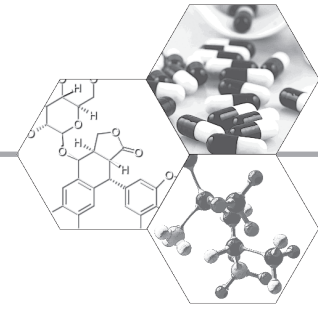

Flexible or fixed: a comparative review of linear and cyclic cancer-targeting peptides

Peptides can serve as versatile cancer-targeting ligands and have been used for clinically relevant applications such as cancer imaging and therapy. A current and long-standing focus within peptide research is the creation of structurally constrained peptides generated through cyclization. Cyclization is envisioned to enhance the selective binding, uptake, potency and stability of linear precursors. This review compares closely related linear and cyclic peptides in these respects. Peptide cyclization generally improves the selective binding and stability of linear precursors; however, not all cyclization strategies and constrained geometries enhance these properties to the same extent. In some instances, linear analogues actually have better cancer-targeting properties compared with their cyclic counterparts. Although cyclization does not necessarily improve the cancer-targeting properties of linear analogues, cyclic peptides may obtain properties that allow them to be used for additional applications. This review aims to convey the advantages and limitations of cyclic cancer-targeting peptides.

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Peptides are useful receptor-binding ligands; several other classes of targeting ligands sharing this binding property include small molecules, endogenous proteins, antibodies and affibodies. Peptides can be synthesized with relative ease using well-established solid-state Fmoc and Boc synthetic techniques. Compared with proteins and antibodies, this ease of synthesis makes peptides relatively inexpensive to produce and modify for research and commercial purposes. Targeting peptides can be conjugated to small-molecule drugs, imaging contrasts and nanoparticles for a plethora of applications. The geometry of linear peptides can also be modified using a wide variety of traditional and modern chemistries. The type of geometric modification that is currently at the core of peptide research is the creation of cyclic peptides.

As new biomarkers have been, and are continuing to become, identified, peptides have been chosen for targeting these biomarkers. Efficient peptide ligands are discovered by screening random peptide libraries and also by using computer-aided modeling techniques. Optimized peptide sequences have been cyclized for several key reasons. First, it is envisioned that by gaining a fixed geometry, cyclic peptides could bind more efficiently to their respective receptors. If these receptors exist as several subtypes; it is also hoped that the fixed geometry of cyclic peptides will make these constrained sequences selective to particular receptor subtypes compared with their linear analogues. In addition, cyclic peptides are often chosen over their linear analogues due to their enhanced enzymatic stability. Several studies have identified the properties of novel cyclic peptides; however, no review has yet summarized the properties of closely related linear and cyclic peptides. This review will present examples where linear peptides have been compared with their direct cyclic analogues or optimized cyclic derivatives for cancer imaging or cancer therapy. These comparisons will explore the difference between these linear and cyclic peptides in terms of their geometry, binding affinity, biological activity, receptor selectivity and stability. This review will also show that cyclic peptides can be used in ways that linear peptides cannot. Although cyclic peptides often show improvements in some respect(s) compared with their linear precursors, peptide cyclization is not a *panacea*, therefore, we will highlight instances where cyclic analogues did not perform as favorably as their linear counterparts. The goal of this comparative review is to aid current peptide research by conveying the advantages, limitations and unique properties of cyclic cancer-targeting peptides.

Geometry of linear & cyclic peptides

Linear peptides that contain 2–10 amino acid residues are especially flexible in solution. Once the length of linear peptides extends to between 10 and 20 amino acid residues, random linear peptide sequences can begin to obtain secondary structures, including α-helices, turns and β-strands [1]. Computer-aided optimization studies can lead to designing

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Key Terms

VEGF: Overexpression of VEGF proteins promotes vasculogenesis and angiogenesis that can support the growth, survival, motility and differentiation of cancer cells. Pharmaceuticals that inhibit VEGF activation can be used as anticancer agents.

One-bead–one-compound: Solid-state method of creating large peptide libraries where each resin bead contains one particular peptide sequence. This usually involves the 'split and mix' method where separate batches are mixed after a different residue is attached to each separate batch. This mix of beads is then split again so that the next residue is attached to each batch. For example, beads that are conjugated to four residues over 'n' split and mix cycles will produce a library of '4^n' different peptide sequences.

Tyrosine kinase inhibition: Pharmaceuticals that inhibit kinases from phosphorylating various proteins at the tyrosine residue. This inhibition is meant to reduce downstream signal transduction cascades in order to treat tumors.

Integrin: Receptor that regulates cell shape, motility and cell cycle. Also mediates the attachment of cells to other cells or to the extracellular matrix. Particular integrin subtypes are overexpressed by tumor cells and tumor neovasculature.

Phage display: Method of generating large libraries of random peptide sequences. Random DNA that encodes for the peptides of interest is ligated within bacteriophages. These phages express these peptides at their outer protein coat. Panning studies attempt to bind these random phages to targets of interest in order to discover novel targeting peptides.

linear peptides that contain such secondary structures; for example, D'Andrea *et al.* have designed the linear 15-amino acid peptide, QK-Ac-KLTWQELYQLKYKGI-NH_2, which mimicked the α-helix **VEGF**$_{17-25}$ and was more biologically active than the unstructured isolated sequence of VEGF$_{17-25}$ (VEGF$_{15}$, Ac-KVKFMDVYQRSYCHP-NH_2) [2]. These secondary structures impose constraints that reduce the free energy of linear peptides and limit their conformations to those that may be more biologically active.

In lieu of indepth computer modeling, the geometry of short peptide sequences can be easily constrained by cyclization. Several groups have reviewed the geometries that result from peptide cyclization [3–8]. The constraints imposed by cyclization will force cyclic peptides to adopt a limited number of molecular conformations in solution. When peptides are to be used as ligands for targeting disease biomarkers, only a limited number of conformations will allow the ligand to bind efficiently. While refining a Monte Carlo simulation method for improving ligand–receptor docking, Deem and Bader noted that if the conformations of cyclic peptides are limited, the time spent in each conformation in solution is proportional to the inverse exponential of the free energy of the conformation [9]. Cyclic peptides may continue to lose entropy upon interacting with a target [1]; the result is a compounded reduction of the free energy of cyclic peptides when they are used as targeting ligands. Generally, if cyclization limits conformations to those required for optimum receptor binding, these cyclic peptides would be more useful compared with their linear counterparts that can adopt more conformations, which are not useful for receptor binding.

Cyclization has been shown to increase the propensity for β-turn formation in peptides; the formation of this secondary structure is important since β-turns are often found in native proteins [10,11]. Lau *et al.* generated a cyclic peptide library by the **one-bead–one-compound** technique and discovered several disulfide-cyclized cNGXGXXc (X = interchangeable residues) peptides that bound to the non-small cell lung cancer biomarker α_3 β_1 in A549 cells [12]. 3D modeling of the best hit within the library, cNGQGEQc, suggested that the D-cysteine to L-glutamine motif adopted a β-turn that was responsible for the improved binding affinity. β-turns naturally exist in nine specific conformations, which include types I, I′, II, II′, IV, VIa1, VIa2, VIb and VIII. Although cyclization can support the formation of these reverse turns, it is difficult to create the exact types of β-turn that will match those of native proteins. This is an ongoing avenue of cyclic peptide research that is being refined. Several groups have summarized the various synthetic methods used to generate cyclic peptides [13–16]. It is possible that particular synthetic methods can support the formation of specific geometries.

Although peptide cyclization generally induces structural constraints, the site of cyclization within the sequence can affect the binding affinity of cyclic peptides. Kumar *et al.* compared 20 side chain modified linear peptides and 11 cyclic analogues to the original linear sequence Ac-CIYKYY for Src **tyrosine kinase inhibition** [17]. The cyclic derivatives included the original CIYKYY sequence but were cyclized by either head-to-tail, N- or C-termini-to-side chain or sidechain-to-sidechain coupling strategies. This study found that the head-to-tail cyclized peptide had an inhibitory potency that was 62.5-fold higher (IC_{50} = 6.4 μM) compared with the original linear peptide (IC_{50} = 400 μM). This cyclic peptide was also more potent than the seven other cyclic peptides with the same sequence, which were cyclized by the different aforementioned chemistries (IC_{50} = from 16 to >400 μM). Two other cyclic peptides with the same sequence had low to submicromolar IC_{50} values (1.9 μM and 0.28 μM) due to their constrained geometry attained by cyclization through the sidechains of residues 3 (tyrosine) and 4 (lysine) by extended linkers. This study illustrates that peptide cyclization can improve the biological activity of linear analogues, but that the position of cyclization will constrain particular motifs and modulate the biological activity of the active motif.

