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Bicyclic Peptides: Types, Synthesis and Applications

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Bicyclic peptides form one of the most promising platforms for drug development owing to their biocompatibility, similarity and chemical diversity to proteins, and they are considered as a possible practical tool in various therapeutic and diagnostic applications. Bicyclic peptides are known to have the capability of being employed as an effective alternative to complex molecules, such as antibodies, or small molecules. This review provides a summary of the recent progress on the types, synthesis and applications of bicyclic peptides. More specifically, natural and synthetic bicyclic peptides are introduced with their different production methods and relevant applications, including drug targeting, imaging and diagnosis. Their uses as antimicrobial agents, as well as the therapeutic functions of different bicyclic peptides, are also discussed.

**Introduction**

Small chemical molecules with molecular weight (MW) <500 Da are the most usable FDA-approved drugs with the ability to regulate a biological process. However, small molecules are generally limited to targeting proteins with well-defined binding pockets (e.g., enzymes, receptors and ion channels). Small molecules cannot affect proteins involved in protein–protein interactions (PPIs) because these proteins are not easily available. To overcome the limitations of small molecules, many macromolecules with MW >5000 Da, such as antibodies, have arrived in the clinic as therapeutics since the 1980s. These macromolecules can interact with challenging targets but, unlike small molecules, they are too large to enter the cell and are thus limited to extracellular targets [1]. Peptides, as the next-generation therapeutics, represent unique features – molecularly they are between small molecules and macromolecules, but are biochemically and therapeutically distinct from both [2].

Peptides have a more specific function compared with small molecules. Therapeutic peptides are categorized as linear or cyclic. Cyclic peptides do not have amino and carboxyl termini and are resistant to hydrolysis by exopeptidases. Also, owing to the conformational rigidity, cyclic peptides usually have a better biological activity compared with linear ones. The rigidity of cyclic peptides decreases the Gibbs free energy, thus allowing the enhanced binding toward targets [3]. Cyclic peptides can be used as a therapeutic, drug delivery tool, surfactant, imaging agent, biosensor or antimicrobial agent. Bicyclic peptides, compared with monocyclic peptides, have additional advantages, including higher conformational rigidity, target binding affinity and selectivity, improved metabolic stability and better membrane permeability [4].

Natural bicyclic peptides are produced by different organisms. Romidepsin, actinomycin D, moroidin and celogentin C are some examples of natural bicyclic peptide that have different activities – like inhibition of tubulin polymerization, prevention the polarization of actin filaments, inhibition of RNA polymerase II, among others 4, 5, 6. Bicyclic peptide libraries include many bicyclic peptides with high diversity. Combinatorial library technologies have recently been developed to rapidly synthesize and screen large bicyclic peptide libraries for ligands against specific targets. Two categories can be mentioned for bicyclic peptide libraries: biologic and synthetic. Some peptide libraries are restricted to peptidogenic amino acids, whereas other libraries can contain nonpeptidogenic amino acids, such as β- or γ-amino acids, o-amino acids or non-natural amino acids. Nowadays, different methods like phage display method, split-intein circular ligation of peptides and
proteins (SICLOPPS) and mRNA display are used for bicyclic peptide library fabrication [7]. In this review, we will discuss types, preparation and application of bicyclic peptides as next-generation therapeutics.

Bicyclic peptide features and types

Bicyclic peptides are a class of polypeptides with two macrocyclic rings that have advantages over linear and monocyclic peptides. Their size, structure, functionality and increased conformational rigidity improve their proteolytic stability in the blood circulation, binding properties and offer better tissue penetration, widening their application routes [1]. In addition, each ring in this bicyclic structure can function independently, allowing these peptides to be bifunctional [8]. Bicyclic peptides potentially have some of the favorable properties of monoclonal antibodies (high target specificity and affinity) and small molecules (stability and good tissue penetration) and, therefore, might be appropriate candidates for the progress of therapeutics with low toxic profiles against a diverse set of targets [9]. These molecules are classified into two groups: natural and synthetic. There are different types of bicyclic peptides in nature, exhibiting various biological activities. Moroidin, celogentins A–K, phallotoxins and amatoxins are typical examples of natural bicyclic peptides. Also, the various activities of naturally occurring bicyclic peptides suggest that synthetic bicyclic peptides can mimic some biological activities [1].

