Chapter 3
Biological Activities of Lasso Peptides and Structure–Activity Relationships

3.1 Biological Activities

Microcin J25 (MccJ25), which is currently considered as the archetype of lasso peptides, has been discovered in connection with its potent and narrow spectrum antibacterial activity directed mainly against enterobacteria and *Escherichia*. By contrast, several lasso peptides have been discovered through screening of specific biological activities against human targets, such as hormone receptor antagonism and enzyme inhibition. A detailed scope on this broad spectrum of biological activities including receptor antagonism, enzyme inhibition and antiviral and antimicrobial properties is described in this chapter. These activities are summarized in Table 3.1.

3.1.1 Receptor Antagonists

The lasso peptide RES-701-1, produced by *Streptomyces* sp., is a selective antagonist of the endothelin type B receptor ET_B (Tanaka et al. 1994). The endothelins (ETs) are a family of vasoactive peptides distributed in vertebrates and highly conserved within mammals (Yanagisawa and Masaki 1989; Masaki 2004), which share structural and functional homologies with the snake venom sarafotoxins (Ducancel 2005; Fig. 3.1a). In human, there are three members of this family, each with distinct gene and tissue distributions, the ET 1 (ET-1), 2 (ET-2) and 3 (ET-3; Yanagisawa et al. 1988; Dhaun et al. 2007). They all consist of 21 amino acid residues with two disulfide bridges at Cys3–Cys11 and Cys1–Cys15. ET-1, the predominant cardiovascular isoform, has been most extensively studied (Drawnel et al. 2013). It is involved in the physiological control of systemic blood pressure and body sodium homeostasis (Kohan et al. 2011), but also plays a role in several other processes such as vascular remodelling, angiogenesis or extracellular matrix synthesis (Rodriguez-Pascual et al. 2011). The ET system has been associated with a number of pathologies, in particular cardiovascular diseases (Ohkita et al. 2012; Kaoukis et al. 2013), kidney disease (Dhaun et al. 2012) and cancer (Rosano et al. 2013). The biological effects of ETs are mediated by at least two receptor subtypes,

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Table 3.1 Biological activities reported for lasso peptides

Name	Producer	Class[a]	Biological activities	References
Lasso peptides from actinobacteria				
Siamycin *I*/MS-271/ NP-06	*Streptomyces* sp.	I	Anti-HIV Antibacterial Inhibitor of myosin light chain kinase	(Chokekijchai et al. 1995; Detlefsen et al. 1995; Tsunakawa et al. 1995; Lin et al. 1996; Yano et al. 1996)
Siamycin II	*Streptomyces* sp.	I	Anti-HIV Antibacterial	(Constantine et al. 1995; Tsunakawa et al. 1995)
RP 71955/ Aborycin	*Streptomyces sp.*	I	Anti-HIV Antibacterial	(Helynck et al. 1993; Potterat et al. 1994)
Sviceucin/ SSV-2083	*Streptomyces sviceus*	I	Antibacterial	(Ducasse et al. 2012a)
Anantin	*Streptomyces coerulescens*	II	Atrial natriuretic factor antagonist	(Weber et al. 1991)
Propeptin	*Microbispora* sp.	II	Prolyloligopeptidase inhibitor Weakly antibacterial	(Kimura et al. 1997a)
Lariatin	*Rhodococcus jostii*	II	Antimycobacterial	(Iwatsuki et al. 2006)
Sungsanpin	*Streptomyces* sp.	II	Inhibitory activity in a cell invasion assay with a lung cancer cell line	(Um et al. 2013)
BI-32169		III	Glucagon receptor antagonist	(Potterat et al. 2004; Knappe et al. 2010)
Lasso peptides from proteobacteria				
RES-701-1 RES-701-3	*Streptomyces* sp.	II	Endothelin type B receptor antagonist	(Tanaka et al. 1994; Ogawa et al. 1995)
Microcin J25 (MccJ25)	*Escherichia coli*	II	Antibacterial RNA polymerase inhibition	(Salomón and Farías 1992; Bayro et al. 2003; Rosengren et al. 2003; Wilson et al. 2003)
Capistruin	*Burkholderia thailandensis*	II	Antibacterial RNA polymerase inhibition	(Knappe et al. 2008; Knappe et al. 2009)
Astexin-1	*Asticcacaulis excentricus*	II	Antibacterial	(Maksimov et al. 2012)