Peptides containing the same residues, which are cyclized by the same chemistry but at different positions, can create alternate geometries that elicit different affinities to targeted biomarkers. Assa-Munt *et al.* created two synthetic derivatives of the native RGD-containing peptide sequence, ACDCRGDCFCG (RGD-4C) [18]. These were cyclized by oxidizing two different pairs of cysteine residues to create two isoforms labeled RGD-A (C^1–C^4 and C^2–C^3) and RGD-B (C^1–C^3 and C^2–C^4). Competitive binding studies found that the RGD-A isoform bound to $\alpha_v\beta_3$ (K_d ~100 nM) and $\alpha_v\beta_5$ (K_d ~100 nM) **integrin** receptors while the binding of RGD-B was approximately tenfold less

efficient for these receptors. Modeling analysis showed that RGD-A contained a hydrophobic pocket and type I β-turn while the RGD-B isoform contained type II′ β-turn **(FIGURE 1)**. It was not clear which structural components were responsible, but results showed that the geometry of the RGD-A isoform allowed for better integrin receptor binding.

The type of chosen cyclization chemistry and even the orientation of the cyclizing amide bond can affect the resulting biological activity of cyclic peptide analogues. García-Aranda *et al.* attempted to mimic the β-hairpin fragment of the $VEGF_{81-91}$ protein by creating cyclic peptides that were constrained by either disulfide or amide cyclization [19]. They found that the cyclic peptide Ac-M-c(CH_2-NH-CO-CH_2)^{2,10}GIKPHQGQGI-NH_2 had a twofold lower IC_{50} (87.6 μM) for displacing the $VEGF_{165}$ protein from the D1–D3 domains of VEGFR-1 compared with the linear H-MRIKPHQGQHI-OH peptide, which had a similar sequence. At 100 μM concentrations, this cyclic peptide displayed the best inhibition of biotinylated $VEGF_{165}$ (53%) from the whole extracellular domain compared with both the derivative with a reversed amide components (16%) and the derivative that was disulfide-cyclized (11%) but contained the same sequences. Although biological activity was improved, molecular modeling and 2D NMR studies showed that the whole native β-hairpin structure was not stabilized. However, amide cyclization favored the formation of a β-turn mimic of the $VEGF_{81-91}$ β-hairpin. These constrained structures were likely responsible for improving the binding affinity of the amide-cyclized sequences compared with the original linear sequence.

Library screenings & structure–activity studies

Novel cancer-targeting peptide ligands can be identified by screening libraries generated by **phage display** or combinatorial methods. Several groups have reviewed the topic of peptide library generation [20–23]. Some studies generated and screened libraries of linear sequences, while others created cyclic analogues of the most promising linear peptides in order to study the effect that cyclization had on peptide binding activities **(FIGURE 2)**. Bonetto *et al.* generated a library of peptides using phage display to discover novel ligands that mimicked the functional epitope of the crystallizable fragment of immunoglobulin G (IgG1-Fc) for targeting the Fc-γ receptor 1 (FcγRI) [24]. The FcγRI target has several complex biological roles and a potent targeting ligand was proposed to have therapeutic properties against cancer and inflammatory disorders. After phage screening, they identified the conserved sequence, TXXCXXΘPXLLGCΦXE (Θ = hydrophobic residues; Φ= acidic residues; X = any interchangeable residue), and found that two of the 27 selected sequences were linear. When these two linear peptides were compared with 15 of the most promising cyclic sequences at 50 μM peptide concentrations, they found that most of the cyclic sequences were able to inhibit the binding of IgG1 to FcγRI better than the linear peptides. The remaining cyclic peptides that had a similar binding activity to FcγRI as the linear peptides were originally found in low (<3%) frequencies during round three of the phage biopanning studies. Since there were no cyclic peptides that contained the same sequence as either of the two linear peptides, it cannot be concluded that cyclization was solely responsible for improving binding to FcγRI. However, since

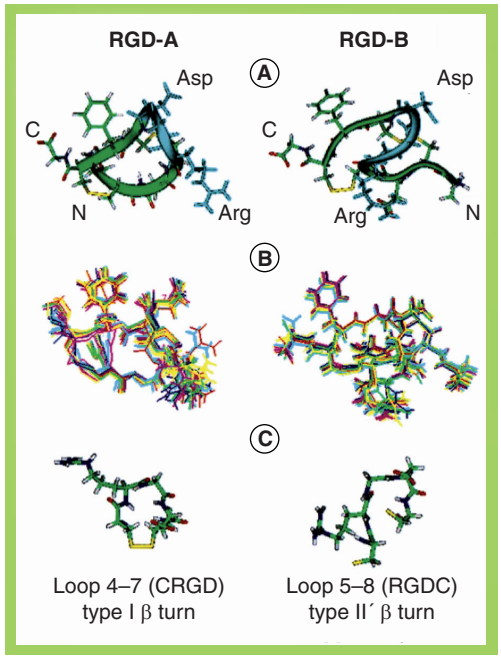

Figure 1. The complete and partial structures of RGD-A and RGD-B.
(A) Ribbon representations of the NMR structures of the RGD-A and RGD-B peptides. **(B)** The superimposed solution structures of 19 structures of RGD-A and RGD-B. **(C)** The NMR structures of the distorted type I β-turn for RGD-A (C_2RGD segment) and the type II′ β-turn for RGD-B ($RGDC_3$ segment). Reproduced with permission from [18] © American Chemical Society (2001).

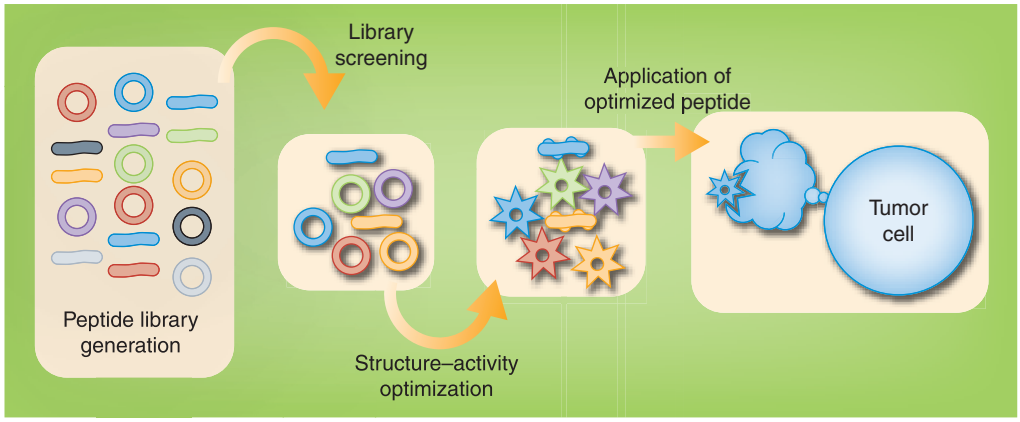

Figure 2. Typical strategies involved in creating a novel cyclic peptide with a high binding affinity to cancer biomarker receptors.

there were approximately five-times more cyclic peptides discovered containing the aforementioned conserved sequence, it is possible that these linear peptides would target FcγRI more efficiently if each were cyclized.

Phage display was used by Hsiao *et al.* to identify promising ligands with high binding affinities to the $\alpha_v\beta_6$ integrin receptor on human oral squamous cell carcinoma and, by extension, to major head and neck squamous cell carcinomas [25]. This cyclic peptide phage display library generated 7-amino acid binding sequences with N- and C-terminal cysteine residues that allowed disulfide cyclization. Biopanning found that the best hit included the sequence CRGDLASLC (cRGD). Among their comparative studies, the cyclic cRGD peptide was compared with the cyclic CRGELASLC peptide. The cRGD peptide reduced the number of HSC-3 cells to 41% compared with the PBS control sample at 300 µM concentrations, while the cyclic CRGELASLC had a similar activity at 300 µM concentrations as the cRGD peptide had at 100 µM concentrations. In a separate experiment, neither the linear RGDLASL peptide (RGD) nor the linear RGELASL peptide (RGE) could reduce the number of HSC-3 cells compared with the PBS control, even at 1 mM concentrations. These comparisons showed that the $\alpha_v\beta_6$ binding affinity of the RGDLASL sequence was improved by disulfide cyclization. It was further shown that even the RGE-containing disulfide-cyclized peptide could attain $\alpha_v\beta_6$ binding activity due to cyclization.