Natural bicyclic peptides

Many bicyclic peptides with various biological activities are naturally produced by different organisms (Table 1). One of these naturally occurring peptides is romidepsin, which is extracted from the bacterium Chromobacterium violaceum [10]. Because this bicyclic peptide inhibits histone deacetylase, it is used as an anticancer agent in T cell lymphoma [11]. There is also a class of bicyclic heptapeptides consisting of at least seven compounds (phalloidin, prophalloin, phalloin, phallisin, phallacidin, phallacin and phallisacin) isolated from Amanita phalloides (the death cap mushroom) [12]. Phallotoxins bind actin, specifically at the interface between F-actin subunits, and inhibit the depolarization of actin filaments 13, 14. Fluorescent derivatives of phalloidin have mainly been used to localize and visualize actin filaments in cells [15]. Vitilevuamide, a cytotoxic compound isolated from two marine ascidians, is a bicyclic 13-amino-acid peptide. This naturally occurring bicyclic peptide is very toxic in mammalian cell lines and inhibits tubulin polymerization [5]. Owing to the appealing features of bicyclic peptides, several strategies have been examined to generate synthetic bicyclic peptides for various applications.

Table 1. Examples of naturally occurring bicyclic peptides

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Mechanism of action</th>
<th>Activity</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D</td>
<td>Streptomyces parvulus</td>
<td>Interfering with mRNA synthesis</td>
<td>An antineoplastic antibiotic</td>
<td>[6]</td>
</tr>
<tr>
<td>Moroidin</td>
<td>Celosia argentea</td>
<td>Inhibitor of tubulin polymerization</td>
<td>Antimitotic activity</td>
<td>[57]</td>
</tr>
<tr>
<td>Celogentin C</td>
<td>C. argentea</td>
<td>Inhibitor of tubulin polymerization</td>
<td>Antimitotic activity</td>
<td>[58]</td>
</tr>
<tr>
<td>Phalloidin</td>
<td>Death cap mushroom (Amanita phalloides)</td>
<td>Preventing the polarization of actin filaments</td>
<td>Inhibition of cytokinesis and cytotaxis</td>
<td>[15]</td>
</tr>
<tr>
<td>α-Amanitin</td>
<td>A. phalloides</td>
<td>Inhibition of RNA polymerases, in particular RNA polymerase II</td>
<td>Cytotoxicity</td>
<td>[59]</td>
</tr>
<tr>
<td>Theonellamide F</td>
<td>Marine sponge (Theonella)</td>
<td>Inhibits growth of various pathogenic fungi</td>
<td>Cytotoxicity</td>
<td>[60]</td>
</tr>
</tbody>
</table>
Synthetic bicyclic peptides
The diverse biological functions of naturally occurring bicyclic peptides and their potential in the development of novel therapeutics have encouraged scientists to generate synthetic bicyclic peptides with desired activities. Consequently, the first synthetic bicyclic heptapeptide was synthesized in 1978, which was structurally similar to phallotoxins 1, 16. Vancomycin, a branched tricyclic glycopeptide antibiotic, is produced by the actinomycete Amycolaptosis orientalis and is commonly used for the treatment of serious bacterial infections. Recently, Yang et al. reported a bicyclic tripeptide synthesis as a mimic of the ABC ring system of vancomycin using the ruthenium-based cyclization chemistries [17]. In another study, Rodriguez and co-workers designed and synthesized a polymyxin-based bicyclic peptide by developing a new solid-phase synthetic approach and showed that this synthetic bicyclic peptide has the potential to be used as a source for the design and generation of novel and more-effective carbohydrate receptors for bioanalytical or medicinal applications [18].

Also, Dehua Pei and colleagues improved the cell permeability of protein tyrosine phosphatase 1B (PTP1B) inhibitor 2 by a bicyclic system in which the cyclo(FΦRRRRQ) (cFΦR4, where Φ is L-naphthylalanine) as a novel class of cell-penetrating peptide (CPP) is placed in one ring and the target-binding sequence constitutes the other ring. Fusion of cyclic PTP1B inhibitor 2 with cFΦR4 as the cyclic cell-penetrating peptide produces bicyclic peptides that are cell permeable and retain the ability to recognize specific intracellular targets [19].