[a] The classification refers to the number of disulfide bridges that further stabilize the lasso structure and has been described in Chap. 2. Classes I, II and III are characterized by two, zero and one disulfide bridge(s), respectively

ET_A and ET_B. The ET_A and ET_B receptors are G protein-coupled receptors (GPCRs) with seven transmembrane domains. The ET_A receptor binds ET-1 and ET-2 with an affinity two orders of magnitude higher than that for ET-3 (K_i ~0.01–0.1 and 1–3 nM, respectively), while the ET_B receptor binds all three isoforms with similar affinity (K_i ~0.01–0.02 nM; Williams et al. 1991; Schiffrin 2001). In blood vessels,

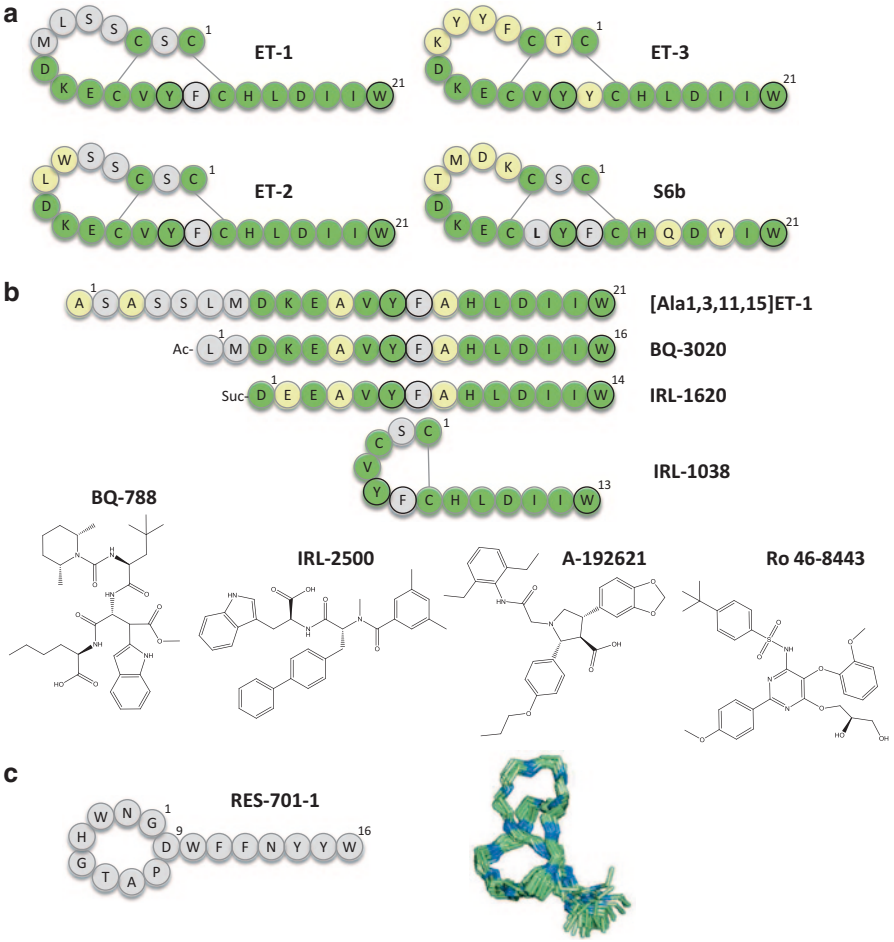


Fig. 3.1 ETs and selective antagonists of the ET_B receptor. **a** Primary structures of ET-1, ET-2, ET-3 and related sarafotoxin b (S6b; adapted from Fagan et al. 2001). **b** Selective antagonists of the ET_B receptor (adapted from Mazzuca and Khalil 2012). The amino acids conserved between ET-1, ET-2 and ET-3 are coloured in *green*. The residues proposed to constitute the pharmacophore of ET-1 are circled in *bold*. The amino acids different from those of ET-1 are shown in *yellow*. **c** Primary and secondary structures of RES-701-1. (The latter has been kindly provided by Tomoaki Kuwaki, Kyowa Hakko Kirin Company; Katahira et al. 1995)

the ET_A receptor, mainly produced in vascular smooth muscle cells, mediates vasoconstriction and cell proliferation, while the ET_B receptor, mainly produced by endothelial cells, mediates vasodilatation and ET-1 clearance (Mazzuca and Khalil 2012; Ohkita et al. 2012).

The general structure of ETs contains a cystine-stabilized α-helix motif in the N-terminal region of the 21-residue sequence, which consists of a β-turn followed by an α-helix (Tamaoki et al. 1991; Takashima et al. 2004a). Comparison

