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Review Article

Designing βpep peptides: a rational approach to the discovery of novel pharmaceutical agents and small molecules

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Abstract

Approaches to drug discovery are varied and range from high-throughput screening of small molecule databases to rational design. This review is focused on our use of structural biology and principles of protein folding to rationally design novel peptides (βpep peptides) as potential pharmaceutical drugs. βpep peptides generally fold as β-sheets that can be used as a scaffold with which to present amino acid residues in a functionally relevant fashion, thereby eliciting a desired biological activity. Presently, βpep peptides can function as antibacterials, antiangiogenics, as well as antitumor and anticoagulation agents. Our design approach also allows for relatively easy development of structure-activity relationships, identification of pharmacophore sites and reduction of peptides to smaller molecules.

Introduction

Approaches to drug discovery are varied and range from the full "shotgun" approach centered around high-throughput screening (HTS) of small-molecule databases, generally favored by the major pharmaceutical companies, to the more efficient and more often more productive approach of rational design. In itself, rational design means different things to different researchers; however, in its basic formulation it involves designing a new molecule using information from various sources, the most important of which are molecular structure and structure-activity relationships (SARs) to identify functionally key residues. Advances in structural analysis using X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, combined with advances in molecular biology to mutate and to produce proteins in quantity, as well as in peptide synthesis have enabled researchers to design and to construct peptides for investigating basic folding principles, making novel structural motifs, modifying functional activities and developing pharmaceutical drugs.

The focus of this review is on the design and biological activities of a novel class of peptides, βpep peptides. βpep peptides generally fold as β-sheets that can be used as a scaffold with which to present amino acid residues in a functionally relevant fashion, *e.g.*, to some receptor, thereby eliciting a desired activity that depends upon the spatial relationships of key residues built into βpep peptides. Presently, βpep peptides can function as antibacterials, antiangiogenics, as well as antitumor and anticoagulant agents.

Designing βpep peptides

The initial goal in designing βpep peptides was to make a relatively small peptide that could form good

β-sheet structure and remain in solution under physiological conditions. Peptide design has been used for some time to aid in understanding the principles behind protein folding and in designing proteins with new and improved functions (1-12). Studies on β-sandwich peptides (*e.g.*, betabellin and betadoublet) (6, 9, 13) provided guidance in the design of βpep peptides. When designing β-sheet folds in short peptides where considerably more β-sheet and/or side-chain surface (particularly hydrophobic surface) is exposed to solvent, solubility can be problematic. For example, peptides in the betabellin series (6), as well as betadoublet (9), showed limited solubility in water and folded poorly into β-sheets, primarily because too many polar amino acid residues like threonine, which statistically based structure propensity scales showed are favored in β-sheet domains of well-folded proteins, were incorporated into these sequences. When insufficient hydrophobic core is present, polar side chains tend to oppose hydrophobically mediated folding. Although some short, water-soluble β-hairpin (14, 15) and α/β (16) peptides have been shown to form β-sheet structures and remain monomeric, larger designed β-sheet-forming peptides, like βpep peptides, are inherently designed as amphipaths to aggregate through their otherwise solvent exposed hydrophobic surface. One of the keys to designing βpep peptides was to recognize that solubility and structure must be considered simultaneously with the desired goal being to bury as much of the hydrophobic surface within the folded structure in order to drive folding and to improve solubility.

Biophysical studies on α-chemokine-derived peptides (17, 18), as well as those on betabellins (6, 13, 19) and betadoublet (9), led to several observations that were key to designing βpep peptides (18). In particular, comparison of amino acid sequences and compositions of platelet factor-4 (PF4), IL-8, Gro-α, betabellin 14D and betadoublet, helped to formulate the following guidelines as a recipe in the design of water soluble β-sheet-forming peptides (18). To optimize peptide solubility and the potential for β-sheet folding in aqueous solution, one should maintain: (i) positive (K, R) to negative (E, D) residue ratio between 4/2 and 6/2; (ii) noncharged polar residue (N, Q, T, S) composition less than about 20%; (iii) aliphatic hydrophobic residue (I, L, V, M and A) composition between 40% and 50%. I/L appear to work best. Although aromatic hydrophobic residues (W, F, Y) generally do not favor tight packing between β-sheets, they may work equally as well in specific cases; (iv) proper placement and pairing of residues, particularly hydrophobic ones, in the sequence; and (v) specific turn character.

Based on this recipe and using the basic β-sheet scaffold from α-chemokines, a series of βpep peptide 33mers was designed (18) that remain soluble in water under physiological conditions and form β-sheet structure. C-terminal residues are nearly the same as those from the C-terminus of the IL-8 β-sheet domain in order to maintain the α-chemokine folding initiation site (20). In addition, most of the hydrophobic residues in βpep peptides were positioned sequentially according to basic folding principles in order to promote optimal amphipathic β-sheet folding.