Chemical methods for cyclization of peptides
To date, several strategies have been used to form synthetic bicyclic peptides. The first strategy was reported to generate a bicyclic tryptathionine heptapeptide in 1978 by Zanotti et al. [16]. In this method, the first step of cyclization involved formation of a thioether bond between a cysteine residue and a derivative of L-tryptophan, which was generated by oxidation with peroxyacetic acid. The second cyclization (head-to-tail cyclization) was performed by the mixed anhydride method.

Kemp and McNamara described syntheses of the conformationally restricted cyclic nonapeptides cyclo-(Gly-L-Cys(Bzl)-Gly)3, cyclo-(L-Pr-Gly-L-Cys(Meb))3 and cyclo-(L-Cys(Meb)-LL-Acp)3, in which Meb = p-methoxybenzyl and Acp = 3-amino-2-piperidone-6-carboxylic acid. In this method, a ring-forming reaction was performed by 1,3,5-tris(bromomethylene)benzene after removal of the S-blocking groups from each compound [20].

Sun et al. reported an efficient approach to synthesize amphipathic bicyclic peptides in 2001. The linear peptide precursors were produced using the Boc/BOP protocol [21]. The sequences of these precursors contained an N-terminal cysteine, a C-terminal thioester and an internal cysteine residue. First, cyclization of unprotected peptides was performed using an on-resin intramolecular thioester ligation reaction between N-terminal Cys and C-terminal thioester (N-to-C cyclization). Finally, bicyclic peptides were obtained by forming an off-resin intramolecular disulfide bond between N-terminal cysteine and an internal cysteine residue by DMSO oxidation (Fig. 1a).
Various strategies for chemical synthesis of bicyclic peptides. (a) Synthesis through an on-resin intramolecular thioester ligation and an off-resin DMSO-mediated disulfide formation. (b) Synthesis by ring-closing metathesis. (c) Synthesis by formation of C–C bond (through an intramolecular palladium-catalyzed C–H activation process) between tryptophan and iodinated tyrosine or phenylalanine. (d) Synthesis through ring-closing alkyne metathesis. (e) Synthesis through the formation of an amide bond between the α- or γ-carboxyl group of glutamate and the N terminus, then subjected to amide-bond formation conditions to perform the second cyclization. Abbreviations: RCM, ring-close metathesis; RCAM, ring-close alkyne metathesis.

In another strategy, Ghalit et al. synthesized bicyclic mimics of the lantibiotic nisin by ring-closing metathesis. In these alkene-/alkane-bridged bicyclic peptides, the disulfide bond and thioether bridge were replaced with alkene staples (Fig. 1b) [22]. Later, Mendive-Tapia et al. reported a new stapling methodology for the synthesis of peptides containing a C–C bond between tryptophan and phenylalanine or tyrosine residues via a selective palladium-catalyzed C–H arylation process (Fig. 1c) [23]. Grandas and colleagues reported a straightforward synthesis of bicyclic peptides from cysteine-containing peptides. In this approach, they formed bicyclic peptides via an intramolecular Michael addition reaction between thiols and maleimides [24].

Also, Teixido et al. described solid-phase synthesis and characterization of N-methyl-rich peptides. Synthesis of high N-methylamino acid content peptides is challenging because the coupling of protected N-methylamino acids with N-methylamino acids generally occurs with low yields. They described (7-azabenzotriazol-1-yl oxy)tris(pyrrolidino) phosphonium hexafluorophosphate (PyAOP) or PyBOP/1-hydroxy-7-azabenzotriazole (HOAt) as the most promising coupling reagents for these couplings. Also, they showed that the time of cleavage of the peptide from the resin affected loss of Ac-N-methylamino acid when a peptide contains an acetylated N-methylamino acid at the N-terminal position [25].

Bartoloni et al. showed that bicyclic homodetic peptides can be obtained by solid-phase peptide synthesis and two subsequent cyclizations. The first on-resin cyclization was performed through the formation of an amide bond between the α- or γ-carboxyl group of glutamate and the N terminus. The resulting monocyclic peptides were then subjected to amide-bond formation conditions under high dilution to perform the second cyclization and to form bicyclic peptides (Fig. 1e) [26]. This strategy was also used to prepare bridged bicyclic peptides (BBPs) corresponding to the topology of bridged bicyclic alkanes, such as norbornane [27]. Recently, in 2016, Cromm and co-workers applied a chemically orthogonal ring-closing olefin (RCM) and alkyne metathesis (RCAM) system to generate GTPase-targeting bicyclic peptides (Fig. 1d) [28]. In addition to the above methods, a simple way to synthesize bicyclic peptides is the linking of two monocyclic peptides by different linkers. For
example, Oh et al. synthesized two different bicyclic peptides through linking monocyclic peptides using triazole and β-alanine (β-Ala) linkers [29].