of the structures provided by nuclear magnetic resonance (NMR) and X-ray crystallography has revealed important differences in conformation, especially in the C-terminus (Wallace et al. 1995). X-ray indicated an α-helix structure in the 9–20 region (Janes et al. 1994; Janes and Wallace 1994), while NMR showed that the 16–21 C-terminal region has an extended β-structure and is loosely looped back to the 9–15 α-helix by a turn (Takashima et al. 2004a). The C-terminus is crucial for the activity of ET-1 (Kimura et al. 1988; Nakajima et al. 1989). Several spectroscopic studies have indicated that this residue in close proximity to the rings of Tyr 13 and Phe 14 forms a hydrophobic core (Takashima et al. 2004a; Takashima et al. 2004b), which could be critical for the mechanism of action.

In the past 20 years, numerous antagonists of ETs have been developed for the treatment of cardiovascular diseases (Dhaun et al. 2007; Kaoukis et al. 2013) and for cancer therapy (Rosano et al. 2013). In particular, the mixed $ET_{A/B}$ receptor antagonist bosentan and the selective ET_A receptor antagonist sitaxsentan have been used clinically for the treatment of pulmonary artery hypertension (Anderson and Nawarskas 2010), while the ET_A receptor antagonists atrasentan and zibotentan or the mixed $ET_{A/B}$ receptor antagonist macitentan have demonstrated potential anticancer activity in preclinical and ongoing clinical studies (Rosano et al. 2013). Many antagonists developed have close assembling of their aromatic rings, suggesting that the residues Trp21, Phe13 and Tyr14 of ET-1 define a pharmacophore (Remuzzi et al. 2002; Funk et al. 2004; Takashima et al. 2004b; Fig. 3.1a).