Structure of βpep peptides

Nuclear magnetic resonance (NMR) structural analysis of well-folded βpep-4 (18) indicates that the peptide 33mer folds relatively compactly as a tetramer with its monomer subunits having essentially the same three-stranded antiparallel β-sheet fold (21). Within tetramers, however, there is conformational heterogeneity in that two equally populated dimer states are present. Each dimer is formed by continuing the monomer β-sheet into a six-stranded sheet, with dimer heterogeneity arising primarily from a two-residue shift in the alignment of interfacial strands. Figure 1 shows a schematic for the folding of βpep-4 dimers, along with the superposition of calculated structures using NOE restraints (21). The surface of

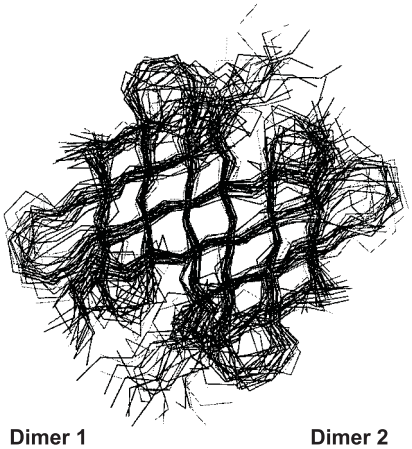

Fig. 1. The βpep peptide β-sheet fold is shown. As presented using the single letter amino acid code, two different dimers (1 and 2) arise primarily from a two-residue shift in the interfacial strand 1-strand 1 alignments (21). In either case, dimers form by extending their antiparallel β-sheets via interactions between strand 1 from two monomers. The hydrophobic faces of two amphipathic dimers then associate to form a β-sheet sandwich tetramer. Twenty-five structures for dimer 1 and dimer 2, calculated by using NMR data and dynamical simulated annealing, have been superimposed using Cα atoms (21) and are shown modeled as a tetramer at the top of the figure.

either β-sheet is amphipathic with the central residues on the hydrophobic side consisting of V7, M9, L11, W18, I20, V22, L24, L30 and L32 and the hydrophilic side being highly positively charged, *i.e.*, net positive charge of +20. This makes βpep-4 and other βpep peptides good candidates for binding to anionic biomolecules like heparin and cell surface heparan sulfate and possibly for modulating various cellular activities (22).

In most βpep peptides, however, compact structure is lacking, and transient β-sheet conformation is characterized by a strong CD band at 217 nm and a more "random coil" NMR spectrum (18, 23). βpep folding is thermodynamically linked to self-association, and tetramers are usually the highest oligomer state formed. Pulsed field gradient (PFG)-NMR diffusion measurements allowed derivation of fractional oligomer populations and equilibrium constants, which for βpep-4 at 313 K are 2.5 x 10^5 M^{-1} for the monomer-dimer equilibrium and 1.2 x 10^4 M^{-1} for the dimer-tetramer equilibrium (24). These values decrease only slightly, if at all, with decreasing temperature indicating a hydrophobically mediated association/folding process. Conformational analyses using NMR and CD provide a picture where "random coil" monomers associate to form β-sheet sandwich dimers, which further associate and fold as well-structured tetramers. For βpep peptide monomers, this "random coil" state includes about 10-20% β-sheet, which is in relatively rapid equilibrium with a broad spectrum of other conformational states. For most βpep peptides, equilibrium dissociation constants are larger, indicating a shift to lower aggregation states. In this regard, most βpep peptides exist primarily as monomers at concentrations in the micromolar range used in the *in vitro* and *in vivo* assays described in the following sections. This should be kept in mind and will later beg the question of what then is the bioactive conformation of βpep peptides.

Bioactivities of βpep peptides

Currently, various βpep peptides exhibit a range of bioactivities from being bactericidal, to promoting the production of the natural anticoagulant activated protein C, to inhibiting angiogenesis and tumor growth. From the outset, it should be noted that unlike most any recombinant protein drug, βpep peptides have long shelf-lives. βpep peptides remain active after being in solution at room temperature for at least several months, being boiled in solution, standing as a dry powder for over 6 months or being stored as a frozen (–20 °C or –80 °C) solution for months. The following subsections discuss the bioactivities of βpep peptides.

Antibacterial activity

Initially, βpep peptides were designed to be bactericidal and to neutralize the bacterial endotoxin lipopolysaccharide (LPS). Residues 82-108 from bactericidal/permeability increasing (BPI) protein, thought to be functionally key to killing bacteria and to neutralizing LPS (25, 26), were, in part, designed into the βpep presentation scaffold (27) following the guidelines given above. In BPI, however, this sequence forms an extended loop (28), whereas in βpep peptides, residues exerpted from this BPI sequence are part of a β-strand (21). Although bactericidal activity (submicromolar range) is similar to that in BPI, endotoxin neutralizing activity is about 100-fold less, suggesting that a larger area on the surface of parent BPI is required for effective binding to LPS and that this surface is not being presented in βpep peptides. By varying the positions of residues in βpep peptides, activities could be optimized to some extent (27). However, improvement of bactericidal activity usually led to a reduction in endotoxin neutralizing activity and *vice versa*. Furthermore, unlike most presently used antibiotics that are aimed at inhibiting enzymes involved in bacterial cell membrane/wall construction, βpep and βpep-derived peptides fall into a well-known class of membrane disintegrating agents, which function by disrupting the bacterial cell membrane/wall and causing it to become highly permeable and thereby killing the bacteria. In this regard, they are probably not subject to bacterial resistance.