The phage display method was also recently used by Duncan *et al.* to discover novel ligands for targeting the phosphorylation-dependent peptidylprolyl isomerase, Pin 1 [26]. Since Pin 1 overexpression has been observed in cervical, colon, lung and oral cancers, a potent Pin 1-targeting ligand would have the potential for diagnosing and treating these cancers. A library was generated that contained 7-amino acid binding sequences that were flanked by cysteine residues to allowed disulfide cyclization. The most promising sequence within this library contained the residues CRYPEVEIC. They compared the binding of the cyclic and reduced linear analogue of CRYPEVEIC by inhibiting Pin 1 phosphorylation-dependent PPIase activity at various concentrations of the substrate peptide *cis*-Suc-AEPF-*p*NA (Suc = 3-carboxypropanoyl). They found that the cyclic analogue had a 85-fold higher receptor binding affinity (K_i = 0.52 µM) compared with the linear peptide. The geometry induced by disulfide cyclization was required for this activity since amide cyclization, afforded by replacing the N- and C-terminal cysteines with lysine and glutamic acid (respectively), did not inhibit Pin 1 PPIase activity. These results suggest that disulfide cyclization was essential for improving the binding of the CRYPEVEIC sequence to Pin 1 and that this sequence can be pursued for cancer therapy.

The binding activity of cyclic cancer-targeting peptides can be optimized using structure–activity studies. Pomilio *et al.* have compiled an extensive review on this topic [27]. One such structure–activity study investigated the effect that cyclization had on the binding of the modified cyclin recognition motif, R-Xaa-L-Yaa-Zaa (Xaa = diamino residues; Yaa and Zaa = hydrophobic residues), to the cyclin binding groove. Andrews *et al.* compared linear and cyclic peptides for the inhibition of cyclin A- and E-associated CDK2 activities to discover

new ligands that could be useful for inducing apoptosis in tumor cells [28]. They synthesized seven cyclic peptides containing the Xaa-LFG sequence that were cyclized by tail-to-side chain coupling by the C-terminal glycine and Xaa (lysine, ornithine [Orn], α,γ-diaminobutyric acid [A_2bu] or 2,3-diaminopropanoic acid [A_2pr]) and contained 1–5 residues towards the N-terminus beside Xaa. The binding affinities of these peptides were compared with four linear peptides using a competitive cyclin A binding assay. Results showed that the cyclic peptide Ac-AA-Abu-R(KLFG) (Abu = 2-aminobutyric acid) had the lowest IC_{50} (0.63 μM), which was 94-fold lower compared with a similar linear analogue with the sequence Ac-AA-Abu-RNLFG-NH_2. This suggested that the cyclization and replacement of asparagine with lysine were key factors that improved binding.

Receptor subtype specificity of linear & cyclic peptides

The cyclization of linear peptide sequences can create constrained geometries that can alter the specificity of cyclic peptides to different isoforms or subtypes of targeted receptors **(FIGURE 3)**. The linear RGD-motif can bind to several different integrin subtypes (generally with IC_{50} values >100 nM) and, therefore, binds to integrins with little specificity [29]. However, the cyclization of RGD peptides can create derivatives that are specific to particular integrin receptors. Pfaff *et al.* conducted a structure–activity study where they compared the binding affinity of 16 cyclic RGD peptides to the linear GRGDS and RGDFv peptides with respect to several different integrin receptors [30]. Of all of those compared, the cyclic RGDFv had a 3.3-fold greater binding affinity (IC_{50} = 11.3 μM) to soluble $\alpha_v\beta_3$ and a 5.8-fold greater binding affinity (IC_{50} = 0.4 μM) to immobilized $\alpha_v\beta_3$ compared with the linear RGDFv peptide. Interestingly, the linear RGDFv showed a 1.3-fold and 1.5-fold better binding affinity to the soluble and immobilized $\alpha_{IIb}\beta_3$ integrin, respectively, compared with the cyclic RGDFv. This linear RGD also had a 4.3-fold greater binding affinity to immobilized $\alpha_5\beta_1$ compared with the cyclic sequence. Although cyclization did not improve the binding affinity of RGD sequences to all integrin receptors, this study showed that cyclization created a fixed geometry that made the RGDFv sequence more specific to $\alpha_v\beta_3$ compared with its linear counterpart.

A thorough structure–activity study by Haubner *et al.* compared the specificity of 27 cyclic RGD-containing pentapeptides to the linear GRGDSPK standard, in terms of binding affinity to $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ [31]. They found that almost every tested cyclic RGDxV, RGDfX, RGDXv and RGDFx (X = interchangeable L-amino acid and x = interchangeable D-amino acid) derivative bound with a better binding affinity to $\alpha_v\beta_3$ compared with the linear standard. However, the affinities of these cyclic peptides were similar to the linear reference in terms of $\alpha_{IIb}\beta_3$ integrin binding. Interestingly, Bach *et al.* suggested that if particular cyclic RGD peptides can be tuned to contain a type I instead of a type II′ β-turn, the resulting constrained cyclic RGD peptides can allow preferential binding to $\alpha_v\beta_3$ compared with the $\alpha_{IIb}\beta_3$ integrin [32]. This is not the case for all RGD-containing sequences. Wermuth *et al.* synthesized 18 derivatives of the cyclic RGDFV sequences, which included five parent sequences and their respective inverso, retro and retro-inverso isomers [33]. When the binding affinities of each peptide were compared with the linear reference GRGDSPK for the $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins, they found that the cyclic RGDFv (parent), RGDfV (parent) and vfdGR (retro–inversion) peptides had 40- to 250-fold greater binding affinities to $\alpha_v\beta_3$, but did not show any improvements for $\alpha_{IIb}\beta_3$ binding. Structural analyses continued to show that the aforementioned cyclic peptides contained a type II′ β-turn. In addition, the retro–inverso isomers of RGDFv and RGDfV contained type II β-turns and had a fivefold and 3.5-fold lower binding affinity (respectively) to $\alpha_v\beta_3$ compared with the linear standard. This suggests that the RGDFV-peptides preferentially bound to $\alpha_v\beta_3$ integrins and that this may have been partially due to the presence of a type II′ β-turn. It is

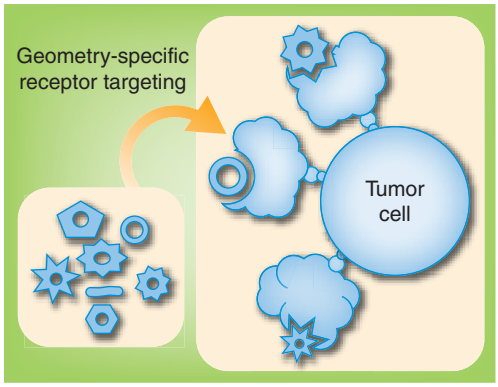

Figure 3. Peptides can specifically bind to different receptor subtypes due to particular constrained peptide geometries.

possible that constraining the RGD-motif to other types of β-turns can further modulate its specificity to other integrin subtypes.

Stability of linear & cyclic peptides

Short linear peptides exist in fast equilibrium of interchanging conformations while in solution. Of these rapidly interchanging conformations, only few will attain an orientation that has receptor selectivity. Although some conformations will preferentially interact with desired receptors, particular conformers can also fit into active sites of proteolytic enzymes. Peptides can be cyclized in order to reduce the overall numbers of interchanging conformers in the hope of limiting them to those selective for desired receptors while avoiding degradation by not forming conformers susceptible to interacting with proteolytic enzymes **(Figure 4)** [34]. Although the theory is intuitive, achieving both goals is not at all trivial. In general, however, cyclization often increases the stability of peptides, which can prolong their biological activity. This prolonged activity may even be the result of additional resistance to enzymatic degradation by exoproteases that preferentially cleave near the N- or C-termini of peptide sequences [1].

A combination of computer modeling and experimental evaluations can deduce important conclusions about cyclic peptide conformations and their effects on stability compared with linear derivatives. A study by Bogdanowich-Knipp *et al.* modeled the structures of cyclic and linear RGD peptides with the sequence cyclo-(1,6)-Ac-CRGDF-Pen-NH_2 (Pen = 8,8-dimethylcysteine) and H_2N-RGDF-OH, respectively [35]. Their modeling suggested that a salt bridge at the guanidinium of arginine and the carboxyl of aspartic acid within the disulfide-cyclized RGD could stabilize the labile aspartic acid and reduce degradation of the cyclic peptide compared with the linear RGD. A second study by Bogdanowich-Knipp *et al.* tested these models experimentally [36]. The cyclo-(1,6)-Ac-CRGDF-Pen-NH_2 peptide was synthesized and the solution stability of this sequence was compared with the linear H_2N-RGDF-OH peptide while in solutions ranging from pH 2 to 12. They found that the cyclic RGD peptide was 30-fold more stable at pH 7 compared with linear RGD, and was also generally more stable from pH 3 to 7. Once the pH ranged from 8 to 12, the disulfide bond likely became reduced and facilitated in the higher degradation rate of the cyclic RGD compared with the linear peptide. This experimental evidence supported the suggestion that the disulfide bond constrained the cyclic RGD analogue and could stabilize the labile aspartic acid residue from degradation at biological pH ranges.