Selective ET_B receptor antagonists appear less promising for therapeutic applications, although certain positive effects have been reported (Lahav et al. 1999). Such inhibitors provide anyway a very important tool to better understand the physiological and physiopathological role of this receptor (Mazzuca and Khalil 2012; Ohkita et al. 2012). Several peptidic and non-peptidic selective antagonists of the ET_B receptor have been described (Mazzuca and Khalil 2012; Fig. 3.1b). In 1994, Tanaka et al. reported the potent antagonist effect of RES-701-1 (Fig. 3.1c) on the ET_B receptor (IC_{50} 10 nM). This effect was measured from competitive experiments in the presence of ^{125}I-labelled ET-1 on bovine cerebellar membranes as well as on membranes from Chinese hamster ovary (CHO) cells expressing the ET_B receptor. RES-701-1 was also shown to block the ET_B receptor-mediated responses such as (1) increase in the intracellular calcium concentration (in COS-7 cells expressing the ET_B receptor) and (2) blood pressure response to exogenously administered ET-1 in anaesthetized rats. By contrast, RES-701-1 did not show any antagonist effect on the ET_A receptor ($IC_{50} > 5$ μM) as well as on various receptors (for adrenaline; dopamine; histamine; acetylcholine; serotonin; atrial natriuretic peptide (ANP), angiotensin II; $IC_{50} > 1$ μM; Tanaka et al. 1994). The antagonist effect of RES-701-1 on the ET_B receptor was confirmed on different animal models (dog, rabbit, pig, guinea pig, rat; Tanaka et al. 1995). However, the IC_{50} value was much weaker in rats (in the 1 μM range), which rendered this animal model delicate for examining the role of ET_B receptor using RES-701-1 as antagonist. The use of RES-701-1 participated in different advances in the understanding of the physiology of the ET_B receptor (Conrad et al. 1999; Miasiro et al. 1999; Gandley et al. 2001; Yamaguchi et al. 2003; Gardner et al. 2005; Cervar-Zivkovic et al. 2011; Ji et al. 2013). The

peptide RES-701-3 showed an antagonist activity similar to that of RES-701-1 (Ogawa et al. 1995), but was not used very much in further studies.

Although there is no amino acid sequence similarities between RES-701-1 and the ETs, they share several properties (Tanaka et al. 1994): (1) a C-terminal tryptophan residue, which is crucial for the activity of ET-1 (Kimura et al. 1988); (2) a hydrophobic core near the C-terminus; and (3) a highly restrained structure (lasso scaffold for RES-701-1, two-disulfide bridge scaffold for the ETs, see Fig. 3.1a, c). The peptidic nature of certain ET antagonists has limited their therapeutic applications due to proteolytic degradation in the gastrointestinal tract and circulatory system (Attina et al. 2005). The sequence of RES-701-1 has been used to design bioactive peptides with higher stability towards proteolysis (Shibata et al. 2003). In addition, hybrid peptides constructed from RES-701-1 and ETs permitted to modulate the selectivity towards ET receptors (Shibata et al. 1998). This suggests a high biotechnological interest of this peptide. This aspect will be developed in Chap. 5.

The lasso peptide anantin, produced by *Streptomyces coerulescens,* was described as the first microbially produced antagonist of the ANP (Weber et al. 1991). Natriuretic peptides (NPs) are hormones involved in the maintenance of osmotic and cardiovascular homeostasis (Brenner et al. 1990; Drewett and Garbers 1994; McGrath et al. 2005; Potter et al. 2006; Pandey 2011). They are distributed in vertebrates including mammals, amphibians, reptiles and fishes (Takei 2000) and homologous peptides are found in plants (Vesely and Giordano 1991; Gehring and Irving 2013). In human, there are three main members of this family, whose precursors are encoded by separate genes: ANP, a 28-residue peptide also known as atrial natriuretic factor (ANF), B-type NP, a 32-residue peptide also known as brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), composed of 22 amino acids (Potter et al. 2006). The three peptides contain a well-conserved 17-residue disulfide-linked ring (Fig. 3.2a).

