Award Number: DAMD17-01-1-0029

TITLE: Comprehensive Development Program of Hunter-Killer Peptides for Prostate Cancer

PRINCIPAL INVESTIGATOR: Howard M. Ellerby, Ph.D.

CONTRACTING ORGANIZATION: Buck Institute for Age Research
Novato, California 94948

REPORT DATE: May 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
### Comprehensive Development Program of Hunter-Killer Peptides for Prostate Cancer

**Prostate cancer** is now the most common cancer among men in the United States. Angiogenesis is required for prostate tumor survival, growth, and metastasis. We proposed to design novel Hunter-Killer Peptides (HKPs), each representing a chimeric peptide of an angiogenesis-targeting peptide and a mitochondrial membrane-disrupting peptide. While non-toxic in the circulation, the HKPs will be preferentially toxic to mitochondria once internalized into angiogenic cells, via the targeting domain. As we reported in Ellerby et al., *Nature Medicine*, 5, 1032-1038, 1999, our prototypes contain only 21 and 26 amino acid residues, are selectively toxic to angiogenic endothelial cells and show strong anti-cancer activity in mice (breast carcinoma xenografts). In the work described here, we evaluated the HKPs for efficacy and toxicity in a xenograft model of human prostate carcinoma, and in the TRAMP (transgenic adenocarcinoma mouse prostate) model for prostate cancer. The central theme of this research is to develop and appraise this new chemotherapy with the goal of producing both a safer, and more effective, treatment of advanced prostate cancer.

---

**Subject Terms**

No subject terms provided.

---

**Security Classification**

- **Of Report**: Unclassified
- **Of This Page**: Unclassified
- **Of Abstract**: Unclassified

---

**Price Code**

- **NSN**: 7540-01-280-5500

---

**Distribution Code**

Approved for Public Release; Distribution Unlimited

---

**Number of Pages**

35

---

**Security Classification of Report**

Unclassified

---

**Security Classification of This Page**

Unclassified

---

**Security Classification of Abstract**

Unclassified

---

**Limitation of Abstract**

Unlimited
# TABLE OF CONTENTS

Front Cover ......................................................................................................................... 1  
Standard Form (SF) 298, Report Documentation Page ....................................................... 2  
Table of Contents .................................................................................................................. 3  
Introduction .......................................................................................................................... 4  
Body ..................................................................................................................................... 4  
Key Research Accomplishments .......................................................................................... 6  
Reportable Outcomes .......................................................................................................... 7  
Conclusions .......................................................................................................................... 8  
References ............................................................................................................................ 8  
Bibliography ......................................................................................................................... 9  
List of Personnel .................................................................................................................. 9  
Appendices ........................................................................................................................... 9
Introduction

The subject of our research is prostate cancer. The purpose of this research is the development of a more effective and less toxic treatment for prostate cancer. The currently used chemo-therapeutic agents are drugs with the narrowest therapeutic index in all of medicine. Therefore, effective doses of a wide variety of anti-cancer agents are restricted by their non-selective, highly toxic effect on normal tissues. In response to this, we designed short peptides composed of two functional domains, one a tumor blood vessel 'homing' sequence and the other a programmed cell death-inducing sequence, and synthesized them by basic peptide chemistry. The ‘homing’ domain was designed to guide the peptide to targeted cells and permit its internalization. The pro-apoptotic domain was designed to be non-toxic outside all cells, but toxic when internalized into just the targeted cells by the disruption of mitochondrial membranes.

Thus our approach was to create non-toxic anticancer peptides, which we named Hunter-Killer Peptides (HKP), designed to only destroy tumor blood vessels while leaving normal blood vessels unharmed. As presented in Ellerby et al., 1999, we succeeded in the development of HKPs, demonstrating that although our 2 prototypes contained only 21 and 26 amino acid residues, they were selectively toxic to angiogenic endothelial cells and had strong anti-cancer activity in mice. Furthermore, in Arap et al., 2002, we showed that HKPs delayed the development of the cancers in prostate cancer-prone transgenic mice (TRAMP mice). Moreover we elaborate on our previous report that HKPs can be given IP (intraperitoneal) in addition to IV (intravenous; tail vein injection in mice), opening up the possibility to administer our peptides more than once a week. We found that IP injection of 2 of our prototypes was more effective at reduces tumor volumes than IV. A manuscript on this work is now in preparation. We also discuss problems and solutions to those problems encountered this year with our CNGRC-based peptides, involving peptide quality, and receptor expression. We conclude this part of the report with a discussion of a study that we have just begun to treat TRAMP mice that spontaneously get prostate cancer, with a dual therapy designed to attack the prostate tumors blood vessels and the prostate cells as well. These studies will be completed at the end of August, 2004.

We also review and elaborate on our previous report concerning a new anti-cancer therapy that complements our work on targeted antiangiogenic peptides. We have discovered that certain membrane-disrupting/pore-forming peptides can be quite effective as direct anti-neoplastic agents (directly killing cancer cells). Specifically, we review here, as described in our recent published paper (Ellerby et al., 2003), that a 69 amino acid peptide, Small Globular Protein (SGP) can reduce tumor volume (eliminating some tumors), and increase survival, in a xenograft model of human prostate carcinoma. We conclude this report with a discussion of a study that we have just begun to treat TRAMP mice that spontaneously get prostate cancer, with a dual therapy designed to attack both the prostate tumors blood vessels and the prostate cells.

Body

Our work to date in designing, synthesizing, and testing Hunter-Killer Peptides (HKPs), and Small Globular Protein (SGP), is described in the accompanying reprints by Ellerby et al., 1999, and Arap et al., 2002, and in our newly published paper Ellerby et al., 2003, in the appendix. In the following, we re-state the approved Statement of Work, and then summarize how we have met our goals so far.
Statement of Work

Task 1, Specific Aim 1, (months 0-18). Optimize the dose of current HKPs in the TRAMP C model.

- Establish human PC3-derived tumor xenografts in nude mice (60 mice).
- Establish murine TRAMP C-derived tumors in C57BL/6 mice (60 mice).
- Treat the mice in 1 and 2 with HKPs to determine optimal doses, and dosing schedule.
- We have acquire/breed TRAMP C mice in the appropriate background to carry out survival studies (60 mice). Since only the male mice get the disease this took longer than anticipated to set up again at the Buck Institute. Lifespan is shortened by using an FVB background which will allow us to complete our studies shortly.
- Treat the mice in 4 with HKPs, with our optimal doses, and dosing schedule. Histopathological studies will also be performed, to look for efficacy and side effects.

Task 2, Specific Aim 2, (months 0-24). Design HKPs with improved therapeutic indices.

- Design new HKPs using the guiding principles of anti-mitochondrial peptide chemistry.

Task 3, Specific Aim 3, (months 6-24). Evaluate in vitro efficacy and toxicity of new HKPs.

- Evaluate the efficacy of new HKPs in mitochondrial swelling assays,
- Evaluate the efficacy of new HKPs in mitochondrial in tissue culture.
- Use the results of 1 and 2 to find approximate doses and therapeutic indices. HKPs with high therapeutic indices will then be evaluated in TRAMP mice, Specific Aim 4.

Task 4, Specific Aim 4, (months 18-36). Evaluate the in vivo efficacy of new HKPs in the TRAMP model of prostate cancer.

- Acquire/breed TRAMP C mice (60 mice).
- Treat the TRAMP C mice with new HKPs to determine optimal doses.
- Treat the TRAMP C with new HKPs to determine dosing schedule.
- Histopathological studies will also be performed, to look for efficacy and side-effects.

Task 5, Specific Aim 5, (months 24-36). Determine the in vivo pharmacokinetics of HKPs in the TRAMP model of prostate cancer.

- Acquire/breed TRAMP C mice (60 mice).
- Radiolabel HKPs and treat mice.
- Evaluate tissues, and cells in culture (using our in vitro angiogenesis assays, Ellerby et al., 1999), for presence of HKPs. Determine concentrations and locations of HKPs inside cells. Determine concentrations of HKPs in tumor blood vessel endothelial cells. Determine if there are any in vivo locations HKPs build up in that might create unwanted side effects.

Summary of Results/Data:

Task 1. As discussed previously, we have established human PC3-derived tumor xenografts in nude mice, and have evaluated the efficacy of SGP (see introduction) in this model (see below and
Ellerby et al. 2003, for a discussion of these results). Furthermore, we also reported in Arap et al., 2002, that HKPs delayed the development of the cancers in prostate cancer-prone transgenic mice (TRAMP mice). Thus we are already ahead of schedule on this task, having already demonstrated the efficacy of HKPs in this transgenic mouse model. We have now begun work on the process of optimizing the doses of HKPs in the TRAMP model, and have proceeded to formulate a study in TRAMP mice involving the dual use of HKP's to simultaneously target the angiogenic blood vessels of prostate tumors and the normal prostate cells. This work in progress and will be completed in the end of August. Furthermore, based on these initial results, we plan later this year to also include in some combination with HKP therapy, targeted nanoparticles containing SGP.

Task 2. We are currently designing new HKPs, so this Task is in progress, and will continue throughout the life of these studies. In particular, a new killer sequence has been developed, ALLLAIRRRKKK, based on an antibacterial peptide produced in moths. Initial tests of the efficacy of this peptide in our in vitro tissue culture models (see Ellerby et al. 1999), demonstrate that this killing sequence is 1,000-5,000 times more effective than our original killing peptide KLAKLAKKLAKLAK, as measured by the therapeutic index calculated as the ratio of the concentration required to kill normal untargeted cells divided by the concentration required to kill targeted cells. In addition, we have discovered that SGP (see introduction) can be used as a complementary therapy to HKPs. While HKPs target and destroy tumor vasculature, SGP directly kills tumor cells when injected intratumorally. This data is summarized below, and in our recent paper, Ellerby et al., 2003.

Task 3. We are currently testing new HKPs, so this Task is always in progress, and as described above, we have been focusing on HKP's with a new ALLLAIRRRKKK killing domain, for which initial tissue culture studies suggest that this new HKP will be over 1,000 times more effective.

Task 4 and Task 5. These tasks are in progress. There was some delay this past year in beginning these studies because we had problems of peptide purity (the peptides are manufactured commercially), and were informed by a colleague that the CNGRC targeting moiety might not be the most optimal targeting peptide to employ, due to variances in receptor expression. This peptide had been selected over more complex targeting peptides such as our ACDCRGCFC (bicyclic with 2 disulfide bonds) targeting peptide, due to issues involving the cost and complexity of synthesis of the RGD-based targeting peptide. We were fortunate to discover the above before embarking on our complex and expensive TRAMP studies. We will begin our TRAMP study this month using the RGD-based targeting sequence, to target the tumor blood vessels, and the SMS-based targeting sequence (see Arap et al., 2002). In addition, we have been forced by the quality issues to switch to another custom peptide synthesis company. We have also discovered that CNGRC is more effective at targeting in a particular chemical form and have a paper in progress on the receptor based mechanism of this discovery.

Key Research Accomplishments

* We successfully designed and tested a Hunter-Killer Peptide, SMSSIARL-GG\(_{D}(KLAKLAK)_2\), in the TRAMP Mouse Model of Prostate Cancer. This work is described in Arap et al., 2002.

* We successfully published a paper on our design and evaluation of SGP. As described above, we successfully tested the anti-tumor effects of SGP in human prostate carcinoma xenografts in nude mice. The data show that SGP can reduce tumor volume and extend survival. This data is discussed in detail in Ellerby et al., 2003, which is attached.
• We worked out issues involving peptide quality (commercial synthesis), and made the decision to switch to a different RGD-based targeting peptide. We discovered this year after a 3 month study that we were having a problem with the peptide company we dealt with, involving issues about timely delivery and quality of peptide. We also decided to switch to our RGD-based targeting peptide, in part because some of our in vitro tests were inconsistent from peptide batch to peptide batch. This situation was clouded by real concern over quality, but even so, colleagues reported to us that they were just getting a better result with our RGD-based targeting peptide. Thus, in the studies we are about to begin on TRAMP mice, we will be using the RGD-based targeting peptide for HKP’s designed to target angiogenic vasculature.

Reportable Outcomes

Papers, manuscripts, abstracts, presentations


Patents and licenses applied for and/or issued

We will be applying for a patent on the use of SGP. This has been reported in the Inventions Report to the DAMD.

Degrees obtained that are supported by this award

None.

Development of cell lines, tissue or serum repositories

None.

Informatics such as databases and animal models, etc

See ref. (2) above by del Rio et al.

Funding applied for based on work supported by this award

None at this time.
Employment or research opportunities applied for and/or received on experiences/training supported by this award

None at this time.

Conclusions

We have designed short peptides, Hunter Killer Peptides (HKP), composed of two functional domains, one a tumor blood vessel ‘homing’ motif and the other a programmed cell death-inducing sequence, and synthesized them by simple chemistry. The ‘homing’ domain was designed to guide the peptide to targeted cells and allow internalization. The pro-apoptotic domain was designed to be non-toxic outside cells, but toxic when internalized into targeted cells by the disruption of mitochondrial membranes. We demonstrated in Ellerby et al. 1999, that HKPs show strong anti-cancer activity in mice (xenografts of human breast carcinomas and melanomas). We also reported here and in Arap et al., 2002, that HKPs delayed the development of the cancers in prostate cancer-prone transgenic mice (TRAMP mice). The publication of this paper was a milestone for us, and meant that we were ahead of schedule with our proposed research (this research was not planned until 2003). Finally, we now have demonstrated the feasibility of using membrane-disrupting/pore-forming peptides/proteins as anti-neoplastic agents by evaluating the efficacy of Small Globular Protein (SGP). This work is being published this month, Ellerby et al., 2003. We now proceed this year to our TRAMP studies, involving the use of HKPs targeted to tumor blood vessels, and HKPs targeted to normal prostate cells. Work will then begin on the consideration of an SGP-targeted therapy, probably involving nanoparticle technology, designed to encase SGP so that it can be injected directly into the blood stream, targeted by a surface coating of targeting peptide. The implications of our work are that it is now possible for humans to engineer targeted and untargeted artificial peptides and proteins to be used systemically and locally as effective anti-cancer agents.