To assess the *in vivo* endotoxin neutralizing effect of βpep peptides, Weiss *et al.* (upublished data) coinjected LPS and several βpep peptides into Swiss/Webster mice and monitored TNF-α and IL-6 release. Bacterial infection can lead to sepsis and septic shock via LPS-mediated triggering of overproduction and release of cytokines, like TNF-α and IL-6, from macrophages. Whereas LPS alone injected into mice induced very high levels of TNF-α (600 units) and IL-6 (7000 units), the presence of βpep-8, for example, greatly diminished serum levels of both TNF-α (30 units) and IL-6 (200 units). This is consistent with TNF-α release data from macrophages in cell culture (29).

In a related study, a series of dodecapeptides, which "walk through" the sequence of βpep-25, were investigated for their ability to kill Gram-negative and Gram-positive bacteria and to neutralize endotoxin (30). One of these dodecapeptides, SC-4 [KLFKRHLKWKII-NH_2], was highly effective, more so than βpep-25, at killing Gram-negative bacteria with LD_{50}s in the single-digit nanomolar range. Against Gram-positives, SC-4 also showed good activity with submicromolar LD_{50}s. Leakage studies indicate rapid bacterial membrane permeability with $t_{1/2}$ values of about 10 min or less. SC-4 in the micromolar range also effectively neutralized endotoxin and was not hemolytic at concentrations at least up to 10^{-4} M. NMR-based computational modeling yielded an amphipathic helix structure with positively charged residues K1, K4, R5 and K8 arrayed on the same face of the molecule. Relative to other known bactericidal peptides in the linear peptide, helix-forming category, SC-4 is the most potent, broad-spectrum antibacterial identified to date. The spatial relationships of hydrophobic and positively charged

residues within the folded structure are key to maximizing the antibiotic activity of these novel peptides and should assist in the design of a small-molecule mimetic, which is currently under way.

Antiangiogenic activity

Angiogenesis is crucial to numerous biological functions in the body, from normal processes like embryogenesis and wound healing to abnormal processes like tumor growth, arthritis, restenosis and diabetic retinopathy. The search for angiogenesis inhibitors which could be therapeutically useful has been mainly concentrated on controlling two of the processes promoting angiogenesis: endothelial cell (EC) growth and cell adhesion (31, 32). Targeting drugs to ECs is considered a potential anticancer strategy primarily because ECs are more accessible than other cells to pharmacologic agents delivered via the blood, are genetically stable and are not easily mutated into drug-resistant variants. Most antiangiogenic agents have been discovered by identifying endogenous molecules, primarily proteins, which inhibit EC growth. This traditional approach has produced a number of antiangiogenic agents, such as PF4 (33), thrombospondin-1 (34), interferon-γ inducible protein-10 (35), angiostatin (36), endostatin (37), vasostatin (38) and bactericidal/permeability increasing (BPI) protein (39). The use of antiangiogenic agents in *in vitro* and *in vivo* studies, particularly those applied to antitumor research (37, 39), has strongly suggested that antiangiogenic therapy will be a promising therapeutic modality in the future.

Because most proteins known to be antiangiogenic are structurally and compositionally similar to βpep peptides in that they are comprised primarily of antiparallel β-sheet structure and contain a high incidence of hydrophobic and cationic residues, for example, endostatin (40), PF4 (41), tumor necrosis factor (TNF) (42) and BPI (28), we initially tested all βpep peptides in our library in a tritiated-thymidine incorporation assay in EC and found that a few βpep peptides were quite effective at inihibiting endothelial cell (HUVEC) proliferation (23). In fact, βpep-25 (also referred to as anginex) was the most effective, more so than other known antiangiogenic proteins like PF4, endostatin, IP-10, as well as the fumagillin derivative TNP-470. Furthermore, although the growth inhibitory potency of βpep-25 was found to be similar against human (HUVEC and microvascular ECs), bovine and mouse EC, the peptide was most effective against human ECs, suggesting that there may be some species specificity in the sequence. This observation, however, requires additional investigation.