The activities of NPs are mediated by three dimeric single-span transmembrane receptors, mainly NPR-A, NPR-B and NPR-C (Potter et al. 2006). NPR-A and NPR-B contain an intracellular domain consisting of a protein kinase-like, adenosine triphosphate (ATP)-dependent regulatory domain and a guanylyl cyclase catalytic domain (Misono et al. 2011). NPR-C has a short 37-amino acid intracellular domain with no guanylyl cyclase activity and has been proposed to be a clearance receptor modulating the plasma levels of NPs (Maack et al. 1987; Fuller et al. 1988). NPR-C is the most promiscuous of the three receptors, binding to all NPs with high affinity, while NPR-A and NPR-B are more specific towards their own spectrums of ligands (He et al. 2005). The rank order of affinities between NPs and their receptors are ANP (K_d in the pM range) > BNP » CNP (K_d > 500 nM) for NPR-A, CNP (K_d in the pM range) » ANP > BNP (K_d in the nM range) for NPR-B and ANP ($K_d \sim 2$ pM) > CNP > BNP ($K_d \sim 15$ pM) for NPR-C (Bennett et al. 1991; Koller and Goeddel 1992; Suga et al. 1992). The well-conserved 17-residue disulfide-linked ring is required for activity of NPs (Bovy 1990; Brenner et al. 1990), while the flanking residues outside the ring can modulate their affinity to receptors (Cunningham et al. 1994; Schoenfeld et al. 1995). The crystal structure of the NPs has been solved in complex with the extracellular domains of their receptors

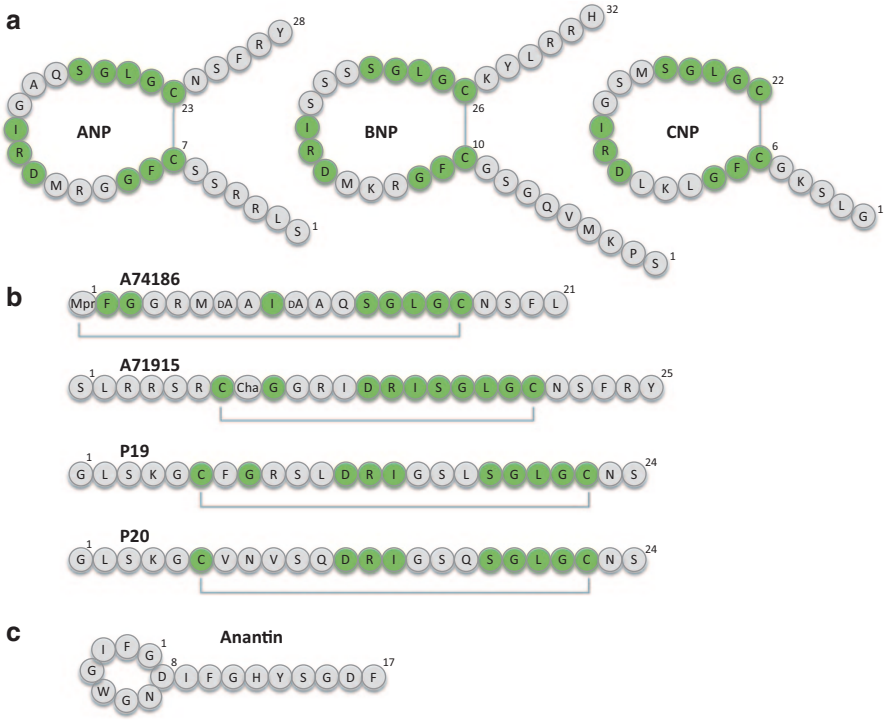


Fig. 3.2 Natriuretic peptides and antagonists of their receptors. **a** Primary structures of ANP, BNP and CNP (adapted from Potter et al. 2006). **b** Selected other peptidic antagonists of natriuretic peptide receptors: A74186 (von Geldern et al. 1990), A71915 (Delporte et al. 1992), P19 and P20 (Deschênes et al. 2005). Cha: cyclohexylalanine. Mpr: 3-mercaptopropionic acid. The amino acids conserved between natriuretic peptides are coloured in *green*. **c** Primary structure of anantin

(He et al. 2001; Ogawa et al. 2004; He et al. 2005; He et al. 2006). It shows a disk-like shape in an extended conformation, with not remarkable stabilizing intramolecular interactions (He et al. 2006). Only a few structural data are available for the free peptides, which are mostly unordered in aqueous solution and display a high conformational variability (Papaleo et al. 2010).