Screening of bicyclic peptides from a random library

A bicyclic peptide library contains a great number of bicyclic peptides and provides a powerful tool for the isolation of bicyclic peptides against a target of interest for various applications. To build large libraries and increase the chances of identifying bicyclic peptides with desired properties, various chemical and biological methods have been developed.

Chemical methods

As mentioned in the previous section, the first chemically synthesized library of nine bicyclic peptides was created by Sun et al. using intramolecular thioester ligation and DMSO-mediated disulfide formation [21]. To synthesize larger libraries and increase the likelihood of finding peptides with desired properties, other methods should be developed. The one-bead-two-compound (OBTC) approach is a simple method used to design bicyclic libraries, in which each cyclic peptide is encoded on the same bead by the corresponding linear peptide [9].

One of the main advantages of chemical synthesis of bicyclic peptides is the possibility of the incorporation of nonproteinogenic amino acids and nonpeptidic moieties into a peptide structure, which can increase the diversity and stability of the peptide structure. Upadhyaya and co-workers devised an approach for generating and screening large bicyclic peptide libraries displayed on rigid small-molecule scaffolds. Bicyclic peptides produced by this method contained random residues of 24 different amino acids (including ten proteinogenic amino acids, four nonproteinogenic α-L-amino acids and ten α-D-amino acids) in each ring. After screening of a chemically synthesized library against tumor necrosis factor (TNF)α, they recognized an element that inhibits the TNFα–TNFR interaction [9]. In another study, Upadhyaya et al. synthesized a bicyclic peptide library in OBTC format according to the previous approach, screened against the K-Ras G12 V mutant and finally discovered several direct Ras inhibitors [30].

Biological methods

Another method for producing bicyclic peptides is biological production through ribosomal synthesis, which can generate large libraries with enormous diversity. Heinis et al. developed a phage strategy for generation of combinatorial chemical bicyclic peptide libraries and affinity selection [31]. In this strategy, they constructed a library of linear peptides using phage display. According to the phage display method, a library of peptides, including two sequences of six random amino acids flanked by three cysteines [Cys-(Xaa)6-Cys-(Xaa)6-Cys], was expressed on the surface of a filamentous phage. These linear phage-encoded peptides were tethered to the trifunctional compound tris(bromomethyl)benzene (TBMB) in a nucleophilic substitution reaction that alkylated the three Cys residues in the peptide sequences and converted the linear sequences into bicyclic peptides. Finally, they isolated potent human plasma kallikrein inhibitors (Ki = 1.5 nM) from a library of >10⁹ members [31].

In another study, Rebollo et al. produced bicyclic peptide phage libraries by combining dissimilar sized macrocyclic rings (Fig. 2). They cloned 14 phage-coded peptide libraries of the format Cys(Xaa)m-Cys-(Xaa)n-Cys, wherein Xaa are random amino acids and the numbers ‘m’ and ‘n’ were 3, 4, 5 or 6. The libraries were exposed to three rounds of affinity assortments against the cancer-associated protease uPA [32]. The phage display bicyclic peptide can also be generated through the formation of two disulfide bonds [33] but, in peptides with several cysteine residues, disulfide isomerization can lead to multiple peptides with various biological actions. Chen et al. developed a dithiol amino acid (Dtaa) that can make two disulfide bridges at a single amino acid site and showed that substitution of two cysteines by Dtaas can prevent the formation of different disulfide bond isomers [34].
Figure 2. Construction of a phage-encoded peptide library of the format CXC(X)5C(X)5C. The CXC motif can direct the folding of randomized peptides on the phage surface toward either a fused or bridged bicyclic structure. After iterative affinity selections, the selected peptide sequence can then be structurally reshaped into a specific bicyclic structure (either fused or bridged) through orthogonal Cys–Pen disulfide pairing technology (only two of the four Pen-substituted analogs were present) [35].