Since angiogenesis is a complex process, which aside from EC proliferation, depends on cell migration and differentiation, the effects of βpep peptides, primarily anginex, on angiogenesis were investigated in an *in vitro* collagen gel-based bFGF-stimulated sprout formation assay. The same βpep peptides that inhibit EC proliferation also inhibit sprout formation, with anginex, once again, demonstrating the greatest inhibition of angiogenesis (23). Similar results were observed when human breast cancer spheroids were used in the gel as the angiogenic stimulus. To study the inhibition of angiogenesis *in vivo*, the chick embryo chorioallantoic membrane (CAM) assay was used. This assay, which measures developmental angiogenesis, is routinely performed prior to the use of an agent in *in vivo* tumor growth models. In CAMs treated with anginex, a profound inhibition of microvessel formation was observed, whereas larger, preexisting vessels were apparently unaffected (43), providing further support that anginex is specific for angiogenically activated ECs. Tumor angiogenesis in the CAM model was mimicked by transplantation of fragments of B16F10 mouse melanoma onto the CAM (44). The melanoma tissue aggressively infiltrated into the CAM and efficiently induced angiogenesis. After 7 daily treatments with anginex, tumor-induced angiogenesis was inhibited.

The antiangiogenic potency of anginex suggests that this agent may be useful in the treatment of various pathological disorders, such as cancer, rheumatoid arthritis, endometriosis, atherosclerosis, psoriasis and ocular neovascularization.

Antitumor activity

Having established that anginex is potently antiangiogenic, we tested the peptide in several tumor growth models in small animals, primarily in athymic (nude) mice using human-derived carcinomas (ovarian, melanoma, glioma and colon).

Initially, anginex was tested in the nude mouse dorsal skin fold chamber model in which the angiogenic potential of human tumor biopsies can be measured and quantified *in vivo* (45). Figure 2 shows that at a dose of 70 μg of anginex/mouse/day (3-4 mg/kg/day), tumor angiogenesis and tumor growth were both strongly suppressed. Panel A (tumor in the control animal) shows that without treatment, the tumor is relatively large and highly vascularized. There is little tumor tissue present following a 10-day administration of anginex (panel B).

The effect of anginex on tumor growth was then studied in three tumor models in mice: (i) colon carcinoma LS174T in nude mice, (ii) ovarian carcinoma MA148 or SKOV-3 in nude mice (46, 47) and (iii) melanoma B16F10 in C57BL/6 black mice (44). In these mouse models, an optimal dose of 120-200 mcg of anginex/mouse/day (6-10 mg/kg/day) administered for 14 days or 28 days, inhibited tumor growth by about 70-80%. Initially, the effect on tumor growth was investigated in the colon carcinoma model wherein 6-10-week old nude mice received the LS174T tumor in the dorsal subcutaneous space. When tumors reached a size of about 50 mm^3, mice received for 14 days a daily loco-regional s.c. injection of anginex (1 mg/mouse/day), negative control peptide βpep-28 (1 mg/mouse/day) or PBS. When tumors in control

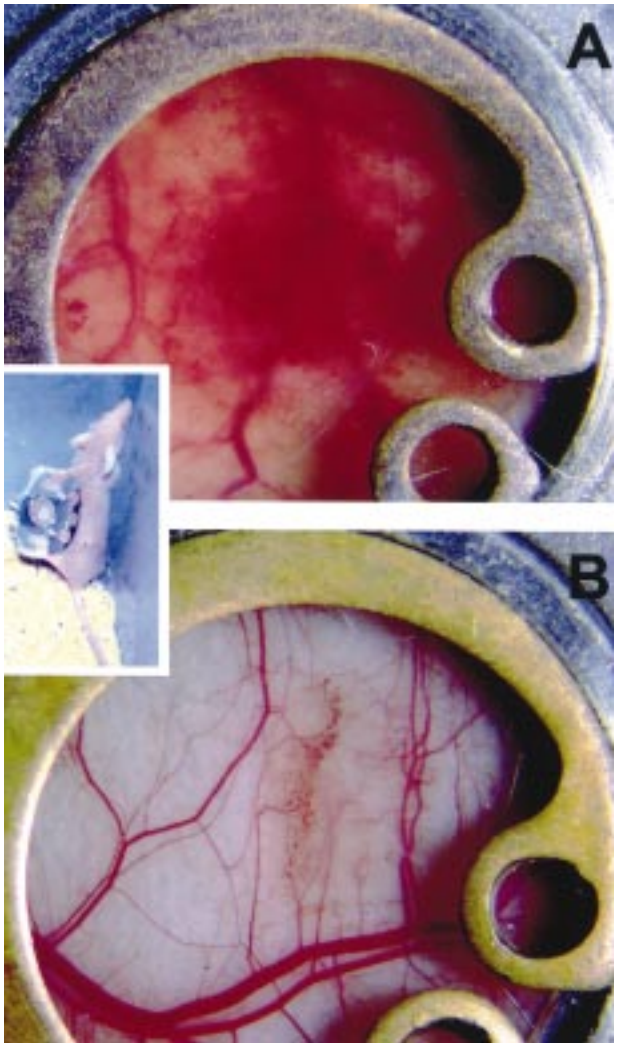

Fig. 2. Nude mouse dorsal skin fold chamber model. In this model, 5 x 10^5 packed cells of the human colon carcinoma cell line LS174T were inoculated in the dorsal skin fold chamber. Within 6 days, the cells had grown to a visible tumor mass and large tumors grew in the chamber after 14 days. In this model, the effect of anginex was tested by using subcutaneously placed osmotic minipumps that administered 70 μg of peptide/mouse/day (3-4 mg/kg/day) resulting in steady-state circulating levels of the peptide. Control mouse not treated with anginex (panel A), and mouse treated with anginex (panel B). Images showing the tumor and surrounding area within the dorsal skin fold chamber, have been enlarged 10-fold.