References


Bibliography


List of Personnel

(5/1/02-4/30/03)

H. Michael Ellerby, Ph.D., PI
Dale E. Bredesen, M.D.
Patricia Spillman (October 2001-Present)

Appendices


Anti-cancer activity of targeted pro-apoptotic peptides


We have designed short peptides composed of two functional domains, one a tumor blood vessel ‘homing’ motif and the other a programmed cell death-inducing sequence, and synthesized them by simple peptide chemistry. The ‘homing’ domain was designed to guide the peptide to targeted cells and allow its internalization. The pro-apoptotic domain was designed to be non-toxic outside cells, but toxic when internalized into targeted cells by the disruption of mitochondrial membranes. Although our prototypes contain only 21 and 26 residues, they were selectively toxic to angiogenic endothelial cells and showed anti-cancer activity in mice. This approach may yield new therapeutic agents.

Tumor cell survival, growth and metastasis require persistent new blood vessel growth1-3 (angiogenesis). Consequentially, a strategy has emerged to treat cancer by inhibiting angiogenesis4. Peptides have been described that selectively target angiogenic endothelial cells5-8. Conjugates made from these peptides and the anti-cancer drug doxorubicin induce tumor regression in mice with a better efficacy and a lower toxicity than doxorubicin alone9. There is also a functional class of cell death-inducing receptors, or ‘dependence receptors’, which have embedded pro-apoptotic amino-acid sequences9-10. These peptide domains are required for apoptosis induction by these receptors. The peptide fragments are thought to be released into the cytosol as cleavage products of caspase proteolysis, where they induce or potentiate apoptosis through unknown mechanisms9,10. However, such peptides, and structurally similar pro-apoptotic antibiotic peptides, although they remain relatively non-toxic outside of eukaryotic cells, induce mitochondrial swelling and mitochondria dependent cell-free apoptosis11,12.

There are more than 100 naturally occurring antibiotic peptides, and their de novo design has received much attention13-14. Many of these peptides are linear, cationic and α-helix-forming. Some are also amphipathic, with hydrophobic residues distributed on one side of the helical axis and cationic residues on the other14. Because their cationic amino acids are attracted to the head groups of anionic phospholipids, these peptides preferentially disrupt negatively charged membranes. Once electrically bound, their amphipathic helices distort the lipid matrix (with or without pore formation), resulting in the loss of membrane barrier function15,16. Both prokaryotic cytoplasmic membranes and eukaryotic mitochondrial membranes (both the inner and the outer) maintain large transmembrane potentials, and have a high content of anionic phospholipids, reflecting the common ancestry of bacteria and mitochondria17,18. In contrast, eukaryotic plasma membranes (outer leaflet) generally have low membrane potentials, and are almost exclusively composed of zwitterionic phospholipids19,20. Many antibacterial peptides, therefore, preferentially disrupt prokaryotic membranes and eukaryotic mitochondrial membranes rather than eukaryotic plasma membranes.

If such nontoxic peptides were coupled to tumor targeting peptides that allow receptor-mediated internalization, the chimeric peptide would have the means to enter the cytosol of targeted cells, where it would be toxic by inducing mitochondrial-dependent apoptosis10,11. Thus, we designed targeted pro-apoptotic peptides composed of two functional domains. The targeting domain was designed to guide the ‘homing’ pro-apoptotic peptides to targeted cells and allow their internalization21,22. The pro-apoptotic domain was designed to be non-toxic outside of cells, but toxic when internalized into targeted cells by the disruption of mitochondrial membranes.

Design of the pro-apoptotic peptide

A computer-generated model and the sequence of one of our prototypes are shown in Fig. 1. For the targeting domain, we used either the cyclic (disulfide bond between cysteines) CNGRC peptide (Fig. 1) or the double-cyclic ACDCRGCFC peptide (called RGD-4C), both of which have ‘homing’ properties23 and for which there is evidence of internalization21,22. We synthesized this domain from all-L amino acids, because of the presumed chiral nature of the receptor interaction. For the pro-apoptotic domain, we selected the synthetic 14-amino-acid peptide KLAKL AKKLAKL (Fig. 1), called (KLAKL)2, because it killed bacteria at concentrations 1% of those required to kill eukaryotic cells24. We used the all-D enan-
Targeted pro-apoptotic peptides induce apoptosis

We evaluated the efficacy and specificity of CNGRC-GG-(KLAKLAK)2 in KS1767 cells, derived from Kaposi sarcoma (Fig. 3a–d), and MDA-MB-435 human breast carcinoma cells (Table 1). We used KS1767 cells because they bind the CNGRC targeting peptide just as endothelial cells do. This may relate to the endothelial origin of the KS1767 cells. We used MDA-MB-435 cells as negative control cells because they do not bind the CNGRC targeting peptide. Although CNGRC-GG-(KLAKLAK)2 was considerably toxic to KS1617 cells, an equimolar mixture of uncleaved CNGRC and (KLAKLAK)2 (negative control), or (KLAKLAK)2 alone, was much less toxic, indicative of a targeting effect (Table 1). In contrast, CNGRC-GG-(KLAKLAK)2 was not very toxic to MDA-MB-435 cells, which do not bind the CNGRC peptide (Table 1). The other targeted peptide (RGD-4C)-GG-(KLAKLAK)2 showed toxic effects similar to those of CNGRC-GG-(KLAKLAK)2 on KS1617 cells, whereas an equimolar mixture of uncleaved RGD-4C and (KLAKLAK)2 used as a negative control, was not very toxic (Table 1; Fig. 3c–d).

Although evidence for internalization of CNGRC and RGD-4C into the cytosol of cells has been published, we directly demonstrated internalization using biotin-labeled peptides. CNGRC-biotin, but not untargeted CARAC-biotin, was internalized into the cytosol of cells (Fig. 3e–f). We also obtained direct evidence for internalization from experiments based on cell fractionation and mass spectrometry. CNGRC-GG-(KLAKLAK)2, but not CARAC-GG-(KLAKLAK)2, was indeed internalized and could be detected in mitochondrial as well as cytosolic fractions (data not shown).

Next, we evaluated the efficacy and specificity of CNGRC-GG-(KLAKLAK)2 in a tissue culture model of angiogenesis. During angiogenesis, capillary endothelial cells proliferate and migrate. Cord formation is a type of migration that can be studied in vitro by a change in endothelial cell morphology from the usual ‘cobblestones’ to chains or cords of cells. We tested the effect of CNGRC-GG-(KLAKLAK)2 on normal human dermal microvessel endothelial cells (DMECs) in the angiogenic conditions of proliferation and cord formation and in the angiostatic condition of a monolayer maintained at 100% confluency.

The treatment of DMECs with 60 μM CNGRC-GG-(KLAKLAK)2 led to a decrease in the percent viability over time compared with that of untreated controls, in the conditions of proliferation (Fig. 4a) or cord formation (Fig. 4b). In contrast, treatment with the untargeted peptide (KLAKLAK)2 as a negative control led to a negligible loss in viability. Furthermore, the LC50 for proliferating or migrating DMECs treated with CNGRC-GG-(KLAKLAK)2 was 10% of the LC50 for angiostatic DMECs maintained in a monolayer at 100% confluency (Table 1). This result indicates that CNGRC-GG-(KLAKLAK)2 kills cells in angiogenic but not angiostatic conditions. The LC50 for the untargeted control (KLAKLAK)2 in angiogenic conditions of proliferation and cord formation and in the angiostatic condition of a monolayer maintained at 100% confluency.
Fig. 2  
KLAKLAK disrupts mitochondrial membranes.  
(a) KLAKLAK, or Ca" (positive control) induced mitochondrial swelling, whereas the non-c-helix-former DLSLARLATARLAI (negative control) did not. As shown by mitochondrial swelling curves (optical absorbance spectrum).  
(b) KLAKLAK, activates cell-free apoptosis in a system composed of normal mitochondria and cytosolic extract, but DLSLARLATARLAI does not. An immunoblot of caspase-3 cleavage from proform (32kDa) to processed forms (18- and 20kDa) demonstrates a mitochondria-dependent cell-free apoptosis (left margin, sizes). Results were reproduced in two independent experiments.  
(c) Morphologic alterations in isolated mitochondria analyzed by electron microscopy. Mitochondria incubated for 15 min with 3 \( \mu\)M DLSLARLATARLAI show normal morphology (left panels). In contrast, mitochondria incubated for 15 min with 3 \( \mu\)M KLAKLAK, show extensive morphological changes. The damage to mitochondria progressed from the stage of focal matrix resolution (short black arrow), through homogenization and dilution of condensed matrix content with sporadic remnants of cristae (long black arrows), to extremely swollen vesicle-like structures (thick black arrows; bottom right, higher magnification); few mitochondria had normal morphology (open arrows). Ultrathin sections are shown. Original magnification, \( \times 4,000-\times 40,000\).

Fig. 3  CNGRC-GG-\( \alpha \)KLAKLAK, and (RGD-4C)-GG-\( \alpha \)KLAKLAK, induce apoptosis.  
(a) KS1767 cells treated with 100 \( \mu\)M of non-targeted CARAC-GG-\( \alpha \)KLAKLAK, (negative control) remain unaffected after 48 h.  
(b) KS1767 cells treated with 100 \( \mu\)M of CNGRC-GG-\( \alpha \)KLAKLAK, undergo apoptosis, as shown at 48 h. Condensed nuclei and plasma membrane blebbing are evident.  
(c) KS1767 cells treated with 10 \( \mu\)M of an equimolar mixture of (RGD-4C) and \( \alpha \)KLAKLAK, (negative control) remain unaffected after 48 h.  
(d) KS1767 cells treated with 10 \( \mu\)M of (RGD-4C)-GG-\( \alpha \)KLAKLAK, undergo apoptosis, as shown at 48 h. Condensed nuclei and plasma membrane blebbing are evident. Scale bar represents 250 \( \mu\)m.  
(e) and (f) KS1767 cells treated with 100 \( \mu\)M of CNGRC-biotin (e) or CARAC-biotin (f) for 24 h and subsequently treated with streptavidin FITC demonstrate internalization of CNGRC-biotin, but not CARAC-biotin, into the cytosol.
Fig. 4 CNGRC-GG-\(,\text{(KLAKLAK)}\), induces apoptosis and mitochondrial swelling in DMECs. a, Proliferating DMECs treated with CNGRC-GG-\(,\text{(KLAKLAK)}\); (filled bars) lose viability (apoptosis) over time (P<0.02), but those treated with the control peptide \(,\text{(KLAKLAK)}\); (gray bars) do not (P<0.05). b, Cord-forming DMECs lose viability (apoptosis) over time (filled bars), but those treated with \(,\text{(KLAKLAK)}\); (gray bars) do not (P<0.05). c, Apoptotic cell death was confirmed with an assay for caspase 3 activity, as shown by the hydrolysis of DEVD-pNA with time. Results were reproduced in three independent experiments. d, Proliferating DMECs show normal nuclear (blue) and mitochondrial (red) morphology after 24 h of treatment with a mixture of 100 \(\mu\text{M} \text{(KLAKLAK)}\), and CNGRC. e-g, Proliferating DMECs treated with 100 \(\mu\text{M} \text{CNGRC-GG-,(KLAKLAK)}\). After 24 h (e), cells show normal nuclear (blue) but abnormal mitochondrial (red) morphology. Mitochondrial swelling and dysfunction is shown by a decrease in fluorescence intensity and a change in morphology from an extended lace-like network to a condensed clumping of spherical structures. Classic morphological indicators of mid- to late apoptosis (for example, condensed and fragmented nuclei) are evident at 48 h (f) and 72 h (g) (arrow).

Caspase 3 activity\(^{10}\). We also tested a caspase inhibitor for its effect on cell death induced by CNGRC-GG-\(,\text{(KLAKLAK)}\). We used Kaposi sarcoma cells, as these cells bind CNGRC. The inhibitor zVAD.fmk, at a concentration (25 \(\mu\text{M}\)) that inhibits caspas but not non-caspase proteases, inhibited the cell death induced by CNGRC-GG-\(,\text{(KLAKLAK)}\) (data not shown). This result is compatible with the earlier demonstration that the CNGRC-GG-\(,\text{(KLAKLAK)}\) peptide is pro-apoptotic. Although the relatively early mitochondrial swelling is consistent with the putative mechanism of action, that is, a direct activation of the apoptotic machinery, we cannot rule out the possibility that the peptides actually kill by inducing some irreversible damage to cells which then activates the apoptotic program.

In addition to the fluorescence studies shown above, we studied cultured cells by electron microscopy to confirm that CNGRC-GG-\(,\text{(KLAKLAK)}\) induces abnormal mitochondrial morphology in intact cells (Fig. 5). Kaposi sarcoma-derived KS1767 cells treated with the control peptide CARAC-GG-\(,\text{(KLAKLAK)}\) for 72 hours showed no overall changes, with no or very minor changes in the mitochondria (Figs. 5a-c). In contrast, the mitochondria in KS1767 cells incubated for 12 h with 100 \(\mu\text{M} \text{CNGRC-GG-,(KLAKLAK)}\) begin to show a condensed appearance and vacuolization despite a relatively normal cell morphology (black arrows). Original magnifications: a, \(\times4,000\); b, \(\times25,000\); c, \(\times45,000\). d-f, In contrast, the mitochondria in KS1767 cells incubated for 12 h with 100 \(\mu\text{M} \text{CNGRC-GG-,(KLAKLAK)}\) begin to show a condensed appearance and vacuolization despite a relatively normal cell morphology (black arrows). Original magnifications: d, \(\times12,000\); e, \(\times20,000\); f, \(\times45,000\). g and h, Progressive damage to KS1767 cells is evident after 24 h, when many mitochondria show typical large matrix compartments and prominent cristae, ultrastructural features of low level of oxidative phosphorylation. Original magnifications: g, \(\times12,000\); h, \(\times40,000\). Some of the swollen mitochondria (g, black arrows) are similar in appearance to those in isolated mitochondria treated with 100 \(\mu\text{M} \text{,(KLAKLAK)}\), (Fig. 2c, bottom right). i, In some cells, this process progressed to a final stage, with extensive vacuolization and the pyknotic, condensed nuclei typical of apoptosis. Original magnification, \(\times8,000\).
with CNGRC-GG-\((\text{KLAKLAK})_2\) showed abnormal condensation and vacuolization despite a relatively preserved cell morphology (Fig. 5d-f, black arrows). Progressive cellular damage could be seen after 24 hours, when many mitochondria showed ultrastructural features of low-level oxidative phosphorylation (Fig. 5g and h); in later stages, some of the damaged mitochondria (Fig. 5g, black arrows) showed profound changes, as seen in the isolated mitochondria treated with \((\text{KLAKLAK})_2\) (Fig. 2c, right lower panel). In some cells, this process progressed to a late apoptotic stage. Typical vacuolization and condensed nuclei became evident (Fig. 5i). These results show that the mitochondria underwent changes in morphology and function that were well-represented by a progression from a state of normal morphology and normal oxidative phosphorylation (Fig. 5a) to a state of condensed morphology and a high rate of oxidative phosphorylation (Fig. 5d) to a final edemac state (Fig. 5g) associated with a low energy level.