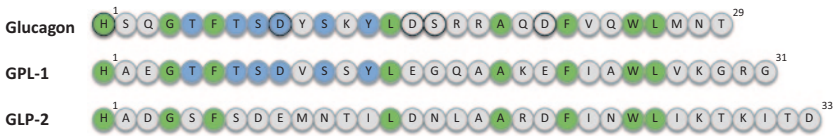
ANP is secreted by the atrium of the heart in response to blood volume expansion. It elicits natriuretic, diuretic and vasorelaxant effects, thereby reducing blood volume and pressure (Potter et al. 2006; Misono et al. 2011; Pandey 2011). It also displays anti-fibrosis, anti-proliferative and anti-hypertrophic effects and is involved in the remodelling of the heart and vascular system. ANP binding to NPR-A leads to the activation of the guanylyl cyclase catalytic domain, yielding accumulation of cyclic guanosine monophosphate (cGMP; Duda 2010). The physiological effects of the peptide are then elicited through three classes of cGMP-binding proteins: cGMP-dependent kinases, cGMP-regulated phosphodiesterases and cyclic nucleotide-gated ion channels (Potter et al. 2006). ANP (together with BNP) has expanding applications in diagnosis and biomarkers-guided therapy for cardiovascular and kidney diseases (Silver 2006; Motiwala and Januzzi 2013).

The main antagonist described for NP receptors are analogues to this class of hormones (von Geldern et al. 1990; Delporte et al. 1992; Cunningham et al. 1994; Deschênes et al. 2005; Fig. 2.2b). In addition, several non-peptidic inhibitors have been proposed, such as the fungal polysaccharide HS-142-1 (Morishita et al. 1991; Poirier et al. 2002), the indole derivative isatin (Glover et al. 1995) and the monoclonal antibody 3G12, the latter being specific to NPR-B (Drewett et al. 1995). Given the positive effects of NPs, the interest of receptor antagonists resides mainly in providing a tool to better understand the physiology of the natriuretic system. In 1991, Weber et al. showed that anantin (Fig. 3,2c), a 17-residue peptide predicted as having the lasso topology, but for which the three-dimensional structure has not been published, binds competitively to ANP receptors from bovine adrenal cortex and inhibits the intracellular cGMP accumulation in bovine aorta smooth muscle cells, in a dose-dependent manner (Weber et al. 1991). This effect was measured from competitive experiments in the presence of ^{125}I-labelled ANP on bovine adrenal cortex membranes. The IC_{50} value was 1 µM, which is 4,000-fold less potent than rat ANP (103–126), and the K_d deduced from the competition curves was 0.61 µM. Des-phe-anantin, a side product of anantin missing the C-terminal Phe17, was 50 times less potent than anantin. In 1993, Trachte reported that anantin had no antagonist properties on the neuromodulatory effects of ANP, showing that this activity does not rely on cGMP production (Trachte 1993). This effect was later attributed to the receptor NPR-C (Trachte 2005). Therefore, anantin is recognized as a selective antagonist of the guanylyl cyclase NP receptor of ANP, i.e. NPR-A.

Anantin has been used extensively as an antagonist to investigate the role and molecular mechanisms of the natriuretic system (recent selection: Citarella et al. 2009; Abraham et al. 2010; Hrometz et al. 2011; Baetz et al. 2012; Bian et al. 2012; Maeda et al. 2013; Vilotti et al. 2013) and is cited in more than 100 patents. However, most of the studies reported have used commercially available versions of anantin, which are peptides obtained by solid-phase synthesis. Depending on the providing company, these peptides are either linear or branched cyclic (i.e. with the macrolactam ring), but they cannot display a lasso topology since this specific fold has never been obtained by chemical synthesis, as described in Chaps. 2 and 4. Although the three-dimensional structure of anantin has not been resolved, it is highly probable that it adopts a lasso structure. Therefore, the activities reported for the linear and branched-cyclic variants cannot be attributed to anantin sensu stricto. These synthetic peptides display antagonist activities on NPR-A. Therefore, the lasso topology appears not to be a requisite for this activity. However, a comparative study of the affinities of the lasso, branched-cyclic and linear variants of anantin for NPR-A would be necessary to better understand the structure/activity relationships of these peptides and use the most relevant form as an antagonist in the future.