groups reached a size of 2,000 mm^3, animals were sacrificed. Treatment with anginex resulted in a 70% reduction of tumor volume. In addition, in tumors of control groups, ulcerations were observed, probably due to the rapid growth of the tumors. In anginex-treated mice, no ulcerations were observed, and in 40% of the animals, tumors were nonpalpable at the end of treatment and remained apparently dormant until treatment was halted. Renewed growth of tumor tissue was then observed. Administration of anginex by osmotic mini-pumps gave the best results, compared to loco-regional injections, once- or twice-daily subcutaneous injections or slow-release alginate bead strategies (46). At inhibiting tumor growth in the ovarian carcinoma model in mice, anginex was also found to act synergistically with another antiangiogenic protein, angiostatin (36), as well as with the chemotherapeutic agent carboplatin (47).

Anginex was also tested against xenograft tumors from glioma 9L cells grown in immunocompetent Fisher 344 rats (Low, Ni, Mayo, unpublished results). Figure 3 shows that the 14-day treatment with anginex (1 mg/rat/day) results in a significant reduction (about 80%) in tumor volume. During the posttreatment period, all control animals eventually showed ulcerated tumors and had to be sacrificed, whereas anginex-treated animals demonstrated only moderate tumor growth for about 1-2 weeks whereafter tumors regressed and within about 1 month were no longer palpable. Because inhibitors of angiogenesis block angiogenesis-mediated ICAM-1 and VCAM downregulation and induce the reexpression of ICAM-1 and VCAM following bFGF- and VEGF-induced downregulation (48), it may be that anginex can surmount the effect of tumor-released angiogenic factors that act to reduce leukoctye infiltration (49). Improved leukocyte infiltration into tumors may help to explain why in the long term, tumors in immunocompetent rats regressed.

1) Conjugation to a carrier protein improves bioavailability

Since anginex is a small peptide that may be cleared rapidly via filtration through the kidney, an attempt to

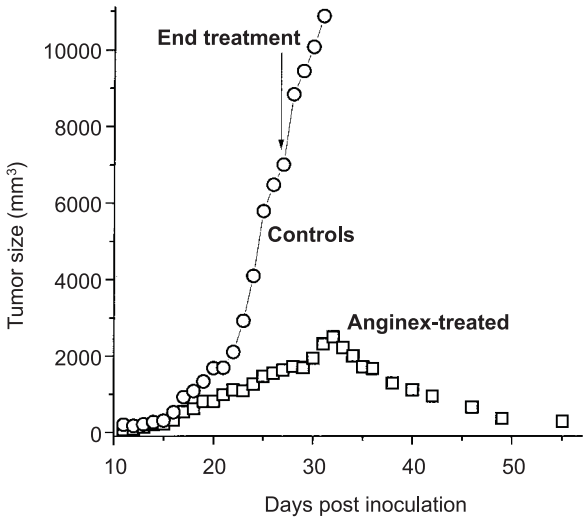

Fig. 3. Tumor growth curves for 9L glioma in rats. Anginex was tested against tumors from 9L glioma cells injected s.c. in the flank of immunocompetent Fisher 344 rats (5 per group). In this experiment, treatment with anginex (1 mg/mouse/day) was compared to treatment with βpep-28 (1 mg/mouse/day) and PBS alone. Growth curves are plotted as tumor volume (mm^3) *vs.* days after innoculation. 14 days of treatment was initiated on day 11 and halted on day 25.

improve bioavailability was made by conjugating anginex to human serum albumin (HSA-anginex, HSA:anginex molecule ratio of 1:5). *In vivo* activity of the conjugate in the MA148 xenograft tumor model was significantly improved over free anginex. Molar equivalent doses (free anginex at 2.5 mg/kg/day and the HSA-anginex conjugate at 41.5 mg/kg/day) demonstrated a significant improvement in efficacy with the conjugate (46). Moreover, tumors in animals treated with HSA-conjugated anginex remained significantly smaller (approximately 25% of controls) than those in control animals treated with free HSA for at least 30 days posttreatment when the animals were sacrificed.

2) Anginex is antiangiogenic in tumors

Immunohistochemical staining of cross-sections from any of these tumor tissues using fluorescently labeled anti-CD31 antibodies showed that the tumor inhibitory effect from anginex was the result of angiogenesis inhibition. This is exemplified with the study of ovarian carcinoma tissue (Fig. 4). In anginex-treated mice, both microvessel density and vessel length in tumors were decreased by at least 50% relative to controls. Together with an increase in the number of vessel endpoints, this is indicative of a change in vessel architecture in tumors treated with anginex.