Not all cyclization chemistries will provide peptide sequences with the same degree of stability. Somatostatin-14 (SRIF) is an endogenous disulfide-cyclized peptide with the sequence AG(CKNFFWKTFTSC) that plays a role in the regulation of several hormones, notably insulin, glucagon and growth hormone [37]. There are comparative studies that investigated the hormone-regulating ability of closely related linear and cyclic SRIF derivatives, particularly by Diel *et al.* and Vécsei *et al.* Surprisingly, Diel *et al.* found that the linear SRIF analogue was better able to protect histamine-containing gastric mucosal mast cells from ethanol-induced erosion in rats [38]. Interestingly, Vécsei *et al.* also found that the linear SRIF derivative was more able to treat electroshock-induced amnesia in rats [39]. It is important to note that these and several other early investigations of SRIF in the 1970s and 1980s significantly contributed to the advancement of cyclic peptides for medicinal applications. In addition to hormone-regulating investigations, SRIF and its analogues (most notably octreotide, f[CFwKTC]T [40]) were subsequently tested for the treatment of hormone-secreting tumors. A review of the anticancer applications of SRIF was provided by Evers *et al.* [41] and the anticancer applications of octreotide have been

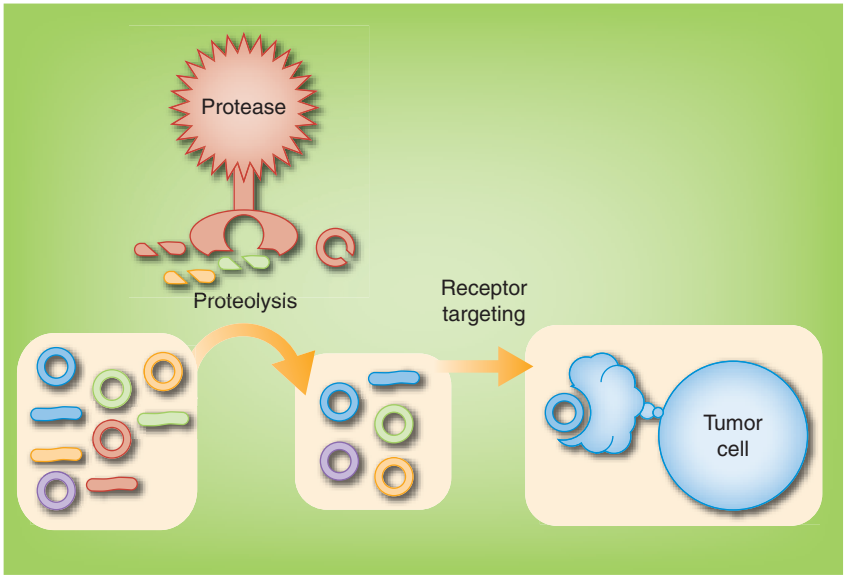

Figure 4. Cyclic peptides have high proteolytic stability compared with linear peptides and are, therefore, more useful for cancer biomarker targeting.

reviewed by Anthony and Freda [42]. Although the disulfide-cyclized SRIF and its subsequent derivatives often displayed the desired biological activities, Besser *et al.* found that amide cyclization could create SRIF derivatives that were more stable than the original SRIF peptide [43]. They created nine derivatives of the somatostatin receptor-binding octapeptide with the sequence fFYwKVFT-NH_2 and cyclized these sequences by amide coupling. The derivatives were all cyclized at the D-phenylalanine and valine residues, but they differed in terms of the orientation of the amide bond and the use of extended linkers. The *in vitro* somatostatin-14 ($[^{125}I]Y^{11}$-SRIF) displacement studies with BON-1 cells showed that the IC_{50} values of these cyclic peptides were moderate and ranged from 10 µM to 0.1 µM. More importantly, the amide-cyclized peptide J1738 generated in this study was stable for 15 h in homogenized rat liver while the disulfide-cyclized SRIF standard degraded within 1 h. This showed that the amide-driven cyclization was responsible for improving enzymatic stability and suggested that the disulfide bridge in the native SRIF is prone to enzymatic reduction.

In some cases, both the biological activity and the stability of peptides can be improved by cyclization. Pakkala *et al.* studied the activity and stability of linear and cyclic human glandular kallikreain (KLK2)-targeting peptides with the sequences ARRPAPAPG (KLK2a) and GAARFKVWWAAG (KLK2b) [44]. The KLK2 protein is a highly prostate-specific serine protease that can be inhibited to reduce the metastasis of prostate cancer. They found that disulfide-cyclized derivatives of the KLK2b sequence obtained enhanced KLK2 inhibition compared with the linear sequence while the cyclic KLK2a peptides were all inactive. They created a series of head-to-tail cyclized KLK2b peptides and found that the inhibitory activity of these peptides was also greater compared with the linear derivative. Next, they tested the stability of the linear, disulfide-cyclized and the head-to-tail cyclized KLK2b derivative in trypsin and human plasma. These stability studies found that 57% of the head-to-tail cyclized KLK2b ht1 peptide ARFKVWWN remained stable after a 4 h treatment with trypsin while the disulfide-cyclized GCAARFKVWWACG peptide and the linear ARFKVWWG and Ac-ARFKVWWGG peptides each completely degraded after 30 min. They continued to show that the KLK2b ht1 peptide was completely stable in human plasma after 24 h while the other three peptides consistently degraded within 30 min. This study again showed that disulfide cyclization might improve receptor binding affinity but that enzymatic reduction can limit their use *in vivo*. Therefore, amide coupling may instead be used in select cases to improve the biological activity and stability of linear peptides.

Applications of linear & cyclic peptides for cancer therapy & imaging

■ Unconjugated peptides as anticancer therapeutics

Linear and cyclic peptides can elicit biological responses that can result in the treatment of cancer and other diseases. Several groups have reviewed the topic of bioactive peptides [1,45–50]. It was found that the key binding regions of endogenous proteins could be synthesized to create relatively low molecular-weight targeting peptides that mimic the structure and activity of native proteins. These targeting peptides can act as antagonists due to competition with endogenous proteins and can prevent downstream cellular signaling in order to reduce the propagation of cancer growth. The synthetic linear P14 peptide (DFPQIMRIKPHQGQHIGE) and the cyclic CBO-P11 (cyclo-VEGI) peptide (DFPQIMRIKPHQGQHIGE) derived from $VEGF_{79-93}$ were investigated by Zilberberg *et al.* to determine if cyclization could increase the binding affinity of this sequence to the kinase receptor domain of VEGF receptors [51]. Comparative studies showed that cyclo-VEGI successfully displaced ^{125}I-$VEGF_{165}$ from CHO-VEGFR2, CHO-VEGFR1, and BAE cells in a dose-dependent manner with IC_{50} values of 1.3, 0.7 and 12 µM, respectively, while P14 did not. The cyclo-VEGI peptide abolished the $VEGF_{165}$-induced migration of BAE cells in a dose-dependent manner while the linear peptide had no effect on the stimulated cell migration. The cyclo-VEGI peptide also displayed anti-angiogenic activity on chick choriallantioc membrane growth while treatment with P14 resulted in a similar expression of microvessels as treatments with $VEGF_{165}$. This showed that the cyclic analogue cyclo-VEGI successfully inhibited VEGF activity and that this property was the result of peptide cyclization.

The therapeutic activity of peptide sequences can be improved by cyclization. Haier *et al.* induced a colon carcinoma tumor model in rats using 1,2-dimethylhydrazin and compared

linear and cyclic RGD-containing peptides to determine their relative anticancer potencies [52]. Tumors were treated with the cyclic RGDfV, a cyclic inactive control peptide (EMD 135981) or the linear RGDS peptide to investigate the therapeutic activity of these RGD derivatives. They found that rats treated with the cyclic RGDfV peptide had an average of seven tumors. In comparison, treatments with either the cyclic control peptide or the linear RGDS peptide resulted in the presence of an average of 15 and 18 tumors, respectively. This study demonstrated that the cyclic RGDfV peptide can effectively treat tumors *in vivo*. Although the anticancer activity of the cyclic RGDfV peptide was greater than that of the linear RGDS peptide, the linear peptide contained different residues at the C-terminus. Therefore, it is not clear if cyclization was the sole reason why the RGD-motif of the cyclic peptide exhibited an improved activity compared with the linear peptide.