**Fig. 7** Treatment of nude mice bearing MDA-MB-435-derived human breast carcinoma xenografts with CNGRC-GG-\((\text{KLAKLAK})_2\). **a**, Tumors treated with CNGRC-GG-\((\text{KLAKLAK})_2\), are smaller than control tumors treated with CARAC-GG-\((\text{KLAKLAK})_2\), as shown by differences in tumor volumes between day 1 (○) and day 50 (●). \(P = 0.027\), t-test. One mouse in the control group died before the end of the experiment. **b**, Mice treated with CNGRC-GG-\((\text{KLAKLAK})_2\) (●) survived longer than control mice treated with an equimolar mixture of \((\text{KLAKLAK})_2\), as shown by a Kaplan-Meier survival plot (\(n = 13\) animals/group). \(P < 0.05\), log-rank test.

Tumor volume assessed on day 1 (○) and day 90 (●). \(P = 0.027\), t-test. **b**, Tumor weights (right) and lung metastatic burden (left) are also decreased in mice treated with (RGD-4C)-GG-\((\text{KLAKLAK})_2\); these were measured when the experiment ended, on day 110 (\(n = 9\) animals/group). \(P < 0.05\), t-test.
The peptides were intact up to 1 hour in these conditions. In the second set of experiments, mice were injected intravenously with the two targeted peptides and blood samples were analyzed; the peptides were present at 10 minutes after administration (data not shown). We chose these short circulation times to coincide with the experimental conditions established for 'homing' of targeted peptides in vivo.

Targeted pro-apoptotic peptides represent a potential new class of anti-cancer agents; their activity may be optimized for maximum therapeutic effect by adjusting properties such as residue placement, domain length, peptide hydrophobicity and hydrophobic moment. Beyond this, future targeted pro-apoptotic peptides might be designed to disrupt membranes using a completely different type of pro-apoptotic domain such as β-sheet/sheet-forming peptides. Our results provide a glimpse at a new cancer therapy combining two levels of specificity: 'homing' to targeted cells and selective apoptosis of such cells after entry.

Methods

Reagents. Human recombinant vascular endothelial growth factor (VEGF; PharMingen, San Diego, California), antibody against caspase-3 (Santa Cruz Biotechnologies, Santa Cruz, California), streptavidin FITC (Sigma) and N-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA; BioMol, Plymouth Meeting, Pennsylvania) were obtained commercially. Peptides were synthesized to our specifications at greater than 90% purity by HPLC (DLSLARLATARLAI, Probes, Eugene, Oregon). Mitochondria were then visualized under fluorescence microscopy (100x objective) under an inverted microscope using a triple wavelength filter set (Nikon).

Cell culture. Dermal microvessel endothelial cells (DMECs) were grown in CAMDEG Growth Media™ (media and cells from Cell Applications, San Diego, California). DMECs were then cultured in three experimental conditions: proliferation (30% confluency in a growth media supplemented with 500 ng/ml VEGF); no proliferation (100% confluency in media for-conditions: proliferation (30% confluency in a growth media supplemented with 500 ng/ml VEGF); no proliferation (100% confluency in media formulated to maintain a monolayer); and cord formation (60% confluency). All specimens were fixed with 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.4 for 30 to 45 min at the room temperature, followed by postfixation with aqueous 1% osmium tetroxide and 2% uranyl acetate. After dehydration using a graded series of ethanol rinses, tissues were embedded in resin. Ultrathin sections after additional counterstainings were viewed and photographed on an electron microscope (Hitachi H-600).

Morphological quantification of cellular apoptosis. Percent viability of DMECs was determined by apoptotic morphology. For the percent viability assay, DMECs were incubated with 60 μM active peptide or control peptide. Cell culture medium was aspirated at various times from adherent cells, and the cells were gently washed once with PBS at 37 °C. Then, a 20-fold dilution of the dye mixture (500 μg/ml acridine orange and 100 μg/ml ethidium bromide) in PBS was gently pipetted on the cells, which were viewed on an inverted microscope (Nikon TE 300). The cell death seen was apoptotic cell death and was confirmed by a caspase activation assay. Not all cells progressed through the stages of apoptosis at the same time. At the initial stages, a fraction of the cells were undergoing early apoptosis. At later stages, this fraction had progressed to late apoptosis and even to the necrotic-like stage associated with very late apoptosis (for example, loss of membrane integrity in apoptotic bodies). However, these cells were joined by a new fraction undergoing early apoptosis. Thus, cells with nuclei showing marginalization and condensation of the chromatin and/or nuclear fragmentation (early/mid-apoptosis; acridine orange-positive) or with compromised plasma membranes (late apoptosis; ethidium bromide-positive) were considered not viable. At least 500 cells per time point were assessed in each experiment. Percent viability was calculated relative to untreated controls. LC50 for monolayer, proliferation (60% confluency), and cord formation were assessed at 72 h.

Mitochondrial morphology. DMECs after 24 and 72 h of treatment with peptide were incubated for 30 min at 37 °C with a mitochondrial stain (100 nM MiToTracker Red™ CM-H₂XRos; the nonfluorescent, reduced form of the compound) and a nuclear stain (500 nm DAPI; Molecular Probes, Eugene, Oregon). Mitochondria were then visualized under fluorescence microscopy (100x objective) under an inverted microscope using a triple wavelength filter set (Nikon).

Cell culture. Dermal microvessel endothelial cells (DMECs) were grown in CAMDEG Growth Media™ (media and cells from Cell Applications, San Diego, California). DMECs were then cultured in three experimental conditions: proliferation (30% confluency in a growth media supplemented with 500 ng/ml VEGF); no proliferation (100% confluency in media formulated to maintain a monolayer); and cord formation (60% confluency). All specimens were fixed with 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.4 for 30 to 45 min at the room temperature, followed by postfixation with aqueous 1% osmium tetroxide and 2% uranyl acetate. After dehydration using a graded series of ethanol rinses, tissues were embedded in resin. Ultrathin sections after additional counterstainings were viewed and photographed on an electron microscope (Hitachi H-600).

Human tumor xenografts. MDA-MB-435-derived xenografts were established in female nude mice 2 months old (Jackson Labs, Bar Harbor, Maine) as described. The mice were anesthetized with Avertin as described. The peptides were administered at a dose of 250 μg/week per mouse, given slowly through the tail vein in a volume of 200 μl. Three-dimensional measurements of tumors were made by caliper on anesthetized mice, and were used to calculate tumor volume. Then, tumors and lungs were surgically removed and the wet weights recorded. Animal experimentation was reviewed and approved by the Institute's Animal Research Committee.

Acknowledgments

We thank W.K. Cavenee and G. Salvesen for comments and critical reading of the manuscript. This work was supported by grants CA74238, CA28896 (to E.R.) and 5R01CA73376 and Cancer Center support grant CA30199 (to R.P., D.B. and E.R.) from the National Cancer Institute (USA), and DAMD17-98-1-8581 (to D.B. and R.P.) from the DOD-DCP. H.M.E. is the recipient of a NS10050 NRSA senior fellowship grant. W.A. is the recipient of a CaP CURE award.

RECEIVED 11 MAY; ACCEPTED 30 JUNE 1999

ARTICLES


1038

NATURE MEDICINE • VOLUME 5 • NUMBER 9 • SEPTEMBER 1999
APAP, a sequence-pattern recognition approach identifies substance P as a potential apoptotic peptide

Gabriel del Rio, Susana Castro-Obregon, Rammohan Rao, H. Michael Ellerby¹, Dale E. Bredesen*

Buck Institute for Age Research, 8001 Redwood Blvd., Novato, CA 94945-1400, USA

Received 6 March 2001; accepted 19 March 2001

First published online 30 March 2001

Edited by Gunnar von Heijne

Abstract We have previously described a novel cancer chemotherapeutic approach based on the induction of apoptosis in targeted cells by homing pro-apoptotic peptides. In order to improve this approach we developed a computational method (approach for detecting potential apoptotic peptides, APAP) to detect short PAPs, based on the prediction of the helical content of peptides, the hydrophobic moment, and the isoelectric point. PAPs are toxic against bacteria and mitochondria, but not against mammalian cells when applied extracellularly. Among other peptides, substance P was identified as a PAP and subsequently demonstrated to be a pro-apoptotic peptide experimentally. APAP thus provides a method to detect and ultimately improve pro-apoptotic peptides for chemotherapy. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Apoptosis; Antibacterial peptide; Bioinformatics

1. Introduction

We have previously described the finding that an antibacterial peptide, when targeted intracellularly to the angiogenic vascular tissue [i.e. to the endothelial cells] supplying tumors, can induce apoptosis by swelling their mitochondria [1], leading to the loss of tumor blood supply and consequent tumor regression. We named these chemotherapeutic peptides homing pro-apoptotic peptides. We designed the pro-apoptotic part of the peptides to induce endothelial cell apoptosis through mitochondrial swelling. The peptides are positively charged and the mitochondria, like bacteria, have negatively charged membranes, thus the peptides are attracted to and disrupt the mitochondrial membrane [2,3]. The initial results were obtained with a 21-residue peptide, of which the carboxy-terminal 14 amino acids represented the pro-apoptotic peptide, with the amino-terminal 7 amino acids comprising the targeting peptide and a glycine-glycine bridge. The therapeutic index (TI) of the initial pro-apoptotic peptide is approximately 10. In order to increase the TI and minimize the length of these peptides, we designed a computational approach to detect short, linear and specific pro-apoptotic peptides.

Apoptosis in mammals and other eukaryotic organisms is a characteristic process of cell death, which can, among its other effects, limit the spread of viruses and other intracellular organisms [4]. For example, the difference in viral titer during baculoviral infection with and without apoptosis inhibition is 200–15000-fold [4]. Thus apoptosis is a mechanism of defense against pathogenic infections.

Apoptosis proceeds by the activation of a group of cysteine proteases called caspases [5]. One of these, caspase-9, is activated when cytochrome c is released from mitochondria, which may occur with the disruption of the mitochondrial outer membrane [6]. This cytochrome c release in apoptotic cells may be induced by pro-apoptotic members of the Bcl-2 family, such as Bax and Bid, although the mechanism by which this is achieved is incompletely understood [7]. Nonetheless, the similarities between bacterial and mitochondrial membranes (and membrane potentials) suggested the possibility that there may be similarities between the effect of the antibacterial/pro-apoptotic peptides and pro-apoptotic Bcl-2 family members.

Antibacterial peptides in multicellular organisms are thought to serve as a defense against microbial pathogens. Originally found in invertebrates, antibacterial peptides have now been described in humans and many other organisms [2]. Among these peptides, the most well characterized are the short linear peptides (less than 40 amino acids in length) that do not contain cysteine residues. A characteristic shared by virtually all of these peptides is the presence of an amphipathic α-helical structure, which stabilizes in environments of hydrophobic nature [8] (although this helical structure has been shown not to be necessary for membrane lysis produced by a truncated form of pardaxin, an antibacterial peptide from the sole Pardachirus marmoratus [9]). Another characteristic shared by some of these peptides is selectivity, in that membranes from bacteria are targeted by these peptides more efficiently than mammalian plasma membranes. This selectivity is based on the complementary charge between the peptides, which are characteristically positively charged, and the negatively charged membranes of bacteria [2,3].

Structurally, these peptides typically adopt an unfolded conformation in aqueous solution. On contact with a membrane with a complementary charge, these peptides anchor to the membrane and assume an α-helical conformation. In this conformation, these peptides would either lie over the mem-

---

*Corresponding author. Fax: (1)-415-209 2230.
E-mail: dbredesen@buckinstitute.org

¹ Also corresponding author. E-mail: mellerby@buckinstitute.org.

Abbreviations: APAP, approach for detecting PAPs; PAPs, potential apoptotic peptides; SP, substance P; IP, isoelectric point; M, average helical hydrophobic moment; TI, therapeutic index

0014-5793/01/$20.00 © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

PII: S0014-5793(01)02348-1
brane surface in a carpet-like arrangement (in which the peptide backbone lies parallel to the membrane), or penetrate it according to the barrel-stave mechanism (in which the peptide backbone lies perpendicular to the membrane) [2]. In either case, the integrity of the membrane would be disturbed, eventually leading to membrane lysis.

In order to optimize the homing pro-apoptotic peptide approach to cancer chemotherapy by maximizing the T1 (see Section 2), we have developed a theoretical approach intended to model the properties of the antibacterial peptides that present selectivity for bacteria (and thus have very low toxic effects on mammalian cells when applied extracellularly). It is our goal in this work to develop a sequence-pattern recognition approach to detect peptides that will be toxic towards mitochondria but not to mammalian cells when applied extracellularly. We refer to the peptides identified by this approach as potential apoptotic peptides (PAPs), since they may induce apoptosis by swelling mitochondria when targeted intracellularly, as previously described [1]. We refer to the approach as APAP, as an abbreviation for approach for detecting PAPs. Using APAP, we searched the SwissProt database for PAPs and among other peptides we found that substance P (SP), an extensively studied neuropeptide present in mammals, birds and fish, has all the sequence characteristics of the PAPs. Furthermore, we found that SP is capable of swelling mitochondria and inducing the cleavage of caspase-9 zymogen, a known substrate of the active form of caspase-9 in vitro. As expected, SP demonstrated very low toxicity for eukaryotic cells when applied extracellularly, in addition to displaying toxicity towards bacterial cells. These results support our sequence-pattern recognition approach to identifying new PAPs, and suggest a new role for SP in the brain.

