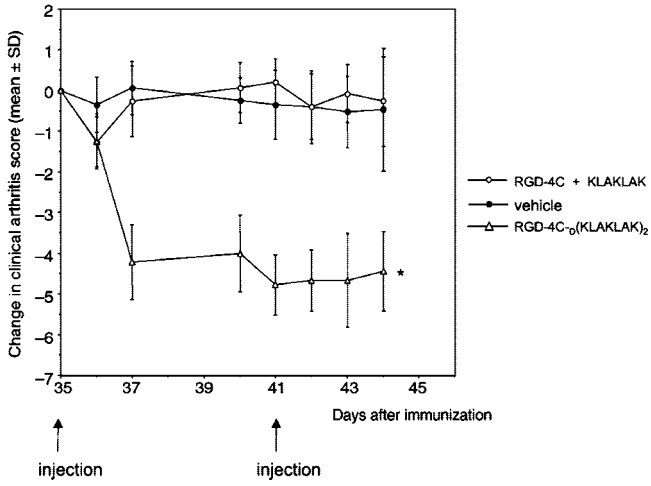

Figure 4. Changes in clinical arthritis scores in mice with collagen-induced arthritis after treatment with HKPs or the vehicle. Mice were injected intravenously on days 35 and 41 with HKP-2 peptide **5** or a mixture of the uncoupled peptides **7** and **8** or the vehicle. Clinical arthritis scores were evaluated using a scale of 0 to 4+ for each paw (maximum = 16). The mean pretreatment score was 10.3 ± 0.5.

HKPs for Obesity Therapy. In the U.S., approximately 65% of the adult population is overweight and over 30% is obese.[43−45] Obesity is associated with increased risk for cancer, diabetes mellitus, and heart disease, and it often shortens human life span.[43,46−48] The treatment of obesity has been rather limited, with few drugs available to control abnormal fat accumulation, based on altering energy balance pathways and appetite by acting on receptors in the brain.[48,49] Some of these drugs (e.g., fenfluramine) have been withdrawn from the market because of toxicity. Recent attempts to develop compounds that inhibit absorption of fat through the gastrointestinal tract (e.g., orlistat) may improve antiobesity treatment. However, even the most effective drugs produce small (5%) reductions in weight loss.[49]

Histological evaluation of adipose tissue reveals that fat is highly vascularized, suggesting the critical importance of blood vessels for the maintenance of the tissue mass.[50,51] Indeed, nonspecific angiogenesis inhibitors can prevent the development of obesity in mice,[52] and the regulation of hepatic tissue mass by angiogenesis has also been reported.[53]

On the basis of these findings, an HKP targeted to the fat vasculature of obese mice resulted in obesity reversal and metabolic normalization.[4,54] Cohorts of wild-type mice in which obesity had been induced by a high-calorie diet received daily subcutaneous doses of the cyclic peptide NH_2-$_c$(CKGGRAKDC)-GG-$_D$(KLAKLAK)$_2$-COOH (**9**), which not only prevented obesity development but also caused a rapid decrease in white fat mass and obesity reversal. Four weeks into the treatment, mice lost an average of over 30% in weight and displayed a reduction in body fat content. Epididymal fat pad size decreased by more than 70% compared with controls. In contrast, control mice receiving an equimolar mixture of the cyclic peptide NH_2-$_c$(CKGGRAKDC)-COOH (**10**) and untargeted NH_2-$_D$(KLAK-LAKKLAKLAK)-COOH (**11**) peptide continued to develop worsening obesity. Kolonin and co-workers[4] have identified prohibitin as the vascular receptor for cyclic-peptide CKG-GRAKDC group in white fat tissue.

Pharmacological Profile of HKPs and Future Development. The first generation HKPs such as **1** have been shown to be intact for at least 1 h when incubated with whole blood at 37 °C. In experiments where mice were injected intravenously with **1** and blood samples were analyzed, the peptides were present at 10 min after administration as measured with an ELISA assay.[1]

The HKP peptide **1** demonstrated anticancer activity (Figure 3) when administered at 250 μg/week per mouse, given slowly through a tail vein injection. Furthermore, when peptide **1** was administered to mice at a dose of 250 μg/week for 8 weeks, no apparent toxicity was observed.[1] However, more recent studies have shown that antimicrobial peptide containing molecules such as the HKPs undergo rapid renal clearance, and the D-amino acid killing domain could be toxic to the kidneys.[55] Hence, modulation of the absorption, distribution, metabolism, and excretion (ADME) properties of the HKPs is important for its further development. Our laboratory is currently involved in