Very recently, Zheng et al. presented the de novo design of cysteine–penicillamine (Cys–Pen)-mixed peptide scaffolds, which can be folded into specific isomers [35]. The resulting sequence-independent peptide scaffolds, which were constrained with two noncanonical disulfide bonds, were isomerically pure and topologically formidable. Another phage-screening strategy for developing bicyclic peptides was reported by Zha et al., in which a phage-encoded peptide library of the format CXC(X)5C(X)5C was synthesized. The CXC motif can direct the folding of phage-displayed peptides toward either a fused or bridged bicyclic structure. Finally, the selected peptide sequences were structurally reshaped into bicyclic structures using orthogonal Cys–Pen disulfide pairing technology. They obtained a library of $4.5 \times 10^9$ independent transformants by transformation of the phagemid vector into Escherichia coli [36]. This strategy can be useful to develop bicyclic peptide ligands (Fig. 2).

Another display technique used for biological production of bicyclic peptides is mRNA display. In this cell-free technique, a covalent bond between each protein or peptide and its own encoding mRNA is formed using puromycin as a mimic of aminoacyl tRNA [37]. The mRNA–protein complex is reverse transcribed to cDNA and employed for in vitro selection. The DNA sequences encoding selected peptides can be analyzed by sequencing [38]. Sako et al. developed a novel approach to construct bicyclic peptide scaffolds by modification of the mRNA display method. They reprogrammed the genetic code to construct a peptide scaffold containing a cysteine (Cys) at the N terminus and three nonproteinogenic amino acids [4-(2-chloroacetyl) aminobutyric acid (Cab), Aha, azidohomoalanine (Aha) and Propargylglycine (Pgl)]. Post-translational cyclizations performed by crosslinking between two pairs of amino acids, Cys–Cab and Aha–Pgl [39]. In a method recently described by Hacker et al., two orthogonal cyclization steps (bisbromomethylbenzene cysteine alkylation and copper-mediated azide–alkyne cyclo addition) were used to make highly constrained bicyclic peptide libraries. In this strategy, the attachment points for each loop are independent, thereby enabling the formation of either manacle or theta-bridged bicyclic peptides, and in vitro selection of bicyclic peptides with desired characteristics can be done using mRNA display (Fig. 3) [40]. SICLOPPS is a split-intein-based technique for the intracellular construction of cyclic peptides [41]. The method of Bionda and Fasan: ribosomal production of bicyclic peptides in E. coli, involved the SICLOPPS method (for initial N-to-C circularization) combined with genetic incorporation
of O-(2-bromoethyl)-tyrosine (O2beY) (for an inter-side-chain thioether bond-forming reaction between a cysteine residue and O2beY to form the bicyclic peptide) (Fig. 4) [42].

![Image of mRNA display scheme.](image1)

Figure 3. mRNA display scheme. Comparative in vitro selections against streptavidin. PURE translation is started with puromycin-linked mRNA. mRNA–peptide fusions are bis-alkylated with α,α’-dibromo-m-xylene (green box) and immobilized on oligo (dT) resin to form the monocyclic peptides. The monocyclic fusion pool is split after reverse transcription and bound to nickel resin, where the CuAAC reaction is performed on the portion of the pool targeted for bicyclic peptide formation (blue box). The monocyclic and bicyclic fusion pools are then used to select for high-affinity binders to streptavidin. Following competitive elution with biotin, functional fusions were amplified via PCR to generate the enriched round 2 DNA library. All steps were performed independently for subsequent rounds [39].

![Image of ribosomal synthesis.](image2)

Figure 4. The approach to ribosomal synthesis of thioether-bridged bicyclic peptides in Escherichia coli. From N to C, the linear precursor polypeptide includes the split intein DnaE C-terminal domain (IntC), a Ser or Cys residue at IntC++1 site, the unnatural amino acid O-(2-bromoethyl)-tyrosine (O2beY or ‘Z’), a variable target sequence comprising the reactive cysteine and a DnaE N-terminal domain attached to a chitin-binding domain (IntN–CBD). The two intended paths to make the bicyclic product are indicated [41].

**Applications of bicyclic peptides in medicine**

Diverse biological activities of naturally occurring bicyclic peptides have inspired scientists to identify and synthesize new bicyclic peptides for various applications, such as therapeutic agents, drug targeting, imaging and diagnostics.