3) Anginex is specific for angiogenically activated tumor ECs

Mechanistically, anginex functions by preventing ECs from attaching to extracellular matrix components (anoikis), thereby inducing apoptosis (43). Anginex accomplishes this by inducing downregulation of adhesion receptors on ECs, rather than by directly blocking interactions between matrix and EC adhesion molecules. This was confirmed by several observations (43). First, anginex acts to downregulate EC adhesion molecules $\alpha_2\beta_1$-, $\alpha_5\beta_1$- and $\alpha_v\beta_3$-integrins and CD44. Second, the caspase inhibitor z-VAD.FMK that completely blocks the process of apoptosis, does not prevent the detachment and anoikis of ECs. And finally, the effect of anginex on migration occurs earlier than the effect on apoptosis and at concentrations that are not inhibitory for the proliferation of ECs. In this regard, anginex functions cytotoxically against ECs, as opposed to agents like PF4, which are cytostatic and arrest ECs in the S-phase (50). In addition, the activity of anginex is specific for angiogeneically activated ECs for several reasons: confluent ECs in culture and other cell types like foreskin and endometrium-derived fibroblasts, smooth muscle cells, PHA-stimulated peripheral blood leukocytes and various neoplastic cells are unaffected by the presence of the peptide, and a double thymidine block experiment showed that ECs arrested in the S-phase of their cell cycle (resting ECs) are 50% less likely to become apoptotic in the presence of the peptide. The fact that anginex is specific for angiogenically activated ECs implies a role for endothelial (cell surface) adhesion molecules (EAMs), possibly integrins, that are upregulated during EC proliferation. Electron microscopic evidence demonstrates that the peptide is present on the matrix binding ("abluminal") side of ECs after 24 h of culture.

Intravenous injection of Oregon Green-labeled anginex in B16F10 tumor-bearing mice revealed a serum half-life of anginex of approximately 50 min. After 1 h, the Oregon Green-labeled anginex appeared in the urine and

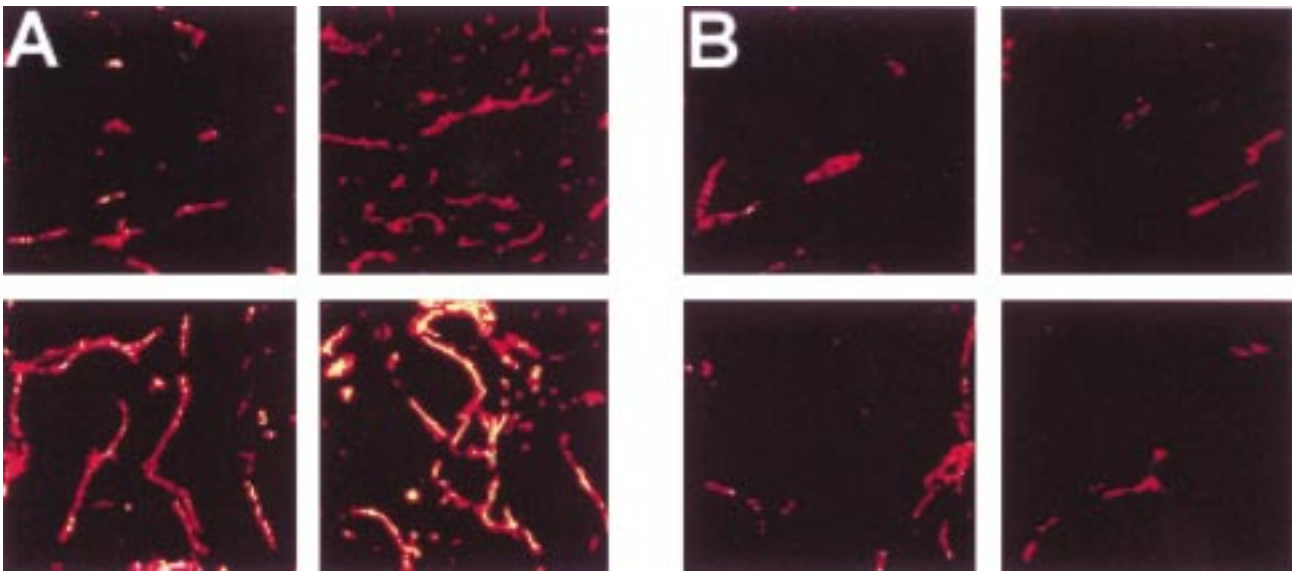

Fig. 4. Cross-sections (5 μM) of the tumor tissue which were treated with fluorescently labeled anti-CD31 (specific for endothelial cells). Tumors were taken from control mice (left 4 panels) and from anginex-treated mice (right 4 panels).

after 4 h so much Oregon Green-labeled anginex was sequestered in the tumor that fluorescence was visible through the skin. In tumors of mice sacrificed 4 h postinjection, Oregon Green-labeled anginex clearly identified the tumor blood vessels (using PE-labeled anti-CD31 antibody), but not the vessels of the hind limb muscle. In the kidney, residual Oregon Green-labeled anginex was present in the tubuli, while blood vessels in the glomeruli, as well as the peritubular blood vessels, were completely devoid of anginex fluorescence. Pharmacokinetic calculations suggest that the serum level of continuously delivered anginex is approximately 2-3 μg/ml (1 μM), which is the concentration where we start seeing biological effects in *in vitro* models. The localization of anginex to tumor blood vessels may, however, lead to higher drug levels in the local microenvironment within the tumor.