The lasso peptide BI-32169, produced by *Streptomyces* sp., is a strong antagonist of the glucagon receptor (Potterat et al. 2004; Knappe et al. 2010). Glucagon is a 29-amino acid peptide hyperglycaemic hormone. Its protein precursor proglucagon is encoded by a gene distributed in vertebrates and highly conserved within mammals (Irwin 2001). In mammals, proglucagon is converted into three distinct structurally related peptides, glucagon, glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2; Fig. 3.3a). These peptides play essential roles in the

a



b

Name	His1	Asp9	Lys12	Cter
Des-His-glucagon	removed			
[1-N α-trinitrophenylhistidine, 12-homoarginine]-glucagon	α-trinitrophenyl histidine		homoarginine	
Des-His^{1}[Glu^{9}]glucagon amide	removed	Glu		amidated

c

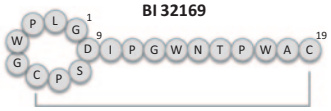


Fig. 3.3 Glucagon peptides and antagonists of their receptors. **a** Primary structures of glucagon, GLP-1 and GLP-2. The amino acids conserved between glucagon, GLP-1 and GLP-2 are coloured in *green*; those conserved between glucagon and GLP-1 only are shown in *blue*. The amino acids in glucagon important for receptor binding and/or signal transduction are circled in *bold*. **b** Table showing the positions modified from glucagon in selected peptidic antagonists of the glucagon receptor (Cho et al. 2012): des-His glucagon (Goldfine et al. 1972), [1-N α-trinitrophenylhistidine, 12-homoarginine]-glucagon (Bregman et al. 1980), des-His^{1}[Glu^{9}]glucagon amide (Unson et al. 1991). **c** Primary structure of BI 32169 (see secondary structure in Fig. 3.2)

regulation of carbohydrate, lipid and amino acid metabolisms and act on separate receptors (Bataille 1996; Drucker 2001). Glucagon is synthesized and secreted mainly by the β cells of the pancreas. It counteracts hypoglycaemia and opposes insulin actions by stimulating hepatic glucose synthesis and mobilization, thereby increasing blood glucose concentration (Quesada et al. 2008). In diabetes, the balance of glucose fluxes is disturbed, partly as a result of inappropriate glucagon secretion (Unger and Orci 1975; Gosmain et al. 2013). As previously mentioned for ETs, discrepancies between the structures of glucagon derived from X-ray crystallography and NMR have been reported. While the crystal structure of glucagon obtained in 1975 revealed a helical conformation (Sasaki et al. 1975), NMR structural analyses indicated that glucagon was unordered in aqueous solution (Braun et al. 1983). It is now established that most class B ligands show little, if any, ordered structure in aqueous solutions but can form α-helices in the presence of organic solvents, or lipids, or upon crystallization (Parthier et al. 2009).

The receptors of glucagon GLP-1 and GLP-2 are seven transmembrane-spanning proteins, all belonging to the class B of GPCRs (Harmar 2001), and more specifically to the glucagon receptor family (Mayo et al. 2003). GPCRs from class B are characterized by (1) a long extracellular N-terminal domain with three conserved disulfide bridges and large extracellular loops that form multiple binding pockets for peptide ligands and (2) a disulfide bond linking Cys residues from the first and second extracellular loops (Harmar 2001; Siu et al. 2013). The N-terminal