**Therapeutic agents**

**Enzyme inhibitors**

Some bicyclic peptides can act as enzyme inhibitors and be used as therapeutic agents in many diseases. For example, Jaulent and Leatherbarrow designed two potent bicyclic protease inhibitors by combining two advantageous features of two naturally occurring inhibitors: Bowman Birk inhibitor (BBI) and sunflower trypsin inhibitor (SFTI)-1. The resulting bicyclic and bifunctional protease inhibitors showed the potent inhibitory properties of the parent structures that are also relatively resistant to proteolysis; but they cannot inhibit two proteases at the same time [43].
In another study, two bicyclic peptide inhibitors of the serine protease urokinase-type plasminogen activator (uPA) were designed rationally by conversion of an established monocyclic peptide (upain-2) to bicyclic peptides. The affinities of two bicyclic peptide inhibitors were similar to upain-2, and isothermal titration calorimetry (ITC) data indicated that the bicyclic peptides reduced loss of entropy upon binding to uPA [44]. Islam et al. synthesized bicyclic tetrapeptide histone deacetylase (HDAC) inhibitors by designing and synthesizing CHAP31-based bicyclic tetrapeptide hydroxamic acids by altering the aliphatic loop length. The potent HDAC inhibitory activity of these nonaromatic bicyclic HDAC inhibitors was evaluated and confirmed in vivo and in vitro [45]. More recently, Teufel et al. discovered stable and long-lasting bicyclic peptide inhibitors against plasma kallikrein (PKal) using a combination of phage display and chemical cyclization. The efficacy of these peptides in inhibition of PKal activity was confirmed by different in vitro assays and in different biological matrices and demonstrated that bicyclic constrained peptides act as potent and highly specific PKal inhibitors that can be useful in the treatment of diabetic retinopathy and diabetic macular edema (DME) [46].

PPI inhibitors
Targeting and inhibition of PPIs has appeared as a novel approach for discovering and developing new therapeutics for complex and multifactorial diseases. Therefore, one of the main goals of developing bicyclic peptides is the development of inhibitors against PPIs; and, in this regard, several bicyclic peptide PPI inhibitors have been synthesized. For example, Grb2, a signaling adaptor, has a key role in receptor tyrosine kinase signaling pathways, and Grb2 inhibitors could be useful as anticancer drugs. A bicyclic peptide inhibitor of the SH2 domain of Grb2 was synthesized by conversion of a monocyclic peptide into bicyclic peptide, enhancing its potency and selectivity for Grb2 SH2 [8].

In another study, Lian et al. identified a bicyclic peptide inhibitor of the TNFα–TNFR interaction by screening of large combinatorial libraries of bicyclic peptides. The resulting bicyclic peptide PPI inhibitor can protect from cell death induced by TNFα [9]. In a recent study, Guardiola et al. designed metabolically stable bicyclic peptides targeting the epidermal growth factor (EGF)–EGFR interaction. EGF contributes to tumor progression and performs its function by binding to its receptor (EGFR), which initiates intracellular events leading to tumor development. A series of bicyclic-constrained peptides mimicking one of the interacting domains of EGFR was designed using a combination of in silico and biophysical methods. The inhibitory effect of these peptides on the EGF–EGFR interaction was established in a specific PPI-disruption assay [47]. These peptides also selectively decrease the viability of human cancer cells expressing a high level of EGFR, with mid-micromolar IC₅₀ values [47]. The efficacy of these peptides, in addition to their metabolic stability, makes them suitable as complementary therapeutics in cancer therapy.