These results indicate that anginex may also be an excellent diagnostic tool for identifying newly forming or micro tumors and for homing/targeting of other agents (chemotherapeutics or radiotherapeutics) directly to the site of the tumor. The combination of targeting tumor blood vessels and having an angiostatic effect combined in one agent may give anginex preferable features for tumor treatment.

4) Anginex is nontoxic in animals

A crucial question in the development of therapeutic drugs is the potential for side effects. In all animal studies, *in vivo* toxicity of anginex was assessed by observing animal behavior, determining body weight, measuring hematocrit and creatinine blood levels, and checking macroscopic and microscopic morphology of internal organs upon autopsy at the end of each study (44, 46, 47). By any of these criteria, anginex appears to be nontoxic. Treated and untreated mice behaved and ate normally. Body weights of treated and untreated mice were essentially the same among any of the groups studied. In addition, prior to the start of treatment, 10 days after the initiation of treatment and the last day of treatment, blood was withdrawn and hematocrit and creatinine levels were determined as an indirect measurement for bone marrow and kidney toxicity. Levels in treated and untreated animals were essentially the same. At the termination of all studies, macroscopic and microscopic morphology of internal organs appeared to be the same among all groups of animals. In addition, no effect from anginex on natural wound healing was observed when minipumps were implanted surgically; wounds healed normally within 4 days even in the continued presence of the peptide.

5) β-Sheet is the bioactive conformation of anginex

At concentrations in the micromolar range, βpep peptides are mostly dissociated into monomers, being present in solution as a broad, mostly "random coil", distribution of conformations that includes some β-sheet (24). Therefore, at the micromolar concentrations used in *in vitro* bioassays that showed anginex to inhibit EC proliferation and to be antiangiogenic, anginex was mostly random coil monomer. Presumably, the bioactive structure of anginex is selected from this conformational distribution and "locked in" upon binding to its as yet unknown receptor on the surface of the ECs. To test the hypothesis that β-sheet is the bioactive conformation of anginex, a series of double cysteine-containing analogs were synthesized and oxidized to form disulfide bonds that maintain the same strand alignment as in the NMR-derived structure of βpep-4 (21). These covalent constraints limit conformational space available to the peptide, such that, β-sheet formation is more highly favored and, for example, helix formation could not occur. Disulfide-bridged analogs were analyzed in three functional assays: endothelial cell proliferation, apoptosis, collagen gel sprouting. Results from all these assays were comparable in that so long as placement of disulfide bonds preserved the β-strand alignment as in the proposed bioactive conformation, activity was preserved. Disulfide-bridged control peptides that shifted the proposed β-strand alignment were much less active. Therefore, the bioactive conformation of anginex is antiparallel β-sheet as found in βpep-4.

Anticoagulant activity

For several reasons, it was hypothesized that βpep peptides may help to modulate the blood clotting cascade via interactions with thrombomodulin. First of all, although no specific cell surface receptor(s) for βpep peptides are known, it is known that these peptides interact specifically with ECs, which actively participate to maintain homeostasis, in part by expressing the transmembrane protein thrombomodulin. Moreover, it is known that PF4 interacts with thrombomodulin to promote thrombin cleavage of protein C and generate activated protein C, a potent natural anticoagulant. Lastly, βpep peptides like PF4 are highly cationic and PF4 is known to modulate the activity and specificity of thrombomodulin-bound thrombin and increase the production of activated protein C (51).

Figure 5 compares the ability of PF4 and βpep-8 to produce activated protein C *in vitro*. While in this assay PF4 exhibits maximal activity at about 100 μg/ml (12.5 μM), βpep-8 stimulates the same maximal activity, but at about 25-fold lower concentration, *i.e.*, 2 μg/ml (0.5 μM) (Slungaard and Mayo, unpublished results). These results suggest that βpep-8 may make for a potent modulator of activated protein C and should be developed as such.