The RGDFV-sequence has been identified as having great potential for treating human cancers that express $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin receptors. The peptide cilengitide (EMD 121974, Cyclo-RGDf(N-Me)V, Merck KGaA, Darmstadt, Germany) has IC_{50} values of 1 and 140 nM for these integrin receptors, respectively, using vitronectin competition assays, and was investigated in a Phase I and pharmacokinetic clinical trial for patients with advanced solid tumors [53]. This study showed that a twice-weekly administration of cilengitide was safe and had a predictable clearance profile. A case study later showed that combined cilengitide and gemcitabine therapy was able to inhibit rapid tumor growth of a patient with highly vascularized head and neck cancer [54]. A recent Phase II clinical trial report by Gilbert *et al.* suggested that cilengitide alone has modest activity for treating recurring glioblastoma but that it may be used in a combination therapy regime for newly diagnosed glioblastoma [55]. These clinical trial studies are promising and show an example of a cyclic peptide with the potential to successfully treat human patients with various types of cancer.

■ Peptide conjugates for cancer imaging & therapy

The conjugation of pharmacokinetic-modifying substituents can improve the properties of cancer-targeting peptides. For example, Haubner *et al.* created cyclic RGD-carbohydrate conjugates to determine if glycosylation could improve the pharmacokinetic and biodistribution properties of cyclic RGD peptides using either melanoma or osteosarcoma-bearing mouse models [56]. During their investigations, they synthesized the glycosylated cyclic c(RGDyK(SAA)) peptide (GP1) to determine if the sugar substituent (SAA) or cyclization of the RGD-motif could improve the binding affinity of the linear GRGDSPK peptide to various immobilized integrins. They found that the cyclic GP1 peptide had a 15-fold lower IC_{50} for the $\alpha_V\beta_3$ integrin and a 2.5-fold lower IC_{50} for the $\alpha_V\beta_5$ integrin compared with the linear standard. However, the IC_{50} of the linear peptide was threefold lower for $\alpha_{IIb}\beta_3$ compared with GP1. A comparison with the cyclic RGDfV peptide showed that this non-glycosylated peptide had a threefold lower IC_{50} for $\alpha_V\beta_3$, a fourfold lower IC_{50} for $\alpha_V\beta_5$ and a threefold lower IC_{50} for $\alpha_{IIb}\beta_3$ compared with GP1. Although the sequences were not identical, this suggested that cyclization improved the receptor binding of the linear derivative for $\alpha_V\beta_5$ and $\alpha_V\beta_3$ but that glycosylation does not improve the *in vitro* integrin receptor-binding properties of all cyclic peptide sequences to the same extent. They continued to radiolabel the GP1 peptide to create GP2 (c[RGD(^{125}I)yK(SAA)]) and compared *in vivo* biodistribution and elimination with the cyclic non-glycosylated P2 peptide (c(RGD(^{125}I)yV)). These studies showed that peptide cyclization improved the binding affinity of the RGD-motif to $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin receptors compared with a linear standard, and that glycosylation contributed to improved tumor retention and allowed faster elimination of the radiotracer from the liver and kidneys.

The conjugation of sugar moieties or contrast agents can have unexpected effects on the receptor binding or biodistribution of linear and cyclic peptides. Interestingly, Achilefu *et al.* discovered that the conjugation of the near-IR dye cypate (Cyp) was responsible for creating a high affinity conjugate with the previously inactive linear GRDSKP (Cyp-GRD) peptide [57]. During these studies, they also compared the cypate conjugates of the RGD-containing linear GRGDSPK (Cyp-RGD) and cyclic RGDfV (Cyp-cyclo-RGD) peptides for targeting the $\alpha_V\beta_3$ integrin receptor of A549 tumors in mouse xenograft models. This investigation showed that the cyclic Cyp-cyclo-RGD conjugate had a threefold higher *in vivo* tumor uptake compared with the linear Cyp-RGD conjugate. Surprisingly, the linear Cyp-GRD had an approximately twofold higher tumor uptake compared with the Cyp-cyclo-RGD and had lower accumulation

in healthy tissues. This comparison showed that the improved tumor uptake of the Cyp-GRD peptide conjugate was due to the rearrangement of glycine from the RGD-motif. This study also suggested that the favorable biodistribution of Cyp-cyclo-RGD compared with Cyp-RGD was due to peptide cyclization.

Peptides can be radiolabeled with radioisotopes to create radiopharmaceuticals capable of imaging and treating cancer and other diseases. This topic has been reviewed by several groups [58–60]. Radiolabeling studies have compared linear and cyclic peptides to investigate the effects cyclization has on radiotracer biodistribution. Fani *et al.* compared the biodistribution of the ^{99m}Tc-radiolabeled linear and cyclic RGDfKH sequence using MDA-MB 435 breast cancer mouse xenograft models [61]. They found that the tumor uptake of cyclic RGDfKH-^{99m}Tc$(H_2O)(CO)_3$ was fourfold higher compared with the linear analogue while the accumulation of these peptides was similar in healthy tissues. Since radiolabeling efficiency and chelation stability (saline containing either 10 mM cysteine or 10 mM histidine) were similar, the improved tumor accumulation of the cyclic RGD peptide was likely due to the constraints imposed by cyclization. It was suggested that the *in vivo* biodistribution of this cyclic RGD peptide could further be improved using multimeric cyclic RGD peptides that could bind to multiple adjacent $\alpha_v\beta_3$ integrin receptors. The topic of multimeric cyclic RGDs has since been reviewed [62].

Radiolabeling studies have investigated peptide cyclization for targeting the α-melanocyte stimulating hormone (α-MSH). Chen *et al.* developed the rhenium-cyclized peptide ReCCMSH that contained three cysteine residues [63]. The *in vivo* biodistribution of the ^{111}In-labeled peptides (from 30 min to 24 h) showed that the B16/F1 murine melanoma tumor accumulation of ^{111}In-DOTA-ReCCMSH (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7-tris[*t*-butyl acetate]-10-acetate) was from 1.2- to 1.5-fold higher compared with the disulfide-cyclized ^{111}In-DOTA-CMSH peptide and the kidney accumulation of ^{111}In-DOTA-ReCCMSH was 2.6–3.9-fold lower compared with ^{111}In-DOTA-CMSH. These results were especially significant since the linear derivative ^{111}In-DOTA-CCMSH had a lower tumor accumulation and higher healthy tissue accumulation compared with the disulfide cyclized ^{111}In-DOTA-CMSH peptide. Although both cyclic α-MSH derivatives performed desirably, these results show that not all cyclization methods are able to equally improve the biodistribution of radiotracers *in vivo*.

Cyclic peptides have been used to create nanoparticles capable of actively targeting cancer biomarkers. Several groups have reviewed this topic [64–66]. In particular, Negussie *et al.* created lysolipid-containing temperature sensitive liposomes (LTSLs), which used the NGR-motif to target CD13/aminopeptidase N – selectively overexpressed in tumor vasculature [67]. They synthesized the linear KNGRG peptide and used on-resin amide cyclization to create the cyclic KNGRE peptide (cKNGRE). These peptides were conjugated to the fluorescent dye Oregon Green (OG) and subsequently to the LTSLs in order to investigate the effect cyclization had on binding to HT-1080 (CD13+) and MCF-7 (CD13-) cancer cells. Their results showed that the cKNGRE-OG (EC_{50} = 61.0 μM), had a 3.6-fold better binding to HT-1080 cells compared with the linear KNGRG-OG peptide (EC_{50} = 219.9 μM), and this comparison was illustrated using epifluorescence microscopy **(FIGURE 5)**. Due to the multivalency effect, the binding affinity of each peptide-OG conjugate increased tenfold when they were conjugated to LTSLs, where the cyclic NGR-OG peptide (EC_{50} = 6.2 μM) maintained a higher binding affinity to HT-1080 cells compared with the linear analogue (EC_{50} = 21.5 μM). These studies

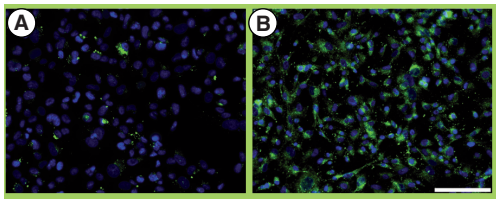

Figure 5. Epifluorescence microscopy showing the *in vitro* binding of Oregon green-conjugated NGR-derivatives to HT-1080 (CD13+) cells following 30 min incubations at 37°C. Much lower Oregon green fluorescence was observed with **(A)** the linear KNGRG-Oregon green (20 μM) compared with **(B)** the cyclic KNGRE-Oregon green (20 μM). Green fluorescence signals show binding of the peptide–Oregon green conjugates and the blue fluorescence signals show the nucleus-staining of 4′,6-diamidino-2-phenylindole. Images were acquired with identical exposure times and displayed consistent window and level (scale bar = 100 μm).
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suggested that cyclization of the KNGRE-motif was responsible for improving EC_{50} values compared with the linear KNGRG sequence. However, it is possible that the glutamic acid residue could have contributed to improving the binding of the cKNGRE derivatives. They continued to show that the cKNGRE-OG-LTSLs could release doxorubicin upon heating as a proof-of-concept experiment to show its potential as a cancer therapeutic delivery system.