2. Materials and methods

2.1. Sequence-pattern recognition approach

We noticed that the known antibiotic peptides fit a pattern, which includes a low likelihood of helicity in aqueous solution, a high likelihood of helicity in the presence of negatively charged membranes, and a high isoelectric point (IP). We therefore calculated the helical probability of monomeric peptides in aqueous solution (AGADIR score), the IP and the hydrophobic moment to account for the characteristics of antibiotic peptides with low toxic activity against mammalian cells. We hypothesized that these characteristics are important in determining the selectivity observed in these peptides towards bacterial membranes and bacterial-like membranes (i.e. mitochondrial membranes).

A subset of 30 antibacterial peptides previously reported in the literature was used for calculations of AGADIR scores (A) [10], IP, and average helical hydrophobic moments (M) [11], (Tables 2A and 2B). The peptide sequences of this subset are shown in Table 1.

The TI of a peptide is here defined as the ratio between the inhibitory concentration observed with mammalian cells and the inhibitory concentration observed with bacterial cells (Tables 2A and 2B). The higher the value of this ratio is, the more specific the peptide is for prokaryotic (negatively charged) membranes.

PAPs were searched for in the SwissProt database, release 38 [12], which contains a total of 80 000 protein sequences. First, all of the peptide sequences of 40 or fewer amino acids in length were extracted from this database. Then all of these sequences (2473 database entries) were used to calculate their corresponding M, IP and AGADIR scores. Protein fragments, as opposed to peptides, were not considered in this study.

2.2. Computational resources

The PEPPLOT and ISOELECTRIC programs from the GCG package (Wisconsin package version 10, USA) were used to calculate M and IP, respectively. We averaged the non-zero r-values calculated by the PEPPLOT program (see Section 2) for windows of eight residues. To calculate the AGADIR score, we used the AGADIR program, which was kindly provided by Dr. Luis Serrano at EMBL. The hydrophobicity of peptide sequences was obtained by calculating the average hydrophobicity of the sequence using the consensus scale reported by Eisenberg [11]. All these programs were run on a SGI Origin 2000 server.

2.3. Caspase-3 activation in a cell-free apoptosis system induced by SP

2.3.1. Preparation of cytoplasmic extracts

Cytoplasmic extracts were prepared as described before [18]. Briefly, non-apoptotic neuronal cells were sonicated and centrifuged at 16 000 × g. This extract was made free of nuclei, mitochondria and did not self-prime.

2.3.2. Preparation of mitochondria

Mitochondria were prepared as described by Houvri et al. [13], with modifications as described previously [14]. Cultured cell mitochondria were prepared as described previously [15].

2.4. Protein electrophoresis and Western blots

Western blot analysis of proteins was carried out using either 8 or 12% SDS-polyacrylamide gels. Equal amounts of total protein were loaded per lane, and the proteins were separated at 4°C at 50 V through the stacking gel, and 90 V through the separating gel.

Western blot transfer of the proteins separated by electrophoresis was carried out at 4°C using PVDF membranes (0.2 mm) (Bio-Rad), at either 200 mA for 2 h. Blots were then blocked for 1 h in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween) containing 5% non-fat dried milk. Finally, the membranes were probed with an appropriate dilution (1:500 to 1:2000) of primary antibody in TBST containing 5% non-fat dried milk for 1.4 h, depending upon the antibody.

Anti-caspase-3 antibodies from mouse, rabbit and goat were purchased from Transduction Laboratories, Inc., Upstate Biotechnology, Inc. and Santa Cruz Biotechnology, Inc., respectively.

2.5. Mitochondrial swelling assays

Rat liver mitochondria were prepared as described above. The peptide concentrations used to swell mitochondria were 50 μM, 10 μM (LSLRATULARML) (negative control), or 200 μM Ca²⁺ (positive control).

The swelling was quantified by measuring the optical absorbance at 540 nm.

2.6. Activity of SP on fibroblasts

10⁵ human embryonic kidney 293 cells per well were seeded into a 96-well plate. After 20 h, different aqueous dilutions (Fig. 2B) of SP, C31 and a peptide used as a control were added to the culture and the cell death was quantified by trypan blue exclusion after 48 h later.

2.7. Toxicity of SP for bacterial cells

DH5α Escherichia coli cells were grown overnight as a pre-inoculum for the bacterial culture used in this assay. When the cells were at the end of their log phase (optical density at 600 nm of 0.8–1.0), 1 μl was used to inoculate 5 ml. Such dilution produced initial concentrations of bacteria capable of forming 10⁵–10⁶ colonies per ml in LB plates at 37°C, that is 10⁵–10⁶ colony forming units. All the bacterial cultures used in these experiments were grown in LB at 37°C. The concentration of SP required to inhibit the cell growth by 60% was determined by following bacterial growth in LB liquid in the presence of varying concentration of the peptide: 0, 1, 10, 20, 50, 125, and 250 μM. Sterilized 96-well plates of polystyrene with flat bottom and low evaporation lid (Costar, USA) were used, in a final volume of 100 μl: 50 μl of LB containing 10⁵–10⁶ colony forming units, and 50 μl of LB with a 2-fold dilution of the peptide. A 10 mM stock solution of the peptide was prepared with 5 μg of SP in 371 μl of water. Inhibition of growth was detected by measuring optical density at 600 nm with a microplate spectrophotometer SPECTRAMax (Molecular Devices,
USA) at varying times: 0, 3, 5, 6, 7 and 8 h. Each IC_{50} was determined from at least two independent experiments performed in triplicate. Additionally, the colonies formed from each experiment were counted in LB plates at 0 and 8 h of growth.

3. Results

The antibacterial peptides analyzed and biophysical properties previously determined are presented in Tables 1 and 2A, respectively.

In order to reproduce these biophysical properties, we calculated three scores from the sequences of these peptides. Table 2A shows a subset of selected antibacterial peptide sequences (see Section 2) and the corresponding experimental values for helix formation in water and in hydrophobic environments, antibacterial activity and cytotoxic activities against mammalian cells. Table 2B shows the corresponding calculated values for M, IP, A and the TI. We observed that the antibacterial peptides presented in Table 1 are more potent against G(+) (MIC = 17.3 μg/ml on average) bacteria than to G(-) (MIC = 44.3 μg/ml), and we used the G(+) values as a reference for the TI.

Peptide sequences with values ranging from 0.4 < M < 0.6, A < 10.0 and 10.8 < IP < 11.7, were found to have the highest TI (highest specificity for bacteria) (Table 2B). These parameters were therefore hypothesized to be the signature of the PAPs. Searching for PAPs in the SwissProt database led us to identify 14 PAPs (Table 3). Two of these peptides have previously been characterized with respect to their toxicity (data not shown). We observed that SP induces the swelling of mitochondria and activating caspase-3 (Fig. 1). In contrast, a peptide chosen as negative control (see Section 2) did not present the properties of PAPs (data not shown) did not display any observable effect on mitochondria (Fig. 1).

3.1. Swelling of mitochondria and activation of caspase-3 by SP

One of the PAPs identified, SP, was tested for its ability to swell mitochondria and induce caspase-3 activation in a cell-free system. This system was developed previously in our group to simulate neuronal apoptosis (see Section 2 and [14]). We observed that SP induces the swelling of mitochondria at 50 μM in our system (data not shown). At such concentration, SP was capable of releasing cytochrome c from mitochondria and activating caspase-3 (Fig. 1).

3.2. TI of SP

The toxicity of SP against bacteria was tested and compared to the effect of SP on fibroblasts when applied extracellularly. SP was able to reduce the growth of E. coli cells with an IC_{50} of 10 μM (Fig. 2B). By comparison, the negative control peptide did not have any toxicity against bacteria. In contrast, Fig. 2A shows that SP did not affect the growth of fibroblasts when applied extracellularly even at a concentration of 1 mM. These results indicate that SP has a TI > 100. Additionally, a peptide from the protein APP (the last 31 amino acids in APP, referred as C31) known to induce apoptosis when expressed intracellularly [16] was tested for its toxicity against bacteria and mammalian cells. This peptide did not present the properties (IP, M, A scores) of PAPs (data not shown). C31 did not present any observable toxicity against bacterial or mammalian cells when applied extracellularly (Fig. 2A,B).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Peptide sequences of a subset of antibacterial peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide name</td>
<td>Peptide sequence</td>
</tr>
<tr>
<td>(K1AKKIA)2NH2</td>
<td>KIAKKIAKAKKIA-NH2</td>
</tr>
<tr>
<td>(K1AKKIA)3NH2</td>
<td>KIAKIAKIAKIAKIAKIA-NH2</td>
</tr>
<tr>
<td>(K1AKLAK)2NH2</td>
<td>KIAKIAKIAKIAKIA-NH2</td>
</tr>
<tr>
<td>(K1AKLAK)3NH2</td>
<td>KIAKIAKIAKIAKIA-NH2</td>
</tr>
<tr>
<td>(KALKALK)3NH2</td>
<td>KIAKIAKIAKIAKIA-NH2</td>
</tr>
<tr>
<td>(KLGKKGK)L3NH2</td>
<td>KIAKIAKIAKIAKIA-NH2</td>
</tr>
<tr>
<td>CecropinA</td>
<td></td>
</tr>
<tr>
<td>Melittin</td>
<td></td>
</tr>
<tr>
<td>Magamin 2</td>
<td></td>
</tr>
<tr>
<td>CA1(1–13)(M1–13)NH2</td>
<td></td>
</tr>
<tr>
<td>CA1(1–8)(M1–18)NH2</td>
<td></td>
</tr>
<tr>
<td>Kla1</td>
<td></td>
</tr>
<tr>
<td>Kla2</td>
<td></td>
</tr>
<tr>
<td>Kla3</td>
<td></td>
</tr>
<tr>
<td>Kla7</td>
<td></td>
</tr>
<tr>
<td>Kla8</td>
<td></td>
</tr>
<tr>
<td>Kla9</td>
<td></td>
</tr>
<tr>
<td>Kla10</td>
<td></td>
</tr>
<tr>
<td>Kla11</td>
<td></td>
</tr>
<tr>
<td>Kla12</td>
<td></td>
</tr>
<tr>
<td>m2a</td>
<td></td>
</tr>
<tr>
<td>W16-m2a</td>
<td></td>
</tr>
<tr>
<td>L2R11A20-m2a</td>
<td></td>
</tr>
<tr>
<td>I6L15-m2a</td>
<td></td>
</tr>
<tr>
<td>I6ASL1517-m2a</td>
<td></td>
</tr>
<tr>
<td>I6R114W16-m2a</td>
<td></td>
</tr>
<tr>
<td>I6OVW12T1517-m2a</td>
<td></td>
</tr>
<tr>
<td>100-m2a</td>
<td></td>
</tr>
<tr>
<td>140-m2a</td>
<td></td>
</tr>
<tr>
<td>160-m2a</td>
<td></td>
</tr>
</tbody>
</table>

The amino acids in the peptide sequence are represented in a one-letter code.
peptides display both antibacterial activity and toxicity. Additionally, it has been previously recognized that hydrophobic peptides are shown to be membrane-associated [11], allowing us to search sequence databases systematically. Caspase-3 antibacterial or pro-apoptotic peptides. Additionally, APAP Proform was originally developed to overcome the problems of lect PAPs. It has been shown previously that antibacterial peptides are known to be membrane-associated [11], and the selectivity for recognizing bacterial-like membranes depends on the composition of the membranes [2,3]. Additionally, it has been previously recognized that hydrophobic peptides display both antibacterial activity and toxicity against mammalian cells [19] (i.e. non-selective toxicity), thus PAPs would be expected not to be simply highly hydrophobic peptides. We observed that in our group of peptides (Table 1), all of the peptides but one were hydrophilic, constituting an appropriate group of peptides from which to select PAPs. It has been shown previously that antibacterial peptides with lower hydrophobicity display higher specificity towards Gram-negative bacteria [20]. In agreement with this notion, all the peptides analyzed in our study presented higher specificity towards G+ bacteria as expressed by the TI values (Tables 2A and 2B).

4. Discussion

In order to optimize our homing pro-apoptotic approach to target and kill angiogenic endothelial cells supplying cancer cells, we have developed APAP, an approach to detect PAPs. APAP was originally developed to overcome the problems of toxicity and synthesis associated with our chemotherapeutic peptides with lower hydrophobicity display higher specificity towards mammalian cells, usually red blood cells or fibroblasts.