Designing mimetics of anginex

Having demonstrated various bioactivities with various βpep and βpep-derived peptides, one of our next goals is to design mimetics or partial mimetics. We have

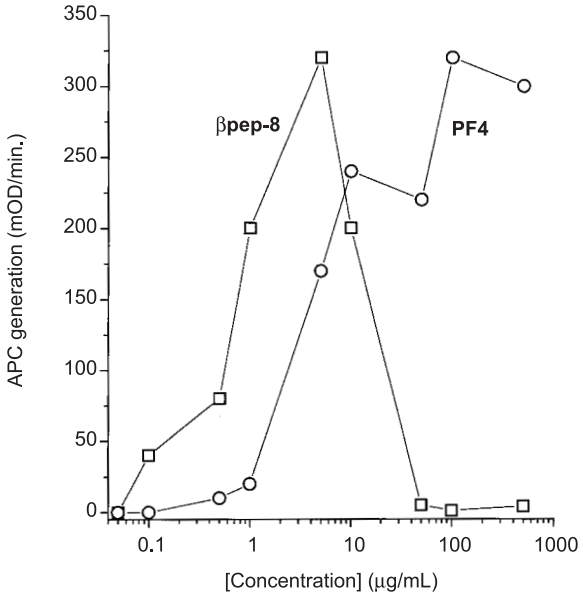

Fig. 5. βpep-8 and PF4 influence on thrombomodulin (TM) protein C cofactor activity. 0.5 nM rabbit TM was suspended in microplates containing 500 nM protein C in 50 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, pH 7.4. βpep-8 and PF4 at the indicated final concentration were added and the mixture incubated at 37 °C for 10 min. 2 nM thrombin was added, and APC (activated protein C) generation was allowed to proceed for 10 min. The reaction was quenched by the addition of 30 μM I-2581 and 1 mM EDTA. APC was quantified spectrophotometrically by assaying cleavage of the chromogenic substrate S-2366 at 405 nm on a V_{max} microtiter plate reader (Molecular Devices Corp., Menlo Park, CA). Results are expressed as ng of APC/min.

used NMR spectroscopy to derive conformational information on both βpep-4 (21) and anginex (43), as well as on the βpep-25-derived antibacterial dodecapeptide SC-4 (30). In addition, we have investigated structure-activity relationships (SARs) in these peptides. Identification of specific amino acid residues and their spatial relationships, which are crucial to promoting bioactivity at the level of molecular interactions, is one of the main goals among structural biologists and pharmaceutical chemists working to develop new drugs. Nevertheless, in antiangiogenic proteins, for example, SARs are sorely needed, and even analyses of high-resolution molecular structures of a number of antiangiogenic proteins, *e.g.*, endostatin (40), PF4 (41) and BPI (28), have not provided this information.

Working with a small peptide like anginex, however, allows more rapid assessment of SARs. By comparing highly homologous βpep sequences in terms of their relative ability to inhibit EC proliferation and promote apoptosis, insight into which amino acid residues are required for activity has been gleaned, and there appear to be only about seven residues that are crucial for antiangiogenic activity: five hydrophobic residues and two lysines (23). Knowing that an antiparallel β-sheet in a particular β-strand alignment is the bioactive conformation, it is interesting to note that all five hydrophobic residues are proximal and are conformed on the hydrophobic face of an amphipathic β-sheet, and the two lysines are positioned on the opposite amphiphilic face, lying diagonally across from one another on β-strands 1 and 2. With the antibacterial dodecapeptide SC-4, SAR information has shown that the peptide forms an amphipathic helix, on one side of which are conformed four positively charged residues, while on the other side are conformed several hydrophobic aliphatic residues (30). We are currently investigating the use of various organic scaffolds off of which can be attached similar functional groups as side chains of those key amino acid residues, that can achieve essentially the same spatial orientation as in parent anginex and in SC-4.

Summary and future directions

Here, we have reviewed work and presented some new results on novel designed βpep peptides. βpep-25 (anginex) is a potent antiangiogenic and antitumor agent. This cytotoxic peptide is specific for angiogenically activated ECs, which forms the basis for its antitumor activity, making it a potentially good therapeutic anticancer agent. Additionally, due to its antiangiogenic activity, anginex also has the potential of becoming a potent therapeutic agent against other pathologic disorders such as arthritis, restenosis, endometriosis and diabetic retinopathy. A βpep peptide-derived 12mer, SC-4, was discovered to be a potent bactericidal agent. Overall, the design of novel peptides is a powerful tool in the development of biologically active molecules.

In the future, the β-sheet scaffold construct will allow other βpep peptides to be designed with other specific functions. To accomplish this in other normal or pathologic disorders, one must first have an understanding of the spatial relationships among residues that will promote the desired activity. Sometimes, analysis of the protein or peptide X-ray or NMR structure alone is sufficient to understand which amino acid residues promote a particular function, but most often this is not the case, and SARs must be derived, usually by substituting residues in the native sequence (for example, site-directed mutagenesis or alanine scanning) and assessing effects on structure and activity. Once a novel peptide is made, tested and found to have some activity, bioactivities can be optimized in a number of ways, *e.g.*, phage display techniques, combinatorial chemistry and/or synthetic peptide libraries, all of which can significantly increase the molecular diversity of these peptides and peptidomimetics. Designing for function is an area of peptide chemistry that is advancing rapidly in the biotechnology field.

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