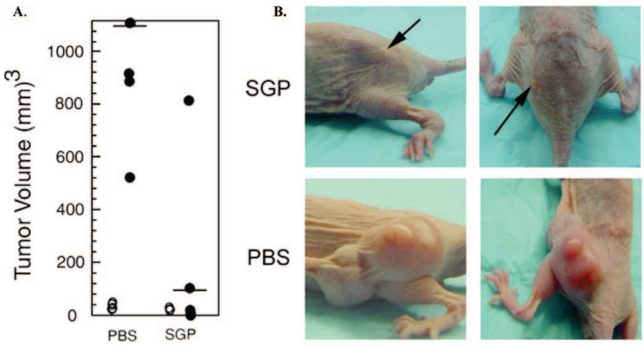

Figure 5. Small globular protein (SGP) treatment of human breast and lung carcinoma xenografts. (A) MDA-MB-435 derived human breast carcinoma xenografts. Direct intratumoral injection of 100 μM SGP results in average tumor volume almost 1000 times less than control (PBS) treated tumors (PBS, phosphate buffered saline). Tumor volumes measured at week 8 (filled black circles). (B) H358-derived human lung carcinoma xenografts. Photographs showing the dramatic differences in tumor volumes between SGP treated (top) and PBS treated (bottom) tumors at 6 weeks. Some tumors disappeared, and the mice appeared cured.

development of second generation HKP analogues with significantly improved ADME properties for evaluation in various therapeutic models and further development as preclinical candidates.

Conclusion

The HKPs represent a novel class of targeted peptides that have demonstrated remarkable efficacy in several basic proof-of-principle paradigms including therapeutics for cancer,[1] arthritis,[3] prostate reduction,[2] and obesity.[4] Although the first generation HKPs hold great promise, they do not yet represent a comprehensive pharmacotherapy that could be brought to the clinic. The modulation of their metabolism and excretion profile would greatly advance their development into clinical candidates. We have designed new hunter−killer "nanostructures/nanospheres" and second generation HKP derivatives that should protect them from proteolytic degradation and modulate their ADME properties. Furthermore, we are using similar technology to encapsulate other promising novel anticancer proteins such as the SGP (small globular protein) developed in our laboratory (Figure 5)[56] into targeted nanoparticles for therapeutic use. Development and evaluation of the next generation of HKPs with improved ADME properties are currently underway.

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Biographies

H. Michael Ellerby is Associate Professor and Chairman of the Pharmaceutical Sciences Department, College of Pharmacy, Touro University, on Mare Island in Vallejo, CA. He was the principal inventor of the HKPs and of the application of SGP (small globular protein) to cancer therapy. He received his Ph.D. in Chemistry (Chemical Physics) from University of California, Santa Cruz, in 1986, and completed postdoctoral fellowships with Professor Howard Reiss at University of California, Los Angeles, and with Professor Dale E. Bredesen at the Burnham Institute for Medical Research in La Jolla, CA. He was also a founding faculty member of the Buck Institute for Age Research in Novato, CA. He has published many peer reviewed scientific articles, holds several patents in his field, and is an award-winning teacher and lecturer.

Dale E. Bredesen, Professor and Founding President of the Buck Institute for Age Research, has spent the past 22 years working in the field of aging and neurodegeneration. He earned a B.S. degree from California Institute of Technology and an M.D. from Duke University. After training in Internal Medicine at Duke and Neurology at University of California, San Francisco, he was an NIH Fellow in the laboratory of Nobel laureate Prof. Stan Prusiner, working on prions and neurodegeneration. After serving on the faculty at University of California, Los Angeles, he became the first director of the Program on Aging at the Burnham Institute and then the founding President and CEO of the Buck Institute. His laboratory discovered a novel class of receptors—"dependence receptors"—that mediates processes as disparate as Alzheimer's disease, the spread of cancer, and fetal development.

Satoshi Fujimura received his Ph.D. from the Department of Chemistry, Faculty of Science, Fukuoka University, Fukuoka, Japan. He is currently a Senior Postdoctoral Research Fellow in the laboratory of Dr. Akira Saito, in the Division of Nephrology and Metabolism, Department of Medicine, Tokai University School of Medicine, Isehara, Kanagawa, Japan. His current research is on the establishment of a bioartificial kidney. Previous to this, Dr. Fujimura was Dr. Ellerby's Senior Postdoc at the Buck Institute for Age Research in Novato, CA, and in the Department of Pharmaceutical Sciences, College of Pharmacy, Touro University, Vallejo, CA, working on the design and development of the next generation HKPs. During this tenure, Dr. Fujimura discovered that the tumor vasculature targeting peptide CNGRC inhibits the enzymatic activity of aminopeptidase N (CD13).

Varghese John is currently Director of the Alzheimer's Drug Discovery Network (ADDN) at the Buck Institute for Age Research in Novato, CA. The Drug Discovery Network is developing novel therapeutic approaches to Alzheimer's disease in collaboration with Professor Dale Bredesen of the Buck Institute. In addition, the Drug Discovery Network is working with Drs. Ellerby and Bredesen to translate the HKP peptides and derivatives into potential clinical candidates. Previously, he was at Elan Pharmaceuticals &Athena Neurosciences for 18 years and led a team of medicinal chemists developing drugs for CNS diseases with a focus on AD. His work at Elan included development of potent inhibitors for BACE and γ-secretase, key enzymes in formation Aβ and amyloid plaques. He has several scientific publications and patents in his field.

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