Table 2A

<table>
<thead>
<tr>
<th>Peptide</th>
<th>CD</th>
<th>Water</th>
<th>Antimicrobial Gram(−)</th>
<th>Activity Gram(+)</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(KLAKKL)2NH2</td>
<td>&lt; 5</td>
<td>24</td>
<td>6</td>
<td>6</td>
<td>&gt; 272</td>
</tr>
<tr>
<td>(KLAKKL)3NH2</td>
<td>&lt; 5</td>
<td>79</td>
<td>4</td>
<td>4</td>
<td>&gt; 11</td>
</tr>
<tr>
<td>(KLAKKL)2NH2</td>
<td>&lt; 5</td>
<td>37</td>
<td>4</td>
<td>4</td>
<td>&gt; 57</td>
</tr>
<tr>
<td>(KLAKKL)3NH2</td>
<td>&lt; 5</td>
<td>79</td>
<td>4</td>
<td>4</td>
<td>&gt; 9</td>
</tr>
<tr>
<td>(KLAKKL)3NH2</td>
<td>&lt; 5</td>
<td>67</td>
<td>4</td>
<td>8</td>
<td>&gt; 11</td>
</tr>
<tr>
<td>(KLAKKL)3NH2</td>
<td>&lt; 5</td>
<td>67</td>
<td>4</td>
<td>8</td>
<td>&gt; 11</td>
</tr>
<tr>
<td>Cetrin A</td>
<td>0</td>
<td>75</td>
<td>0.2</td>
<td>&gt; 300</td>
<td>200</td>
</tr>
<tr>
<td>Melittin</td>
<td>0</td>
<td>75</td>
<td>0.8</td>
<td>&gt; 0.2</td>
<td>400</td>
</tr>
<tr>
<td>Magainin 2</td>
<td>0</td>
<td>44</td>
<td>4</td>
<td>2</td>
<td>200</td>
</tr>
<tr>
<td>CA1(1-13)M(1-13)NH2</td>
<td>0</td>
<td>55</td>
<td>0.5</td>
<td>2</td>
<td>600</td>
</tr>
<tr>
<td>CA1(1-8)M(1-18)NH2</td>
<td>0</td>
<td>63</td>
<td>0.3</td>
<td>1</td>
<td>600</td>
</tr>
<tr>
<td>Kla1</td>
<td>ND</td>
<td>73</td>
<td>5.2</td>
<td>2.6</td>
<td>11</td>
</tr>
<tr>
<td>Kla2</td>
<td>ND</td>
<td>68</td>
<td>11</td>
<td>45</td>
<td>107</td>
</tr>
<tr>
<td>Kla3</td>
<td>ND</td>
<td>59</td>
<td>91</td>
<td>&gt; 91</td>
<td>200</td>
</tr>
<tr>
<td>Kla7</td>
<td>ND</td>
<td>70</td>
<td>5.6</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Kla8</td>
<td>ND</td>
<td>62</td>
<td>5.8</td>
<td>3</td>
<td>2.5</td>
</tr>
<tr>
<td>Kla9</td>
<td>ND</td>
<td>55</td>
<td>6.2</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Kla10</td>
<td>ND</td>
<td>62</td>
<td>6.1</td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td>Kla11</td>
<td>ND</td>
<td>69</td>
<td>5.3</td>
<td>5.3</td>
<td>10</td>
</tr>
<tr>
<td>Kla12</td>
<td>ND</td>
<td>67</td>
<td>6</td>
<td>1.5</td>
<td>10</td>
</tr>
<tr>
<td>m2a</td>
<td>ND</td>
<td>57</td>
<td>40</td>
<td>&gt; 80</td>
<td>428</td>
</tr>
<tr>
<td>w16-m2a</td>
<td>ND</td>
<td>57</td>
<td>40</td>
<td>&gt; 80</td>
<td>509</td>
</tr>
<tr>
<td>l2r11a20m2a</td>
<td>ND</td>
<td>45</td>
<td>75</td>
<td>&gt; 75</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>i6015-m2a</td>
<td>ND</td>
<td>57</td>
<td>38</td>
<td>38</td>
<td>260</td>
</tr>
<tr>
<td>i6a811s17m2a</td>
<td>ND</td>
<td>61</td>
<td>2.4</td>
<td>9.6</td>
<td>32</td>
</tr>
<tr>
<td>i6r11r14w616m2a</td>
<td>ND</td>
<td>52</td>
<td>37.5</td>
<td>&gt; 75</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>i6w9w12r1517m2a</td>
<td>ND</td>
<td>64</td>
<td>2.3</td>
<td>5.6</td>
<td>66</td>
</tr>
<tr>
<td>100-m2a</td>
<td>ND</td>
<td>48</td>
<td>75</td>
<td>&gt; 75</td>
<td>700</td>
</tr>
<tr>
<td>140-m2a</td>
<td>ND</td>
<td>75</td>
<td>13</td>
<td>13</td>
<td>35</td>
</tr>
<tr>
<td>160-m2a</td>
<td>ND</td>
<td>54</td>
<td>19</td>
<td>76</td>
<td>82</td>
</tr>
</tbody>
</table>

Peptide: see Table 1 for the amino acid composition for each peptide described in this table. CD Observed in water or lipid: percent of α-helical secondary structure determined by circular dichroism. Antibacterial activity G(+) or G(−): the minimal inhibitory concentration (μg/ml) for each peptide against Gram(+) and Gram(−) bacterial cells. Cytotoxicity: the concentration (μg/ml) required for inhibiting the growth of mammalian cells, usually red blood cells or fibroblasts.

Fig. 1. Pro-apoptotic activity of SP. The release of cytochrome c from mitochondria and the processing of caspase-3 into the active form are shown for SP and controls (sonication, the detergent Triton X-100 and a non-toxic peptide DLSLARLATAKLAI).
Calculated characteristics of a subset of antibacterial peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>A</th>
<th>M</th>
<th>IP</th>
<th>T1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(KLAKKLAK)2NH2</td>
<td>4.5</td>
<td>0.48</td>
<td>11.5</td>
<td>-0.249</td>
</tr>
<tr>
<td>(KLAKKLAL)2NH2</td>
<td>16.2</td>
<td>0.48</td>
<td>11.7</td>
<td>-0.249</td>
</tr>
<tr>
<td>(KLAKKLKN)2NH2</td>
<td>5.1</td>
<td>0.48</td>
<td>11.5</td>
<td>-0.249</td>
</tr>
<tr>
<td>(KLAKKLKN)2NH2</td>
<td>17.2</td>
<td>0.48</td>
<td>11.7</td>
<td>-0.249</td>
</tr>
<tr>
<td>(KALKLKL)2NH2</td>
<td>16.6</td>
<td>0.48</td>
<td>11.7</td>
<td>-0.249</td>
</tr>
<tr>
<td>(KLGKGLG)2NH2</td>
<td>1.1</td>
<td>0.49</td>
<td>11.7</td>
<td>-0.274</td>
</tr>
<tr>
<td>Cecropin A</td>
<td>1.2</td>
<td>0.44</td>
<td>11.2</td>
<td>-0.123</td>
</tr>
<tr>
<td>Melittin</td>
<td>3.1</td>
<td>0.46</td>
<td>12.6</td>
<td>-0.83</td>
</tr>
<tr>
<td>Magainin 2</td>
<td>0.8</td>
<td>0.56</td>
<td>10.8</td>
<td>-0.036</td>
</tr>
<tr>
<td>CA(1-13)M(1-13)NH2</td>
<td>1.1</td>
<td>0.53</td>
<td>11.1</td>
<td>-0.46</td>
</tr>
<tr>
<td>CA(1-8)M(1-18)NH2</td>
<td>1.3</td>
<td>0.43</td>
<td>11.4</td>
<td>0.065</td>
</tr>
<tr>
<td>Kla1</td>
<td>13.4</td>
<td>0.16</td>
<td>11.4</td>
<td>-0.025</td>
</tr>
<tr>
<td>Kla2</td>
<td>10.6</td>
<td>0.30</td>
<td>11.4</td>
<td>-0.056</td>
</tr>
<tr>
<td>Kla3</td>
<td>7.2</td>
<td>0.17</td>
<td>11.4</td>
<td>-0.087</td>
</tr>
<tr>
<td>Kla7</td>
<td>2.4</td>
<td>0.53</td>
<td>11.4</td>
<td>-0.026</td>
</tr>
<tr>
<td>Kla8</td>
<td>49</td>
<td>0.51</td>
<td>11.4</td>
<td>-0.025</td>
</tr>
<tr>
<td>Kla9</td>
<td>18</td>
<td>0.38</td>
<td>11.4</td>
<td>-0.025</td>
</tr>
<tr>
<td>Kla10</td>
<td>23.5</td>
<td>0.45</td>
<td>11.4</td>
<td>-0.025</td>
</tr>
<tr>
<td>Kla11</td>
<td>14.8</td>
<td>0.16</td>
<td>11.4</td>
<td>-0.027</td>
</tr>
<tr>
<td>Kla12</td>
<td>19.5</td>
<td>0.49</td>
<td>11.4</td>
<td>-0.056</td>
</tr>
<tr>
<td>m2a</td>
<td>0.8</td>
<td>0.56</td>
<td>10.8</td>
<td>-0.036</td>
</tr>
<tr>
<td>w16-m2a</td>
<td>0.9</td>
<td>0.49</td>
<td>10.8</td>
<td>-0.046</td>
</tr>
<tr>
<td>12r11a20-m2a</td>
<td>0.9</td>
<td>0.51</td>
<td>11.1</td>
<td>-0.094</td>
</tr>
<tr>
<td>i6115-m2a</td>
<td>0.6</td>
<td>0.54</td>
<td>10.8</td>
<td>-0.095</td>
</tr>
<tr>
<td>i6811517-m2a</td>
<td>1.1</td>
<td>0.55</td>
<td>10.8</td>
<td>0.016</td>
</tr>
<tr>
<td>i611r14w16-m2a</td>
<td>0.8</td>
<td>0.48</td>
<td>11.7</td>
<td>-0.095</td>
</tr>
<tr>
<td>i69w121517-m2a</td>
<td>0.7</td>
<td>0.56</td>
<td>10.8</td>
<td>-0.035</td>
</tr>
<tr>
<td>100-m2a</td>
<td>1.1</td>
<td>0.46</td>
<td>10.8</td>
<td>-0.045</td>
</tr>
<tr>
<td>140-m2a</td>
<td>0.9</td>
<td>0.57</td>
<td>10.8</td>
<td>-0.049</td>
</tr>
<tr>
<td>160-m2a</td>
<td>0.7</td>
<td>0.57</td>
<td>10.5</td>
<td>-0.017</td>
</tr>
</tbody>
</table>

Peptide: see Table 1 for the amino acid composition for each peptide described in this table. A: AGADIR score. M: average helical hydrophobic moment. IP: calculated isoelectric point. (H): averaged hydrophobicity. TI: calculated therapeutic index.

Table 3
PAPs in the SwissProt database

<table>
<thead>
<tr>
<th>SwissProt name</th>
<th>A</th>
<th>M</th>
<th>IP</th>
<th>∑(H)</th>
<th>Length</th>
<th>Antibacterial activity Gram(−)</th>
<th>Cytotoxicity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boll megpe</td>
<td>7.9</td>
<td>0.52</td>
<td>11.1</td>
<td>0.058</td>
<td>17</td>
<td>&gt;120</td>
<td>80</td>
<td>[24]</td>
</tr>
<tr>
<td>Cec4 bommo</td>
<td>0.5</td>
<td>0.44</td>
<td>11.3</td>
<td>-0.097</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecb_antpe</td>
<td>0.5</td>
<td>0.43</td>
<td>11.5</td>
<td>-0.132</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crbl_vescr</td>
<td>0.7</td>
<td>0.50</td>
<td>11.6</td>
<td>0.144</td>
<td>13</td>
<td>2.5</td>
<td>80</td>
<td>[26]</td>
</tr>
<tr>
<td>Dms3_physa</td>
<td>1.7</td>
<td>0.44</td>
<td>11.1</td>
<td>-0.024</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grar ranru</td>
<td>0.04</td>
<td>0.53</td>
<td>11.6</td>
<td>-0.084</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ranr ranru</td>
<td>1.3</td>
<td>0.44</td>
<td>11.6</td>
<td>-0.239</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Np artrr</td>
<td>4.3</td>
<td>0.45</td>
<td>10.9</td>
<td>-0.297</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sp5_m bacsu</td>
<td>2.5</td>
<td>0.55</td>
<td>11.4</td>
<td>-0.095</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stp_bpt4</td>
<td>1.7</td>
<td>0.43</td>
<td>11.1</td>
<td>-0.278</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tkna_gadmo</td>
<td>0.03</td>
<td>0.48</td>
<td>11.6</td>
<td>-0.190</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tkna horse</td>
<td>0.03</td>
<td>0.51</td>
<td>11.6</td>
<td>-0.201</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tkna_emyred</td>
<td>0.01</td>
<td>0.49</td>
<td>11.6</td>
<td>-0.175</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tkna_secyca</td>
<td>0.03</td>
<td>0.49</td>
<td>11.6</td>
<td>-0.124</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SwissProt name: the accession name in the SwissProt database for that particular peptide. A: AGADIR score. M: average helical hydrophobic moment. IP: calculated isoelectric point. (∑(H): averaged hydrophobicity. Cytotoxicity: the concentration (µg/ml) required for inhibiting the growth of mammalian cells, usually red blood cells or fibroblasts.
been shown to induce apoptosis by an unknown mechanism [16], so we considered it an interesting target for our study since we might provide some hints on the mechanism of action of C31 in addiction to testing our approach. We found that none of these peptides is toxic to bacterial or mammalian cells when applied extracellularly thus confirming our predictions. Based on these results we propose that C31 may induce apoptosis by a different mechanism than PAPs.

In total, 14 sequences were identified as PAPs in the SwissProt database (see Section 2). These 14 peptides can be placed into four different groups based on their known function; i.e. antibacterial peptides, neuropeptides, mast cell degranulating peptides and protein–protein interacting peptides. Two out of these four groups, antibacterial peptides and neuropeptides, represent more than 80% of the total (Table 3). Neuropeptides appear to be over-represented since there were only 48 neuropeptides in the original pool of 2473 peptides in the SwissProt database.

The special need for antibacterial peptides in the mammalian brain has been pointed out previously [21], since these may represent a more immediate line of control for bacterial infection than the immune system (which has a restricted access to the brain). Considering the properties of PAPs, our findings suggest that some previously identified neuropeptides may have antibacterial activity.

Among the neuropeptides identified as PAPs (Table 3), four were homologs of SP: tkna_gadmo, tkna_horse, tkna_oncomy, and tkna_scyca. SP belongs to the tachykinin family. Tachykinins are synthesized as larger protein precursors (usually more than 40 amino acids in length) that are enzymatically converted to their mature forms [22]. In our original search, we were able to detect only those recorded in the SwissProt database in the active form. Analyzing all of the tachykinins deposited in the database (precursors and active forms), we found that 10 out of 61 were predicted to be PAPs (data not shown). Notably, these 10 were SP peptides from different species.