■ Cell-penetrating peptides

Cyclization can create peptides with the ability to penetrate tumors in order to enhance the potency of anticancer drugs. The field of cell-penetrating peptides has been reviewed by several groups [68–71]. A recent study by Sugahara *et al.* described the discovery of the cyclic peptide with the sequence CRGDK/RGPD/EC, labeled iRGD, which was shown to have these properties [72]. When iRGD was injected along with non-conjugated Nab-paclitaxel (ABX), the tumor uptake of ABX was increased by an order of magnitude within orthotopic BT474 and 22Rv1 xenograft mouse models compared with ABX alone. This resulted in a significant reduction in tumor size and weight.

Similar results were found using iRGD in combination with free doxorubicin and with liposomes loaded with doxorubicin using orthotopic 22Rv1 mouse models. The cyclic iRGD was also co-administered with a clinical dose of trastuzumab and resulted in the eradication of tumors in orthotopic BT474 mouse models after 24 days. This report suggested that the mechanism underlying the observed enhancement of anticancer drug activity was due to integrin binding, proteolytic cleavage to CRGDK/R, binding to neuropilin-1 due to the C-end rule motif (R/KXXR/K) (X = interchangeable residue) and cell internalization. Although a linear analogue was not compared with iRGD, this report is the first to show a novel cyclic peptide with the versatile ability to enhance the therapeutic efficacy of several anticancer drugs by cell penetration.

Peptide cyclization can potentially improve the cell-penetrating ability of linear sequences. Mandal *et al.* synthesized 11 cyclic peptides that contained either R, K, E, F or W residues [73]. These residues created peptides that contained charged side chains, which were expected to facilitate the nuclear transport of fluorescein labels and aid the delivery of the anticancer drug, doxorubicin, by enhancing cell penetration. Among their results, the comparative fluorescence-activated cell sorting study showed that the fluorescein-labeled cyclic F-[W4R3K] conjugate had an over twofold higher uptake in CCRF-CEM human leukemia cells compared with the linear F-KR3W4 peptide. Since the charges of these two analogues were the same, the improved cell-penetrating property of the cyclic peptide could be attributed to the constrained geometry obtained by cyclization. However, peptide cyclization does not guarantee an improvement in cell penetration compared with linear analogues. This will be addressed later in this review.

Limitations of peptide cyclization

Cyclic peptides can potentially obtain desirable constrained geometries that are responsible for increasing their binding affinity, specificity or stability compared with their linear counterparts. However, it is important to note that cyclization does not necessarily lead to improvements in all or sometimes any, of these properties. Linear peptides can contain sequences that can support rigid structures without the need for cyclization. Colombo *et al.* synthesized the disulfide-cyclized CNGRC and linear GNGRG peptides, which were subsequently conjugated to the TNF for anti-CD13 targeted melanoma therapy [74]. The anti-tumor therapeutic properties of these conjugates were tested *in vivo* using B16/F1 murine melanoma tumors. They suggested that the anti-tumor activity of the cyclic CNGRC-TNF conjugate was over tenfold higher compared with the linear analogue. This suggestion was made since a tenfold higher dose of the linear GNGRG-TNF (0.1 ng) conjugate was required to reduce tumor volume to the same size as using 0.01 ng of the cyclic CNGRC-TNF conjugate. However, the biological activities of both conjugates were similar after 14 days using 10 ng of either of these agents. Structural characterization of these two analogues surprisingly showed that the uncyclized CNGRC sequence peptide contained a β-turn while the disulfide-cyclized sequence contained a bent configuration. They concluded that the linear NGR-motif itself may induce a rigid conformation to explain why the linear GNGRG sequence still showed desirably anti-tumor activity.

Peptide cyclization can improve biological activity of linear analogues but not in all cases. Mizejewski *et al.* extensively studied the estrogen-dependent anticancer activity of several variations of the linear and cyclic

derivatives of HAFP and compared their ability to inhibit cancer growth in several cell lines. Among these studies, they developed the linear and cyclic 34-amino acid peptides (P149) taken from a fragment of the third loop of HAFP. They reported that the linear P149 peptide could inhibit MCF-7 breast cancer cell growth while the disulfide-cyclized analogue was not as effective [75]. In addition, they found that the linear 149c peptide (EMTPVNPG) fragment of P149 inhibited the growth of MCF-7 tumors to a similar extent (>75%) as the cyclic analogue (>80%) [76]. Similarly, Mesfin *et al.* created truncated linear (EMTOVNOG) and cyclic (cyclo-[EMTOVNOGQ]) derivatives of P149 and found that they provided a similar anti-estrotrophic tumor growth inhibition of MCF-7 tumors [77]. These comparative studies showed that these cyclic derivatives of HAFP fragments generally did not show improved growth inhibitions of MCF-7 tumors compared with the linear analogues.

Although particular native peptides may exist in cyclic conformations, linear derivatives of these native cyclic peptides may display a superior binding affinity to targeted receptors compared with their cyclic counterparts. As previously described, the endogenous disulfide-cyclized SRIF and its analogues were investigated for various endocrine and cancer-related applications. In terms of anticancer therapy, the most clinically relevant binding target of SRIF is the somatostatin receptor subtype 2 (SST_2), since this receptor subtype is responsible for regulating several synthetic and secretory processes within human tumors [78]. Interestingly, Baumach *et al.* showed that a linear derivative of SRIF displayed superior SST_2 binding compared with its cyclic counterpart [79]. They initially screened a synthetic combinatorial linear hexapeptide library to discover a potent SRIF-mimicking peptide with a high binding affinity to SST_2. After library screening, they discovered the promising N-acetylated and C-amidated Ac-hfirwf-NH_2 peptide. Among their studies, they compared the binding affinity of this linear peptide to the amide-cyclized hfirwf peptide and the disulfide-cyclized ChfirwfC peptide. Their competitive binding assay investigated the binding affinity of each peptide to the SST_2 by using the ^{125}I-labeled 14-amino acid somatostatin (^{125}I-S-14) as the competitor. This assay showed that the disulfide-cyclized ChfirwfC peptide surprisingly had a 12-fold lower binding affinity (K_i = 1.95 μM) to SST_2 compared with the lead linear peptide (K_i = 0.16 μM) and that the amide-cyclized analogue displayed a similar binding affinity (K_i = 0.14 μM) to the same linear peptide. Their report thoroughly investigated the *in vivo* biological activity of the linear Ac-hfirwf-NH_2, but the lack of improved binding activity observed with the disulfide-cyclized derivative was not discussed.

In contrast to the previous examples, peptide cyclization has been shown to reduce the degree of cell membrane penetration. Kwon *et al.* compared the cell permeability of nine linear and nine cyclic peptide derivatives with the sequence E-Dpr-Dpr-Dpr (Dpr = 2,3-diaminopropionic acid), which were conjugated to the dexamethasone-derived steroid SDex [80]. These conjugates were incubated with HeLa cells that expressed high levels of an artificial transactivator protein complex comprised of the Gal4 DNA-binding domain, the glucocorticoid receptor ligand binding domain and the VP16 transactivation domain (Gal4 DBD-GR LBD-VP16). This intricate protein complex was created so that the desired binding of SDex to Gal4 DBD-GR LBD-VP16 resulted in luciferase expression. After correcting for IC_{50} variations of the peptide sequences, the luciferase signals indicated the relative cell-penetrating ability of the linear and cyclic peptides. Interestingly, they found that the linear peptides were two- to six-fold more permeable to cells compared with their corresponding cyclic analogues. This was an intriguing result since the sequences compared were the same and the N- and C-termini of the linear analogues were capped to remove charges. A similar observation was noted by Fischer *et al.* while conducting ligand optimization studies of biotinylated penetratin derivatives for HaCaT or A549 cell internalization [81]. Among their results, they found that the linear biotinylated 19-amino acid peptide was internalized to a greater extent compared with its cyclic counterpart that contained the same sequence. Since this linear sequence contained cysteine residues near the N- and C-termini, oxidation may have occurred under physiological conditions to prevent it from becoming internalized better than the original linear 17-amino acid penetratin peptide. It was not clear why these linear peptides were more permeable. It is, however, possible that the lower cell penetration of particular cyclic peptides could be due to reductions in enthalpy, which may be required for stronger membrane interactions [82].