Receptor agonist and antagonist
Some bicyclic peptides act as agonists or antagonists for cell surface receptors, which are either naturally occurring or rationally designed (Fig. 5a). For example, BI-32169 is a naturally occurring bicyclic 19-peptide isolated from Streptomyces sp. (DSM 14996) and is a potent human glucagon receptor antagonist [48]. Jia et al. developed a specific bicyclic peptide antagonist (EG3287) of vascular endothelial growth factor (VEGF)-A165 binding to neuropilin (NP)-1, which is a receptor for VEGF in endothelial cells. By characterization of this antagonist, they reported that the exon 8 of VEGF is very important for NP-1 binding, and NP-1 is needed for optimum kinase insert domain receptor (KDR) signaling [49].
Drug targeting
Bicyclic peptides can be ideal candidates for drug targeting owing to their small size and their ability to bind to different protein targets with good affinity and specificity (Fig. 5b). Moreover, they also exhibit better proteolytic stability and improved plasma stability compared with linear peptides 50, 51. Paclitaxel (PTX), a mitotic inhibitor, binds to tubulin and prevents the disassembly of microtubules and is used as a microtubule-stabilizing drug in cancer treatment. Chen et al. conjugated paclitaxel with a bicyclic peptide E[c(RGDyK)]2 (RGD) and evaluated its antitumor activity in a metastatic breast cancer cell line [52]. Because RGD peptide can selectively bind to αvβ3 integrin receptors, conjugation of PTX to RGD allowed its targeted delivery to tumor cells expressing αvβ3 integrin. Bicycle Therapeutics (Cambridge, MA) developed the bicyclic peptide platform based on bicyclic-peptide–drug conjugates (BDCs) for cell-specific targeting of chemotherapeutic agents. BT1718 is a Bicycle drug conjugate developed by Bicycle Therapeutics to target tumors [53]. BT1718 binds selectively to membrane type 1 matrix metalloprotease (MT1-MMP) with high affinity and is a promising compound to treat solid tumors. MT1-MMP is a tumor-associated membrane-anchored endopeptidase overexpressed in a variety of cancer cells. The results of recent studies show that BDCs can be developed as alternatives to antibody–drug conjugates and a superior strategy for targeted drug delivery [53].

Imaging and diagnosis (PET scan imaging)
Bicyclic peptides can develop into diagnostic imaging agents, especially as positron emission tomography (PET) tracers (Fig. 5c). The main advantages of bicyclic peptides over other protein binders (particularly monoclonal antibodies) are their faster clearance from the blood circulation owing to their smaller sizes, resulting in shorter imaging times delay, and better signal-to-noise ratio (SNR) [1]. Park et al. synthetized two bicyclic RGD peptides: c(RGD-ACP-K) and c(RGD-ACH-K), by incorporating aminocyclopentane (ACP) or aminocyclohexane (ACH) into a monocyclic RGD peptide. After conjugation to DOTA and radiolabeling with radiometal $^{64}$Cu, the efficiency of these peptides as PET radiotracers was evaluated for cancer imaging. Both bicyclic peptides and their DOTA conjugates exhibited high affinities toward U87MG glioblastoma cells, which are better than the monocyclic c(RGDyK). The corresponding $^{64}$Cu complexes showed excellent stability in human and mouse serum and high tumor uptake, as observed by PET, which demonstrated their promise as PET radiotracers for tumor imaging [54]. In another example, Eder et al. identified a bicyclic peptide targeting MT1-MMP (tumor-overexpressed matrix metalloproteinase) with subnanomolar affinity and showed the power of its radioconjugation as a tumor-targeting probe in an HT1080 xenograft mouse model. In vivo studies using different mouse xenograft models showed high affinity to MT1-MMP ($K_d$ at $\sim 1$ nM) and, compared to radiolabeled antibodies directed against the same target, showed rapid tumor penetration characteristics and high potency and chemical versatility [55].

As antimicrobial agents
Di Bonaventura et al. discovered that antimicrobial bridged bicyclic peptides (AMBPs) were active against Pseudomonas aeruginosa and its biofilm. Screening of all AMBPs for antibiofilm activity led to the
identification of two potent *P. aeruginosa* biofilm inhibitors that were enhancers of the activity of polymyxin. They used the concept of chemical space to discover their AMBPs and calculated their chemical space using 2DP – a 136-dimensional molecular fingerprint for peptides. These results suggest that bridged bicyclic peptides can be useful as a new class of antimicrobial peptides [56]. In a recent study, this group identified an antimicrobial bicyclic peptide with potent activity against several multidrug-resistant Gram-negative bacteria. They explored a virtual bicyclic peptide library using the concept of chemical space [57].

**Concluding remarks**

Bicyclic peptides (with two macrocyclic rings) have advantages over linear and monocyclic peptides owing to their size, structure, functionality and increased conformational rigidity. This class of polypeptides has some of the favorable properties of monoclonal antibodies and small molecules and, therefore, can have different therapeutic and diagnostic applications. Various chemical, biological and combinatorial methods have been developed to synthesize large bicyclic peptide libraries and increase the chances of identifying peptides with desired properties. Owing to the similarity of antibodies and peptides in the affinity and specificity to various proteins, it is anticipated that, in the future, peptides will play an important part as therapeutics, diagnostics agents and research tools rather than antibodies, because they have a less complex structure, greater metabolic stability and lower cost of production.

**References**