SP is known to form an α-helical structure in hydrophobic environments but not in aqueous solution [23], while it has a positive charge distribution over its sequence, suggesting the finding that SP is a PAP. Therefore, the neuropeptide SP was tested for its preference for mitochondria-like membranes. The results presented in this work support our predictions that SP is a PAP. However, we did not observe a complete inhibition of E. coli growth, probably because of its well known short half-life in solution (minutes), while our experiments lasted for 8 h. Another possibility is that SP only displays a bacteriostatic activity, since the toxicity displayed by SP on bacterial cells was not markedly affected by the concentration of SP, as in the case of antibacterial peptides.

In developing APAP we focused on the characteristics that define selectivity rather than efficiency to kill bacteria. Therefore, it is not surprising that SP demonstrated bacteriostatic, but not bactericidal, activity. It is noteworthy that SP and most of the antibacterial peptides analyzed in this study (Table 1) are active in the low micromolar concentration range, and that SP is only 11 amino acids long. However, SP was toxic at higher concentrations than the antibacterial peptides in Table 1. We are currently working to use APAP to design more effective antibacterial peptides with higher TI values.

In conclusion, we have described a computational approach, APAP, to identify PAPs. These peptides display selectivity towards bacteria and mitochondria, with little toxic effect on eukaryotic cells when applied extracellularly, thus providing the basis for a new generation of drugs that can be present in the body without toxic effect unless they are taken in by targeted cells as we have shown previously [1]. From a public database, the approach detected mostly antibacterial peptides and neuropeptides suggesting that these neuropeptides may be the first reported with antibacterial activity. In agreement with this idea, we reported that SP is a PAP with a TI > 100. We speculate that these activities have been present in SP during the course of evolution of the tachykinins, which would support the possibility of a biological significance for these findings. APAP provides a method to detect and ultimately improve pro-apoptotic peptides for chemotherapy.

Acknowledgements: G.R., S.C.O. and R.R. are supported by an NIH-Fogarty grant, Pew Charitable Trust Foundation grant and NIH training grant, respectively. This work was supported by NIH Grants R01CA/AG84262-01A1 to H.M.E. and NS33376 and AG12282 to D.E.B. and DoD Grant DAMD17-98-1-8581 to D.E.B.

References


Targeting the prostate for destruction through a vascular address

Wadah Arap*,†, Wolfgang Haedicke*,†, Michele Bernasconi*, Renate Kain§, Daniel Rajotte*,**, Stanislaw Krajewski*, H. Michael Ellerby*,†, Dale E. Bredezen*,†, Renata Pasqualini*†, and Erkki Ruoslahti**

*Cancer Research Center, The Burnham Institute, La Jolla, CA 92037; †Department of Ultrastructural Pathology and Cell Biology, University of Vienna, A-1090 Vienna, Austria

Contributed by Erkki Ruoslahti, December 7, 2001

Organ specific drug targeting was explored in mice as a possible alternative to surgery to treat prostate diseases. Peptides that specifically recognize the vasculature in the prostate were identified from phage-displayed peptide libraries by selecting for phage capable of homing into the prostate after an i.v. injection. One of the phage selected in this manner homed to the prostate 10–15 times more than to other organs. Unselected phage did not show this preference. The phage bound also to vasculature in the human prostate. The peptide displayed by the prostate-homing phage, SMSIARL (single letter code), was synthesized and shown to inhibit the homing of the phage when co-injected into mice with the phage. Systemic treatment of mice with a chimeric peptide consisting of the SMSIARL homing peptide, linked to a proapoptotic peptide that disrupts mitochondrial membranes, caused tissue destruction in the prostate, but not in other organs. The chimeric peptide delayed the development of the cancers in prostate cancer-prone transgenic mice (TRAMP mice). These results suggest that it may be possible to develop an alternative to surgical prostate resection and that such a treatment may also reduce future cancer risk.

Diseases affecting the prostate have gained major significance clinically and economically, primarily because of the increasing average age of the male population in the industrialized countries. Benign prostate hyperplasia affects to some degree most elderly men. Even more serious, the prostate is a frequent site of cancer. Some autopsy studies find that most men older than 70 have occult or overt cancer in the prostate (1). The surgical therapies of prostate hypertrophy and prostate cancer are associated with serious side effects, such as incontinence and impotence.

We have sought to develop a strategy that would provide a less traumatic treatment for prostate disease than is currently available. Our strategy is based on identification of peptides that home to specific sites in the vasculature by in vivo screening of intravenously injected phage libraries. These studies have revealed a surprising degree of specialization in the endothelia of various normal tissues (2, 3). Screening phage libraries for tumor homing yielded a collection of peptides that home to tumor vasculature (4). We and others have used these tumor-homing peptides to direct therapies into tumors in mice (4, 5). We report here the identification of peptides that home to the vasculature of the prostate and the use of one of these homing peptides to deliver a proapoptotic peptide to the prostate.

Materials and Methods

Materials. Peptides were synthesized to our specifications by AnaSpec (San Jose, CA) or by our Peptide Synthesis Facility. The peptides were purified by HPLC and their identity was confirmed with mass spectrometry.

Apoptag Kit for TUNEL staining was purchased from Intergen (Purchase, NY). Testosterone pellets (12.5 mg) and control pellets were from Innovative Research of America (Sarasota, FL), and controlled release pumps from Alzet (Mountain View, CA). The pumps were loaded with peptides following the manufacturer's instructions.

Mice. CD-1 male mice (The Jackson Laboratories) were used for phage screening at an age of 2–4 months. Transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, kindly provided by Norman Greenberg, Baylor College of Medicine, Houston) were bred at our Animal Facility.

Phage Libraries and Library Screening. The phage libraries were prepared in the fUSE5 vector as described (6, 7). The primary library contains about 5 × 10⁹ individual recombinant phage. For the library screening, CD-1 mice were anesthetized with Avertin (0.015 ml/g) and injected intravenously (tail vein) with phage libraries containing 10⁹ transducing units diluted in 200 µl of DMEM. The phage was rescued from tissues by bacterial infection (2), and about 300 individual colonies were grown separately. The bacterial cultures were then pooled and the amplified phage were injected into mice as described above. To test individual phage for homing, 10⁶ colony-forming units (cfu) (fUSE5) or 10¹⁰ plaque-forming units (pfu) (T7), diluted in 200 µl of PBS, were injected. The SMSIARL insert and its scrambled variant were cloned to the T7 phage (TSelect415–1 vector; Novagen), and the resulting phage was tested as described (8).

Results

In vivo screening of a fUSE5 phage heptapeptide library for prostate-homing peptides (6) yielded two phage that accumulated selectively in the prostate. One of these phage, displaying the peptide SMSIARL (single letter code), homed to the prostate 15 times more than nonrecombinant control phage (Fig. 1a). The other prostate-selected phage (VSFLEYR) gave a prostate-homing ratio of ≈10. The homing of the SMSIARL phage to prostate tissue was inhibited when synthetic SMSIARL peptide was injected together with the phage, but not when an unrelated peptide was injected (Fig. 1a). The SMSIARL phage

Abbreviation: TRAMP, transgenic adenocarcinoma of the mouse prostate.

1W.A. and W.H. contributed equally to this work.
2Present address: Departments of Genitourinary Medical Oncology and Cancer Biology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 427, Houston, TX 77030-4095.
3Present address: Ords Biomed, Institut für Pathologie, UKH-Universitätsklinikum Graz, Auenbruggerplatz 25, 8010 Graz, Austria.
4Present address: Department of Pathology, University of Aberdeen, University Medical Buildings, Foresterhill, AB25 2ZD Aberdeen, Scotland, United Kingdom.
5Present address: Biology Department, Research and Development Center, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT 06877-0368.
6Present address: The Buck Center for Research in Aging, 8001 Redwood Boulevard, Novato, CA 94945.
7To whom reprint requests should be addressed: Cancer Research Center, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037. E-mail: ruoslahti@burnham.org.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
homed also to the rat prostate tissue (not shown). The SMSIARL peptide when cloned into the T7 phage (6) showed a similar homing specificity for the prostate.

Phage displaying a scrambled variant of this peptide (LAM-SRIS) showed no homing to the prostate. The T7 SMSIARL phage was not enriched in the brain (Fig. 1b), salivary gland, kidney, testis, thymus, pancreas, skeletal muscle, or lung (not shown). We also confirmed the homing specificity by co-injecting SMSIARL phage and nonrecombinant phage; the ratio of the two types of phage in the prostate was determined by PCR. The SMSIARL phage homed to the prostate 10–15 times more than the nonrecombinant phage. The recovery of the SMSIARL phage was more than 5-fold higher when the tissue was extracted with detergent rather than buffer alone. The brain as a control organ showed no enrichment with or without detergent (Fig. 1b). The greater phage recovery after lysis of the tissue with deter-

Fig. 1. Specific homing of phage to the prostate. (a) Phage selected for prostate homing accumulates specifically in the prostate and the homing is inhibited by soluble peptide. The SMSIARL KUES5 phage, identified by in vivo screening, was tested for prostate homing. This phage and an irrelevant control phage were injected intravenously to male mice [10^6 colony forming units (cfu) per mouse] and the phage were rescued from various tissues based on their ability to infect a host bacteria. As indicated, 200 µg of the SMSIARL peptide or a control peptide (CARAC) was included in the injection to test inhibition of SMSIARL phage homing. (b) The SMSIARL peptide directs specific homing of T7 phage to the prostate. The SMSIARL sequence was cloned to the coat protein of the T7. A 1:10 mixture of SMSIARL and nonrecombinant control T7 phage [10^10 plaque-forming units (pfu)] was injected and allowed to circulate for 7 min. Phage was extracted from prostate and brain with buffer (PBS), or a detergent solution (0.5% Nonidet P-40 in PBS) and plated, and 32 colonies were randomly chosen for PCR. The PCR products of SMSIARL and control phage DNA were distinguished on the basis of a size difference in a 4% agarose gel. (Control tissue was brain.)

Fig. 2. Immunohistochemical staining of phage within prostate endothelial cells after i.v. injection into mice. SMSIARL-phage preparation was injected intravenously into mice. After 7 min circulation, animals were perfused with PBS, the prostate (a–c), brain (d–f), and various control organs were removed, processed for frozen sectioning, and stained with a polyclonal antibody against T7 phage (FITC, a and d) and CD31 (rhodamine; b and e). Merge with nuclear counterstain with DAPI (c and f). Control organs (kidney, spleen, lung; not shown) were negative for the phage, except for liver and spleens, where the reticuloendothelial tissue traps phage nonspecifically (4). (Magnification: ×400.)
Fig. 3. SMSIARL phage binds to endothelium in human prostate. A human prostate tissue section containing both normal and cancerous tissue was overlaid with the SMSIARL phage (10^9 cfu/ml) and the binding of the phage was detected with anti-M13 phage antibody and peroxidase staining. (a) An overview (×200); (b) a detail from a at a higher magnification (×400). Staining of the endothelium is seen. (c) Overlay with phage that contains no peptide insert produces no endothelial staining. (d) The SMSIARL-phage staining is inhibited when soluble SMSIARL peptide is included in the overlay at 0.3 mg/ml.

The SMSIARL phage may have been taken up by tumor vasculature, suggesting that the SMSIARL phage may have been taken up by tumor vasculature. Antibody staining of the phage in tissue sections from mice injected intravenously with the T7 SMSIARL phage revealed staining in the prostate 7 min after an i.v. injection (Fig. 2). The phage staining colocalized with staining for the blood vessel marker CD31, indicating homing to blood vessels in the prostate. No specific staining was seen in control organs, or in prostate or control organs of mice injected with a nonrecombinant control phage. The phage staining appeared to be intracellular, supporting the detergent extraction results shown in Fig. 1b. Overlay of tissue sections from human prostate with the SMSIARL phage indicated that this phage also binds to the endothelium of human prostate blood vessels the same way it binds to the mouse prostate vessels (Fig. 3). Significantly, vessels in hypertrophic human prostate tissue bound the SMSIARL phage. No binding of this phage was detected in the blood vessels in several other human tissues. Similar localization results were obtained with the free SMSIARL peptide coupled to fluorescein (data not shown).

We next studied the ability of the SMSIARL peptide to deliver a biologically active compound to the prostate. D(KLAKLAK)2 is an amphipathic D-amino acid peptide that binds selectively to bacterial, but not eukaryotic cell membranes (9). It has antibacterial activity, but is relatively nontoxic to eukaryotic cells. We have previously shown that D(KLAKLAK)2, if delivered into mammalian cells, disrupts mitochondria (mitochondrial membranes resemble those of bacteria), initiating apoptosis (10). Conjugated through a G-G linker to a homing peptide that homes to tumor vasculature, D(KLAKLAK)2 yields a chimeric compound that is selectively cytotoxic to angiogenic endothelial cells and has antitumor activity in vivo (10). We used the same strategy to prepare a proapoptotic chimera that targets the vasculature of the normal prostate, and studied its ability to cause selective tissue destruction in the prostate.