Key Terms

Chimeric peptide: In terms of cancer targeting, this refers to a single peptide that contains the active sequences of two or more separate motifs. These are envisioned to target multiple cancer-related receptors or the same receptor with a higher affinity than either of the motifs individually.

Prodrug: A compound that does not elicit biological activity until it is metabolized within a cell or organism and is converted into an active drug.

Unique aspects of peptide cyclization

Peptide cyclization allows the use of unique chemistries that can generate conformations not available to linear sequences. Giblin *et al.* engineered cyclic analogues of the α-melanocyte stimulating hormone (α-MSH, Ac-SYSMEHFRWGKPV-NH_2) whereby cyclization involved forming intramolecular rhenium or technecium complexes [83]. They synthesized the rhenium-cyclized peptides, ReMSH (Re–[$C^{4,10}$, f^{7}]-α-MSH_{4-13}) and ReCCMSH (Re–[$C^{3,4,10}$, f^{7}]-α-MSH_{3-13}) as well as the disulfide-cyclized CCMSH ([$C^{3,4,10}$, f^{7}]-α-MSH_{3-13}) peptide. The binding affinity of these peptides were compared with the cyclic APOMSH ([$C^{4,10}$, f^{7}]-α-MSH_{4-13}) derivative of α-MSH. They found that ^{99m}Tc and ^{188}Re gave the cyclic CCMSH sequence a greater stability in solutions of phosphate and cysteine compared with their cyclic counterparts with only two cysteine residues. Although ReCCMSH had a better binding affinity (K_i = 2.9 nM) compared with ReMSH (K_i = 66 nM) and CCMSH (K_i = 7.6 nM), the previously reported APOMSH sequence out-performed all these cyclic compounds (K_i = 0.68 nM). The CCMSH sequence was subsequently radiolabeled with ^{99m}Tc (^{99m}TcCCMSH) to display the *in vivo* tumor-targeting ability of the high-affinity cyclic CCMSH peptide using a γ-camera **(Figure 6)**. This study showed that rhenium cyclization of this α-MSH derivative required three cysteine residues to create a geometry that gave a low nanomolar K_i. Although metal-induced cyclization did not improve the receptor binding property of the cyclic APOMSH sequence, the incorporation of a radiometal during cyclization makes this strategy intriguing for cancer radioimaging and radiotherapy.

Cyclic peptides can have geometries that allow preferential binding to multiple binding sites of targeted receptors. Colangelo *et al.* created synthetic cyclic peptides to target loop 1 and 4 of the NGF by mimicking the geometry of either one, both or the same loop twice to discover new sequences, which can stimulate tyrosine kinase receptor A (TrkA) activation [84]. This activation would lead to a cascade of signals for the differentiation and survival of NGF responsive neurons [85–87]. They found that the bicyclic L1L4, containing the desired mimics of both loops 1 and 4, was able to stimulate the differentiation of the dorsal root ganglia of chick embryos similarly to using the native mNGF protein. The bicyclic L1L4 could also stimulate the differentiation of PC12 rat adrenal medulla cells better than derivatives containing sequences that only mimicked the sequence of one of the targeted loops. The cyclic L1L4 peptide also selectively activated the desired TrkA receptor and not TrkB. This may explain how it improved pain sensitivity and reaction times after chronic sciatic constriction injury almost as well as the dose of rat recombinant β-NGF. They concluded that both loop mimics of the native NGF were required for the desired activity and selectivity. These finding can be useful to cancer research since NGF has been investigated as a potential target for anticancer therapeutics [88].

Peptide cyclization can be used to deliberately reduce the biological activity of peptide sequences. Zhong *et al.* investigated the use of a cyclic 25-amino acid (cycL-25) derivative [82]

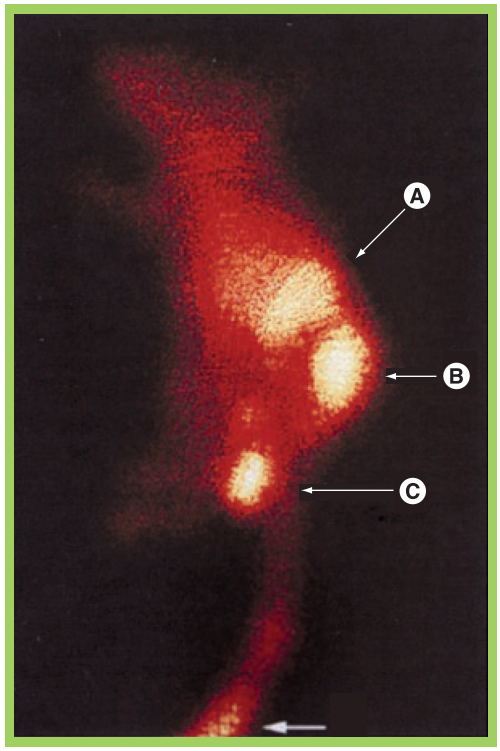

Figure 6. Radioimaging of a mouse bearing a 400-mg B16-F1 melanoma tumor captured with a γ-camera 30 min after the injection of ^{99m}TcCCMSH (25 mCi). Lateral image shows **(B)** high accumulation of the radioactivity in the tumor and **(A)** lower levels in the kidneys, **(C)** bladder and **(D)** the tail vein injection site. The intensity of the γ-emission is color-coded to represent high radioactivity in white-yellow, medium radioactivity in orange and low radioactivity in dark red.
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of the linear 18-amino acid membrane lytic peptide based on the α-helical peptide that was previously developed by Steiner *et al.* (L-18, LRLALKLALKALKAALKL) [89]. The cycL-25 derivative included a membrane type 1-matrix metalloproteinase (MT1-MMP) cleavage site that was expected to cleave upon incubating the peptide with MMP-overexpressing cancer cell lines, thus restoring the activity of the linear membrane lytic sequence. They found that, although the linear L-18 peptide was able to reduce the viability of both MDA-MB-435 (high MT1-MMP expression) and MCF-7 (low MT1-MMP expression) better compared with cycL-25, the cyclic peptide was selectively more toxic to the MDA-MB-435 cell line. In addition, cycL-25 displayed lower undesirable hemolytic activity compared with the linear L-18 analogue due to its constrained structure. Although the desired anticancer toxicity of the cyclic peptide was not as high as the linear analogue, cyclization was able to reduce undesirable hemolytic activity to improve the specificity of the membrane lytic sequence. This strategy of selective activation could be expanded to create novel molecular beacons. For example, it might be possible to create cyclic cancer-specific peptide-based molecular Förster resonance energy transfer beacons by modifying cyclic cancer-targeting peptides to contain cleavable regions, a fluorophore and a fluorescence quencher. This method could potentially combine the improved binding affinity of cyclic peptides with the selective activation offered by enzymes that are overexpressed by cancers for efficient cancer imaging or therapy. The topic of peptide-based molecular beacons has been reviewed by Liu *et al.* [90].

Peptides have the potential to bind to selected targets *in vivo* while not interfering with the immune system to the same extent as their endogenous protein counterparts. Blank *et al.* synthesized a cyclic **chimeric peptide** mimetic of the human IFN- α2b peptide (122-137GG30-35) and compared the apoptosis-inducing activity of this cyclic peptide to the native protein IFN- α2b [91]. The cyclic peptide was previously shown to inhibit 40% of WISH cell growth at a 6 μM concentration, while both the linear chimeric peptide and the $IFN_{122–139}$-α2b peptide only inhibited 30% growth and the $IFN_{29–35}$-α2b peptide gave 10% inhibition at the same concentration [92]. Both the native IFN- α2b protein and the cyclic chimeric peptide induced apoptosis through activation of caspases 8 and 9 and the release of cytochrome C. However, the native IFN- α2b protein induced cell cycle arrest by increasing the distribution of cells in S phase, while the cyclic chimeric peptide had no effect compared with control conditions. These results suggest that the cyclic chimera was able to retain its apoptotic activity due to its rigidity while avoiding induction of an immune response due to its small molecular weight relative to the endogenous IFN-α2b protein.