Mice were injected with 250 µg of the targeted SMSIARL-GG-D(KLAKLAK)2 chimeric compound and the prostates were collected after 1, 4, 8, 12, 16, 24, and 48 h, and after 7 days. Control groups received D(KLAKLAK)2, coupled to a non-homing scrambled peptide (LAMSKIS), SMSIARL and D(KLAKLAK)2 as an uncoupled mixture, or buffer alone. A total of 62 mice treated with SMSIARL-GG-D(KLAKLAK)2 were evaluated. In prostates collected 16 h or later after the injection, histology revealed an unevenly distributed destruction of the prostate glandular epithelial cells that in some areas included epithelial shedding and destruction of entire glandular structures (Fig. 4a and b). These changes were still present 7 days after the treatment and no mitotic figures were observed, suggesting sustained damage and poor regeneration (not shown). Electron microscopy showed extensive destruction of intracellular organelles in the SMSIARL-GG-D(KLAKLAK)2-treated, but not control-treated, mice (Fig. 4c and d). Tissue damage was also evident from an increase in TUNEL-positive vascular and glandular cell nuclei in the prostates of mice treated with SMSIARL-GG-D(KLAKLAK)2 (not shown). The prostates of control animals displayed only rare degenerating epithelial cells and all other organs examined (brain, heart, liver, kidney, lung, urothelium) were histologically normal during or after treatment with each of the compounds (Fig. 4e–h).
were implanted on the peritoneal area opposite the pellets. The peptide binds selectively to the blood vessels in the prostate. Later, controlled release pumps loaded with the peptides the prostate blood vessels after an i.v. injection indicates that this fluctuation in endogenous androgen levels variation in the sensitivity of prostate tissue caused by possible prostate is biochemically distinct. The accumulation of the treatments, we used an implanted peristaltic pump for controlled capable of homing to the blood vessels in the prostate can target x400), We show here that peptides selected from phage libraries for microscopy shows no damage to bladder (e; shows intact cellular structure. (Magnification in c and d, x200). Light microscopy shows no damage to bladder (e; x200), heart (f; x400), kidney (g; x400), or liver (h; x400).

To effect sustained levels of the compounds used in the treatments, we used an implanted peristaltic pump for controlled release. Each pump was loaded with either SMSIARL-GG-D(KLAKLAK)2 or an uncoupled mixture of SMSIARL and D(KLAKLAK)2 as uncoupled peptides (control-treated mice). The mice were killed 24 h after the injection. Prostates were fixed in paraformaldehyde or glutaraldehyde solution and processed for light microscopy by staining with hematoxylin/eosin (H&E) or electron microscopy. Light microscopy showed focal loss of cell borders and epithelial shedding in the ventral lobe of prostates from the SMSIARL-GG-D(KLAKLAK)2 group. (a) H&E-stained micrograph shows massive glandular destruction with nearly complete shedding of the glandular epithelial cells into the lumen. (b) A representative micrograph of normal prostate tissue from a mouse treated with the uncoupled peptide mixture. (Magnification in a and b, x400.) (c) An electron microscopic image of a single epithelial cell from a SMSIARL-GG-D(KLAKLAK)2 group prostate. The cell has sloughed off into the glandular lumen and massive destruction of its organelles is seen. (d) A representative micrograph of normal prostate shows intact cellular structure. (Magnification in c and d, x6,000.) Light microscopy shows no damage to bladder (e; x200), heart (f; x400), kidney (g; x400), or liver (h; x400).

Fig. 4. Targeted proapoptotic peptide to mouse prostate vasculature causes tissue damage in prostate but not in other tissues. Mice received an i.v. 250 μg injection of the SMSIARL-GG-D(KLAKLAK)2 or an equivalent dose of SMSIARL and (KLAKLAK)2 as uncoupled peptides (control-treated mice). The animals were killed after the injection. Prostates were fixed in paraformaldehyde or glutaraldehyde solution and processed for light microscopy by staining with hematoxylin/eosin (H&E) or electron microscopy. Light microscopy showed focal loss of cell borders and epithelial shedding in the ventral lobe of prostates from the SMSIARL-GG-D(KLAKLAK)2 group. (a) H&E-stained micrograph shows massive glandular destruction with nearly complete shedding of the glandular epithelial cells into the lumen. (b) A representative micrograph of normal prostate tissue from a mouse treated with the uncoupled peptide mixture. (Magnification in a and b, x400.) (c) An electron microscopic image of a single epithelial cell from a SMSIARL-GG-D(KLAKLAK)2 group prostate. The cell has sloughed off into the glandular lumen and massive destruction of its organelles is seen. (d) A representative micrograph of normal prostate shows intact cellular structure. (Magnification in c and d, x6,000.) Light microscopy shows no damage to bladder (e; x200), heart (f; x400), kidney (g; x400), or liver (h; x400).

To effect sustained levels of the compounds used in the treatments, we used an implanted peristaltic pump for controlled release. Each pump was loaded with either SMSIARL-GG-D(KLAKLAK)2 or an uncoupled mixture of SMSIARL and D(KLAKLAK)2 as uncoupled peptides (control-treated mice). The animals were killed after 1 week, and their organs processed for histology. In another control experiment, we also implanted s.c. testosterone pellets to eliminate any variation in the sensitivity of prostate tissue caused by possible fluctuations in endogenous androgen levels (11). Seven days later, controlled release pumps loaded with the peptides were implanted on the peritoneal area opposite the pellets.

Fig. 5. Survival of TRAMP mice treated with SMSIARL-GG-D(KLAKLAK)2 or control materials. The treatment was initiated at 12 weeks of age. Male mice (ten per group) received i.v. injections of SMSIARL-GG-D(KLAKLAK)2 peptide (200 μg per dose), or an equivalent dose of SMSIARL and (KLAKLAK)2 as uncoupled peptides (control-treated group). The injections were given once a week for a total of ten doses. The mice in the SMSIARL-GG-D(KLAKLAK)2 group survived significantly longer than the control mice treated with the uncoupled peptide mixture or with buffer.

SMSIARL-GG-D(KLAKLAK)2, consistently produced damage in the prostate (data not shown). The tolerated dose of SMSIARL-GG-D(KLAKLAK)2 was limited by acute toxicity of the compound; the dose could be increased by giving the injection slowly over several minutes. Mice injected with SMSIARL-GG-D(KLAKLAK)2, as well as those injected with equivalent amount of nonconjugated mixture of the homing peptide and proapoptotic peptide, showed marginal elevation of serum parameters of liver (ALT, AST, GGT) and kidney (creatinine and blood urea nitrogen) function. The levels returned to normal 1 week after the treatment. In one experiment, four mice that had been treated with four weekly injections of SMSIARL-GG-D(KLAKLAK)2 were allowed to mate. Vaginal plugs showed that mating had occurred and litters were born in each case. These results suggest that SMSIARL-GG-D(KLAKLAK)2 causes damage in the prostate, while other tissues are spared and the mice remain fertile.

We next analyzed the effect of a systemic SMSIARL-GG-D(KLAKLAK)2 treatment on the longevity of TRAMP mice (12). Two independent experiments gave similar results; one of the experiments is shown in Fig. 5. The SMSIARL-GG-D(KLAKLAK)2 survived significantly longer than the control groups that received the uncoupled peptides or buffer (P < 0.01 for both; Log Rank test).

Discussion

We show here that peptides selected from phage libraries for homing to the prostate vasculature reveal tissue-specific features in the blood vessels of the prostate. We also show that a peptide capable of homing to the blood vessels in the prostate can target a proapoptotic peptide to the prostate, and that systemic treatment with this targeted compound can cause destruction of prostate tissue and delay the development of prostate cancer in mice. Our results show that, like the vasculature of many other tissues analyzed in previous work (2-4), the vasculature of the prostate is biochemically distinct. The accumulation of the SMSIARL phage and fluorescein-labeled SMSIARL peptide in the prostate blood vessels after an i.v. injection indicates that this peptide binds selectively to the blood vessels in the prostate. The
selective destruction of prostate tissue caused by targeting of a proapoptotic peptide to the prostate with the SMSIARL homing peptide supports this conclusion.

The molecular nature of the vascular specialization is incompletely understood. We have identified the receptor for a peptide that homes to lung vasculature as membrane dipeptidase (13). Others have shown that a modified von Willebrand factor promoter is activated in endothelial cells in a tissue-specific manner under the influence of the surrounding parenchymal tissue (14), providing one possible regulatory mechanism for the expression of tissue-specific endothelial markers. Perhaps prostate tissue induces receptors for SMSIARL in the resident endothelium. Although the molecule the SMSIARL peptide binds to in the prostate vasculature remains to be identified, our results suggest some practical applications.

The destruction of prostate tissue by the SMSIARL-targeted proapoptotic peptide is likely to be secondary to loss of blood vessels, the main target of the homing peptide. However, we cannot exclude a direct effect on prostate epithelial cells. The tissue damage was specific for the prostate, suggesting that it may be possible to develop a "medical prostatectomy" procedure based on this principle. Such a procedure could provide an alternative treatment for prostate hypertrophy. Furthermore, the proapoptotic peptide treatment postponed the development of prostate cancer in TRAMP mice. We attribute the effect in the TRAMP mice to a reduction in the number of target cells available for malignant transformation, because the SMSIARL peptide does not home to the vessels in the TRAMP tumors (W.H. and E.R., unpublished result). The lifespan extension in our treated TRAMP mice was 6–8 weeks, close to 20% of the lifespan, even though the treatment works against a tremendous oncogenic pressure in these transgenic mice (12, 15). In human terms, this would mean postponement of prostate cancer development for several years. A medical treatment that reduces the size of the prostate and at the same time delays the development of prostate cancer could be an extremely useful procedure.

We thank Dr. Norman Greenberg for providing TRAMP mice and Eva Engvall for comments on the manuscript. This work was supported by Grants DAMD17-99-1-8164 (to W.A.), DAMD17-98-8581 (to D.E.B.), and DAMD17-98-1-8562 (to E.R.) from the Department of Defense, research awards from CapCURE (to W.A. and E.R.), and Grants CA74238 and CA82713 (to E.R.) and Cancer Center Support Grant CA30199 from the National Cancer Institute.

An Artificially Designed Pore-forming Protein with Anti-tumor Effects*

Received for publication, January 16, 2003, and in revised form, May 7, 2003.
Published, JBC Papers in Press, May 14, 2003, DOI 10.1074/jbc.M300474200

H. Michael Ellerby‡, Sannamu Lee‡, Lisa M. Ellerby‡, Sylvia Chen‡, Taira Kiyota‡, Gabriel del Rio‡, Gohsuke Sugihara†, Yan Sun†, Dale E. Bredersten‡, Wadih Arape‡, and Renata Pasqualini**

From the Program on Cancer and Aging, The Buck Institute, Novato, California 94945, the Department of Chemistry, Faculty of Science, Fukuoka University, Jonan-ku, Fukuoka 814-80, Japan, and The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Protein engineering is an emerging area that has expanded our understanding of protein folding and laid the groundwork for the creation of unprecedented structures with unique functions. We previously designed the first native-like pore-forming protein, small globular protein (SGP). We show here that this artificially engineered protein has membrane-disrupting properties and anti-tumor activity in several cancer animal models. We propose and validate a mechanism for the selectivity of SGP toward cell membranes in tumors. SGP is the prototype for a new class of artificial proteins designed for therapeutic applications.

The tendency of amphipathic peptides to assemble in aqueous solution and of the β-turn to form a loop has been successfully employed to design coiled-coil proteins (1–3), various helix bundle proteins (4–9), and β-structural proteins (10, 11). De novo design of proteins with biological function, such as hemebinding, catalysis, or the formation of a membrane pore or channel, is perhaps the most challenging goal of peptide chemistry (12–19). Much has been done recently in terms of designing membrane proteins that are correctly incorporated into membranes. However, relatively few attempts have been made to design proteins capable of disrupting membranes and subsequently causing cell death in vivo (19, 20).

Small globular protein (SGP)† is a 69-amino acid, 4-helix bundle protein, composed of 3 amphipathic helices, which consist of Leu and Lys residues and surround a single hydrophobic helix consisting of Ala residues, which create a pocket-like structure (Fig. 1, A and B) (21, 22). SGP is monomeric in solution and denatures in a highly cooperative manner, characteristic of native globular-like proteins. SGP was conceived and designed based on the structure of the colicin family of bacteriocins (23–26). Although most naturally occurring, pore-forming proteins maintain their tertiary structure when disrupting membranes, the colicins undergo a spontaneous transition from a native folded state in solution to an open umbrella-like state in membranes. SGP was designed to mimic this membrane insertion mechanism, which was confirmed in synthetic bilayers, where SGP formed a uniform size pore (14 ps) (21). It is still unknown whether or not SGP oligomerizes to form a channel.

Given that SGP forms pores in synthetic membranes, we asked whether it could disrupt biological membranes at the cellular level and whether it could be used successfully in vivo as an anti-tumor agent. We also investigated whether SGP would show any selectivity toward tumor cell lines in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Reagents—SGP, SGP-L, and SGP-E were synthesized according to the Fmoc procedure starting from Fmoc-Leu-PEG (polyethylene glycol) resin using a Miligen automatic peptide synthesizer (Model 9050) to monitor the de-protection of the Fmoc group by UV absorbance (21). After cleavage from the resin by trifluoroacetic acid, the crude peptide obtained was purified by HPLC chromatography with an ODS column, 20 × 250 mm, with a gradient system of water/acetonitrile containing 0.1% trifluoroacetic acid. Amino acid analysis was performed after hydrolysis in 5 M HCl in a sealed tube at 110 °C for 24 h. Analytical data obtained were as follows: Gly, 6.2 (6); Ala, 9.5 (10); Leu, 26.5 (25); Asp, 3.0 (3); Pro, 2.9 (3); Tyr, 3.1 (3); Lys, 18.9 (18). Molecular weight was determined by fast atom bombardment mass spectrometry using a JEOL JMX-HX100: base peak, 7555.1; calculated for C367H638O77N91H+: 7554.8. Peptide concentrations were determined from the UV absorbance of Trp and three Tyr residues at 280 nm in buffer (ε = 8000). Gel filtration HPLC chromatography was performed using Tris buffer (10 mm Tris, 150 mM NaCl, pH 5.0 or pH 7.4) on COSMOSIL 5ODI-300 (Nakalai Tesk, Kyoto, Japan).

Computer Model—The computer-generated model of SGP was made with the program Insight II (Molecular Simulations Inc., San Diego, CA) running on an Octane SSE work station (Silicon Graphics, Cupertino, CA).

Cell Culture—All cell lines were obtained commercially. The Kaposi's sarcoma-derived cell line KS1767 and the breast carcinoma cell line MDA-MB-435 have been described previously (27–29) and were cultured in 10% fetal bovine serum/Dulbecco's modified Eagle's medium, containing antibiotics.