Cyclic peptides can be used to deliberately stimulate an immune response for vaccine research purposes. Witsch *et al.* created a series of cyclic 12-amino acid peptides and their linear analogues that mimicked human *erbb-2* gene protein epitopes that were specific for the L-26 and N-12 antibodies [93]. Flow cytometry studies showed that the cyclic L-26-19 and cyclic L-26-24 peptides could displace the L-26 antibody from binding to the surface of BT474 breast cancer cells overexpressing the *erbb-2* gene. Their linear analogues, in a dendritic octavalent multiple antigenic peptide configuration, did not show this displacement. Similar results were found using the cyclic N-12-1 and cyclic N-12-2 peptides compared with their linear multiple antigenic peptide sequences. In addition to showing that cyclization was responsible for improving receptor binding, this study investigated the use of keyhole limpet hemocyanin conjugates of the cyclic L-26-19 and cyclic L-26-24 peptides. The cyclic peptide-keyhole limpet hemocyanin conjugates elicited an immune response from mice and this study suggested that these cyclic ligands may be useful for creating vaccines against *erbb-2* gene-overexpressing tumors.

Cyclization chemistry can be used to activate linear peptide–**prodrug** conjugates capable of controlled drug-release. Brady *et al.* bonded linear peptides to the anticancer drug vinblastine to create inactive prodrugs [94]. Upon contact with prostate-specific antigen, four of the tested peptide sequences were cleaved at the glutamine-serine residues, which initiated the formation of an intramolecular bond that created a cyclic diketopiperizine. This cleavage and subsequent cyclization was responsible for releasing the active drug cargo. The prostate-specific-antigen-induced proteolytic cleavage was most efficient using the octapeptide 4-*O*-(Ac-Hyp-SS-Chg-QSSP)-dAc (Hyp = hydroxylproline, Chg = α-cyclohexylglycine and dAc = *des*-acetyl) that was conjugated to the 4-position of vinblastine. The topic of cycization-activated prodrugs has since been reviewed by Gomes *et al.* [95].

Executive summary

Geometry of linear & cyclic peptides

- Linear peptides generally require long sequences in order to form constrained secondary structures.
- Peptide cyclization is an easy method of creating constrained structures within short peptide sequences.
- Cyclization can allow peptide sequences to obtain geometries that mimic the geometries of the biologically active portions of large endogenous proteins.
- The type of chosen cyclization chemistry and the position of cyclization within peptide sequences can create constrained structures that elicit different biological activities.

Library screenings & structure–activity studies

- While screening large random libraries of peptide sequences, cyclic peptides that contain capping cysteine residues have often been found to have higher binding affinities to targeted receptors compared with random linear peptides.
- The phage-display method can be modified to create libraries of random peptide sequences that all contain capping cysteine residues. This strategy can create libraries of cyclic peptides that may have improved binding affinities to their targets compared with their linear analogues.
- Structure–activity studies are important for discovering novel targeting peptides, which have improved cancer-targeting properties compared with the original hits discovered from previous library screening studies.

Receptor-subtype specificity of linear & cyclic peptides

- Linear peptides may show little specificity for targeting specific receptor subtypes. Peptide cyclization can create fixed geometries that improve the binding affinity of targeting motifs to particular receptor subtypes that are overexpressed by cancer cells.

Stability of linear & cyclic peptides

- Cyclization can prolong the integrity of linear peptides by stabilizing labile portions of peptide sequences.
- Not all cyclization chemistries will improve the stability of linear sequences to the same extent. Disulfide cyclization is limited by the reduction of the dithiol bond at high pH ranges and also by proteolytic degradation with endogenous enzymes.

Applications of linear & cyclic peptides for cancer therapy & imaging

- Unconjugated peptides as anticancer therapeutics
  - Cyclic peptides that have higher binding affinities to targeted receptors compared with linear peptides can displace endogenous proteins in order to interrupt signaling cascades that are responsible for the propagation of tumor growth.
  - The compound cilengitide is an example of a cyclic peptide that has been investigated in clinical trials for the treatment of head and neck tumors as well as glioblastoma.
- Peptide conjugates for cancer imaging & therapy
  - The conjugation of pharmacokinetic-modifying moieties can improve the *in vivo* properties of cyclic peptides to a greater extent compared with linear peptides with the same sequence. These moieties can potentially elicit unexpected properties whereby they can improve the *in vivo* behavior of inactive peptide motifs.
  - Radiolabeled cyclic peptides can serve as radiotracers, which have higher tumor uptake and lower healthy tissue uptake compared with their linear radiolabeled counterparts.
  - Cyclic peptides can improve the receptor targeting of nanoparticles to a greater extent compared with similar linear peptides.
- Cell-penetrating peptides
  - Cyclization can potentially improve the cell-penetrating ability of linear peptide sequences. Particular cyclic peptides can penetrate tumors in order to improve the potency of anticancer therapeutics.

Limitations of peptide cyclization

- Certain linear peptides can contain fixed geometries that are recognized by targeted receptors without the need of cyclization.
- Linear peptide sequences can have similar or higher binding affinities to receptors compared with their cyclic counterparts.
- Linear peptides can penetrate cells to a greater extent compared with cyclic analogues. It is possible that a higher free energy is required for interactions with cell membranes that facilitate internalization.

Unique aspects of peptide cyclization

- Cyclization attained through metal coordination allows these peptides to be used as radiotracers or radiotherapeutics without the need of additional chelators.
- Multiple loops within endogenous proteins can be mimicked by bicyclic peptides.
- Cyclic peptides can elicit a lower immunogenicity compared with endogenous protein counterparts while successfully targeting desired receptors.
- Cyclic peptides may be useful in the field of vaccine research.
- Cyclization can modulate the activity of peptide sequences whereby biological activity is restored upon proteolysis.

Conclusion

This review has presented studies that have compared closely related linear and cyclic peptide analogues in an attempt to illustrate the advantages, the limitations and the unique properties of cyclic peptides. Cyclic peptides often have higher binding affinities compared with linear counterparts with the same or very similar sequences. Cyclic peptides contain fixed geometries that limit the number of conformations in solution. This property can tune the specificity of particular cyclic peptides compared with linear analogues or even other cyclic analogues, which are cyclized by different chemistries and/or at different sites. Peptide cyclization generally increases enzymatic stability compared with linear peptides, but not all cyclization chemistries can improve this stability to the same degree. There are several aspects of cyclic peptides that are unique from linear peptides and these properties allow cyclic peptides to be used for additional applications. In conclusion, peptide cyclization offers several advantages but cyclization is not guaranteed to induce all intended improvements to linear standards.

Future perspective

There are dozens of chemistries available for creating cyclic peptides. Various new cyclization chemistries offer advantages such as fast reaction times, high yields and/or the incorporation of versatile conjugation handles [16]. However, there has not been a thorough comparison of the geometries, relative binding affinities or stabilities of peptides cyclized by contemporary methods compared with peptides cyclized by conventional amide or disulfide chemistries. Such comparative studies may discover that contemporary cyclization chemistries can create cyclic peptides with particular geometries that can modulate the targeting specificities of peptide sequences to particular receptor subtypes. It is also possible that particular cyclization chemistries can create cyclic peptides with binding affinities that are superior to similar sequences cyclized by conventional cyclization methods. As new chemistries are developed, their advantages should be illustrated by at least comparing novel cyclic peptides to closely related linear analogues.

Linear peptides can be modified to contain multiple cyclic loops [18,84]. These bicyclic peptides can possibly target multiple receptor subtypes that are associated with cancer cells while avoiding binding to particular subtypes not expressed by cancer cells. Similarly, single-looped cyclic chimeric peptides can be used to target multiple unrelated cancer-specific receptors. These two strategies should be further pursued to develop novel high-affinity cyclic peptides that target multiple cancer-specific biomarkers for efficient cancer imaging or therapy. Novel hits discovered through library screening should also be multimerized through biocompatible linkers to test if the multivalency principle that applies to the RGD-motif also applies to other peptides that target cancer cell-surface receptors [62].

Financial & competing interests disclosure

The authors acknowledge support from the NSERC Strategic Network BiopSys, the NSERC Discovery Grant, the CIHR, and the Joey and Toby Tanenbaum/Brazilian Ball Chair in Prostate Cancer Research. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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