Quantification of Cell Death—Cell viability was determined by morphology (29, 30). For viability assays, KS1767 cells were incubated with the concentrations of SGP, SGP-L, SGP-E, or control peptides indicated in the figures and in Table I. Briefly, at the given time points, cell culture medium was aspirated from adherent cells. Cells were then gently washed once with PBS at 37 °C. A 20-fold dilution of the dye mixture (100 μg/ml acridine orange and 100 μg/ml ethidium bromide) in PBS was then gently pipetted on the cells and viewed on an inverted microscope (Nikon TE 300). Cells with nuclei exhibiting margination and condensation of chromatin and/or nuclear fragmentation (early/mid

This paper is available on line at http://www.jbc.org

35311
apoptosis-acridine orange positive) or with compromised plasma membranes (late apoptosis-ethidium bromide positive) were scored as not viable; 500 cells per time point were scored in each experiment. Percent viability was calculated relative to untreated cells.

**Human Tumor Xenografts—MDA-MB-435-, KS1767-, PC-3-, and H358-derived human tumor xenografts were established in 2-month-old female or male (according to the tumor type), nude/nude Balb/c mice (Jackson Labs, Bar Harbor, ME) by administering 10⁶ tumor cells per mouse in a 200 µl volume of serum-free Dulbecco's modified Eagle's medium into the mammary fat pad or on the flank (29). The mice were anesthetized with Avertin as described (29). SGP was administered directly into the center of the tumor mass at a concentration of 100 µM or 1 mM given slowly in 5 µl increments, for a total volume of 40 µl. Measurements of tumors were taken by caliper under anesthesia and used to calculate tumor volume (29). Animal experimentation was reviewed and approved by the Institutional Animal Care and Use Committee.

**Skin Toxicity—**2-month-old female nude mice (Jackson Labs) were anesthetized with Avertin. 10 µl of 100 µM SGP or PBS was injected into the skin. The injected areas were monitored for 2 weeks.

**Cytotoxicity Assays—**Cell viability was determined by morphology (29, 30). KS1767 cells were incubated with SGP at 1 mM in the presence or absence of matrigel or polymeric fibronectin (sFN). The fibronectin polymer was produced as described (31). Briefly, cell culture medium was aspirated from adherent cells. Cells were then coated with matrigel (gently pipetted on each well to completely coat the entire cell layer), or the fibronectin polymer, and incubated at 37 °C for 10 min. SGP was added and the cells were viewed on an inverted microscope (Nikon TE 300). KS1767 cells were also exposed to doxorubicin (20 µg/well) or SGP in the presence or absence of matrigel for 24 h. Cell viability (%) was evaluated after no treatment (medium or matrigel alone), incubation with SGP or doxorubicin. Cell death was evaluated morphologically (29, 30), and cell viability was compared relative to untreated controls (no matrigel) or absence of SGP.

**RESULTS**

**SGP Effects on Cultured Cells—**To evaluate the effects of SGP on cell membranes we treated multiple human cell lines of different origins (Table I). These lines included the Kaposi's sarcoma-derived cell line KS1767, the breast carcinoma-derived cell line MDA-MD-435, and the microvascular endothelial cell line dermal microvessel endothelial cells (27–29). Treatment of KS1767 cells with >10 µM SGP led to rapid non-necrotic, non-apoptotic cell death, characterized by 100% loss of viability within 60 s (Fig. 2A), as determined by Trypan Blue positivity. Such a rapid response suggests that the plasma membrane has been disrupted. Lowering the concentration of SGP to between 5 and 10 µM led to induction of necrosis (scored morphologically), resulting in almost 100% loss of KS1767 cell viability over 60 min (Fig. 2B). SGP levels below 5 µM led to the induction of apoptosis over a 24-hour period (Fig. 2C), which was confirmed by a caspase-3 activation assay. KS1767 cells were unaffected by a 24 h incubation in 100 µM of a control peptide (Fig. 2D). However, the classic morphological signs of apoptosis, such as nuclear condensation (Fig. 2E, short arrow) and plasma membrane blebbing (Fig. 2E, long arrow), were apparent in KS1767 cells after a 24-hour treatment with 3 µM SGP. Similar results were obtained using different cell lines, including several types of malignant cells (solid tumors and leukemic cell lines) and non-neoplastic cells (including endothelial cells and fibroblasts isolated from multiple organs and cells of glial origin, Table I). As negative controls, we used altered forms of SGP (SGP-L and SGP-E). In SGP-L, the central all alanine helix was replaced by an all leucine helix. In SGP-E, lysines have been replaced by glutamic acids, and we had previously determined that the ability of such analogs to...
disrupt synthetic membranes is diminished (22). SGP-L and SGP-E were substantially less toxic to mammalian cultured cells (Table 1). The LC_{50} was increased by at least 10-fold in all cell types tested with SGP-L and SGP-E when these inactive versions of the protein were tested. These observations clearly show that the integrity of the SGP helices is required for SGP membrane disrupting activity. Taken together, these data demonstrate that SGP is a potent membrane-disrupting agent, but also that it is not cell-selective and it will affect tumor-derived cells as well as normal cells at similar concentrations (~3 μM).

**SGP Has Anti-tumor Activity in Vivo**—Given the potent membrane-disrupting activity of SGP, we proceeded to evaluate SGP anti-tumor activity in nude mice bearing human tumor xenografts. We hypothesized that direct administration of SGP might reduce tumor volume and retard metastasis. In the first set of experiments, tumors were allowed to form after injection of a breast carcinoma cell line (MDA-MD-435) and then treated with local injections of SGP. We observed that tumor volume was significantly smaller in SGP-treated mice treated tumor volumes at the end of the experiment are represented as means ± SE (right injection site, arrow) demonstrates that SGP is relatively non-toxic to normal skin. Results represented in C were reproduced in eight independent experiments.

**FIG. 2. SGP treatment of cultured tumor cells.** A, human Kaposi's sarcoma-derived KS1767 cells treated with 10 μM SGP undergo extremely rapid non-necrotic, non-apoptotic cell death within 60 s (black bars), whereas those treated with 100 μM of negative control peptide DLSLARLATARLAI are unaffected (gray bars) (p < 0.04). B, necrosis is observed in KS1767 cells treated with 10 μM SGP within 60 min (black bars), whereas those treated with 100 μM of negative control peptide are unaffected after 60 min (gray bars) (p < 0.03). C, apoptosis is observed after treatment with 3 μM SGP over 24 h, whereas cells treated with 100 μM of negative control peptide are unaffected after 24 h (gray bars) (p < 0.05). Hoffman contrast microscopy of KS1767 cells treated with 100 μM of negative control peptide (D) for 24 h or 3 μM SGP for 24 h (E). Cells with nuclei exhibiting margination and condensation of chromatin and/or nuclear fragmentation (early/mid apoptosis-acridine orange positive) or with compromised plasma membranes (late apoptosis-ethidium bromide positive) were scored as not viable (500 cells per time point were scored in each experiment). Percent viability was calculated relative to untreated cells under all experimental conditions. Classic morphological characteristics of cell death including condensed nuclei (short arrows) and plasma membrane blebbing (long arrows) are evident. Results were reproduced in more than three independent experiments.

**FIG. 3. SGP treatment of nude mice bearing human breast cancer-derived xenografts.** Data are shown for human MDA-MB-435-derived breast carcinomas. Mice had tumor volumes ranging from 100 mm^3 to 600 mm^3 and were divided in similar groups based on matched tumor volumes at the start of the experiment (open circles). A, SGP-treated tumors are smaller than controls (PBS-treated or SGP-treated tumor volumes at the end of the experiment are represented as closed circles). Differences in tumor volumes at 8 weeks are shown (t test, p < 0.05). A total of 10 mice received SGP. B, representative pictures of tumors after 4 weekly treatments with SGP at 40 μl/week, n = 5 for each experimental group. The volume of the PBS-treated tumor is 400 mm^3 (left), whereas 100 μM SGP (middle) and 1 mM SGP (right) treated tumors have flattened and virtually disappeared. These three tumors began at volumes of 100 mm^3, C, lack of skin toxicity of SGP. Subcutaneous injection (40 μl) of 100 μM SGP (left injection sight, arrow) and of PBS (right injection site, arrow) demonstrates that SGP is relatively non-toxic to normal skin. Results represented in C were reproduced in eight independent experiments.
An Artificially Engineered Protein with Anti-tumor Activity

Mice treated with SGP remained tumor-free for up to 4 months after tumor implantation, before being euthanized for histological evaluation. These observations indicate that both primary tumor growth (Fig. 4) and metastases were inhibited. Surgical examination of the tumor sites revealed no sign of tumor cells. Similar results were obtained when xenografts were produced by injection of prostate (Fig. 5A) and lung carcinoma (Fig. 5, B and C) cell lines. By successfully treating a large number of mice and testing the effects of SGP on several different tumor xenograft models (including carcinomas, sarcomas, and melanomas), we firmly established the therapeutic properties of SGP. Our data also show that the anti-tumor effects of SGP are not limited to a specific tumor type. We also determined whether SGP produced adverse side effects such as necrosis when injected under normal skin. Strikingly, in all mice tested, SGP did not produce any surface effect when injected intradermally or subcutaneously (Fig. 3C) when compared with mice that did not receive the active form of SGP.

Histopathological analysis of SGP-treated MDA-MB-435 human breast carcinoma xenografts showed widespread cell death (Fig. 4, upper right panel), as compared with PBS-treated tumors (Fig. 4, upper left panel). Many condensed nuclei were apparent (Fig. 4, upper left panel, short arrows), and there was no effect on the extracellular matrix (Fig. 4B, long arrows). Apoptosis was confirmed by a caspase-3 activation assay (data not shown). It is noteworthy that whereas 100 μM SGP induced almost immediate cell death in vitro that was apparently neither apoptotic nor necrotic, 100 μM SGP induced apoptosis in vivo. Work is underway to evaluate lower concentrations. SGP-treated human KS1767 Kaposi's sarcoma-derived xenografts showed similar effects (Fig. 4, left and right panels). Histological analysis of the major organs of SGP-treated mice showed no overt pathology, confirming that SGP treatments do not affect sites other than the injected tumor area (data not shown). Thus, SGP has anti-tumor specific effects, without showing any tumor cell-specific effects.

**Mechanism of SGP Action and Selectivity toward Cell Membranes**—To determine the mechanisms responsible for selective anti-tumor activity of SGP in vivo, we designed a matrigel...
An Artificially Engineered Protein with Anti-tumor Activity

Fig. 6. SGP treatment of cultured tumor cells in the presence or absence of matrigel or polymeric fibronectin. Treatment of KS1767 cells with 1 mM SGP decreases cell viability and leads to condensed nuclei and plasma cell membrane blebbing (B), whereas cells treated with 1 mM of SGP in the presence of matrigel remain unaffected after 60 min (D). KS1767 cells without (A) or with a layer of matrigel (C) remained healthy for as long as 48 h. Results were reproduced in four independent experiments.

Fig. 7. Cytotoxic assay in vitro and effects of matrigel. A, KS1767 cells were exposed to doxorubicin or SGP in the presence or absence of matrigel for 24 h. Cell viability (%) was evaluated at 24 h after no treatment (medium or matrigel alone), or incubation with SGP or doxorubicin (20 μg/well), as indicated. In contrast to SGP, doxorubicin decreased cell viability (*, p < 0.01) in the presence of matrigel. Shown are S.E. obtained from triplicate wells. Results were reproduced in four independent experiments. B, KS1767 cells were exposed to SGP in the presence or absence of polymeric fibronectin. In contrast to cells exposed to ethanol, cells treated with 1 mM of SGP in the presence of polymeric fibronectin (sFN) remain unaffected (*, p < 0.01). Cell viability (%) was evaluated morphologically. Shown are S.E. obtained from triplicate wells. Results were reproduced in three independent experiments.

Assay (to mimic extracellular matrix). In the absence of matrigel, SGP led to severe disruption of cell membranes, resulting in almost 100% loss of viability over 10 min (Fig. 6B). In contrast, in the presence of matrigel, KS1767 cells were unaffected by incubation with 1 mM SGP (Fig. 6D). This loss of membrane disrupting ability in the presence of a thin matrigel layer could account for the lack of SGP toxicity seen in vivo. Ethanol, as shown in Fig. 7A, or cytotoxic drugs such as doxorubicin (Fig. 7B) damaged the cell layer under similar conditions, regardless of the presence of matrigel, which fails to provide protection from the other toxic agents because these other agents more readily diffuse through the matrix. When matrigel was replaced by polymeric fibronectin (sFN) (31), another form of matrix, SGP was also ineffective and did not interfere with cell viability (Fig. 7A), whereas ethanol induced massive cell death. Fibronectin alone did not prevent SGP activity and was used as a control.

The observations in this model are consistent with the lack of skin toxicity seen with SGP. We propose that the discrepancy between in vitro and in vivo SGP effects (anti-tumor cell activity versus selective anti-tumor activity) results from the potent membrane-disrupting activity of SGP, which is inactivated in the presence of extracellular matrix and connective tissue.

DISCUSSION

SGP represents a novel class of anti-cancer proteins whose therapeutic effects can be optimized by amino acid substitution and by altering helical domain length and hydrophobicity (32).
Although SGP is a nonspecific membrane-disrupting agent, it is selective in the sense that the disruption is limited in vivo. Unlike detergents, which solubilize membranes, SGP physically disrupts membrane architecture, leading to cell lysis. This explains the lack of SGP toxicity when the protein is injected sub-cutaneously or intradermally. Recently published data (22) also suggest that the lipid membrane-disruption properties of SGP are responsible for the anti-tumor activity of the agent.

We report one of the first examples of a pore-forming peptide or protein, natural or synthetic, being applied successfully to treat established human tumor xenografts. It is important to emphasize that SGP is not a bacterial toxin, although such agents (or their natural or recombinant form) have been extensively explored as anti-cancer therapies (33, 34). Several pore-forming peptides and proteins such as SGP.

therapeutic strategies may emerge from the activity of de-

lignant mSalvesen, and Krajewski, S., Lombardo, C.


5. Hecht, M. H., Richardson, J. S., Richardson, D. C., and Ogden, R. C. (1990) Science 249, 884-891


REFERENCES