extracellular domain is responsible for the high affinity and specificity of hormone binding, while the core domain (containing the seven transmembrane helices) is required for receptor activation and signal coupling to the downstream G protein (Hoare 2005; Parthier et al. 2009; Pal et al. 2012). The glucagon receptor family has a highly conserved aspartate at position 63 in the N-terminal extracellular domain and a conserved region within the seventh transmembrane domain (FQG-hydr-hydr-VAx-hydr-YCFx-EVQ, "hydr" being a hydrophobic residue and "x" any amino acid, at position 391–408; the amino acid numbering corresponds to the human sequence of the glucagon receptor; Mayo et al. 2003; Authier and Desbuquois 2008). The N-terminal extracellular domain adopts a globular structure conserved within the family, termed the "glucagon hormone family recognition fold" (Parthier et al. 2009). It consists of one N-terminal α-helix followed by two antiparallel β-sheets and is stabilized by the three intramolecular disulfide bridges. Hormone recognition by class B GPCRs is believed to follow a "two domain model" of binding, in which the C-terminal portion of the ligand is captured by the receptor extracellular domain and the N-terminal portion of the ligand is delivered to the membrane-bound domains of the receptor, where it interacts with extracellular loops and the transmembrane α-helices (Hoare 2005; Parthier et al. 2009).

The glucagon receptor is mainly expressed in liver and kidney (Rodbell et al. 1971; Jelinek et al. 1993; Authier and Desbuquois 2008). Its activation results in the stimulation of the adenylyl cyclase, via the heterodimeric G protein (Birnbaumer 2007), which yields increase of the concentration of intracellular cyclic adenosine monophosphate (cAMP) and subsequent activation of protein kinase A signalling. In addition, glucagon stimulates the phospholipase C-inositol phosphate pathway in hepatocytes, inducing intracellular Ca^{2+} signalling (Wakelam et al. 1986). Extensive structure/activity relationship studies have permitted to identify the residues or the regions essential for ligand binding and specificity, and signal transduction (Carruthers et al. 1994; Buggy et al. 1995; Buggy et al. 1997; Cypess et al. 1999; Unson et al. 2002; Runge et al. 2003a, b). Glucagon binding requires specific segments of the extracellular N-terminal domain (the conserved Asp63 residue together with the segments 102–116 and 125–136), of the first extracellular loop (Arg201, sequence 205–218) and of the third, fourth and sixth transmembrane domains (Authier and Desbuquois 2008). The residues His1, Asp9, Asp15, Ser16 and Asp21 of glucagon are important for either receptor binding or signal transduction (Lin et al. 1975; Unson et al. 1991; Unson et al. 1993; Unson and Merrifield 1994; Unson et al. 1994b). The recent crystal structure reported for the seven-transmembrane helical domain of the human glucagon receptor, complemented by extensive site-directed mutagenesis, and the subsequent structure model proposed for the glucagon-bound receptor (Siu et al. 2013; Fig 3.3b) permitted to confirm these trends. This study proposes that glucagon binding to its receptor has a helix structure, and clearly identifies the binding sites. It reveals that the first transmembrane helix of the glucagon receptor has a "stalk" region, which positions the extracellular domain relative to the membrane to form the glucagon-binding site that captures the peptide and facilitates the insertion of its N-terminus into the seven transmembrane domain, in agreement with the "two-domain" model (Hoare 2005; Parthier et al. 2009).

The therapeutic interest of inhibiting glucagon signalling for the treatment of diabetes and obesity (Bagger et al. 2011; Unger and Cherrington 2012) has led to extensive research of competitive antagonists of the glucagon receptor (Cho et al. 2012). Many peptide antagonists have been described, most of which are analogues of glucagon, modified at positions critical for binding or activation of the receptor, such as [1-N α-trinitrophenyl histidine, 12-homoarginine]-glucagon (Bregman et al. 1980; Johnson et al. 1982) and des-His1-[Nle9-Ala11-Ala16]-glucagon amide (Unson et al. 1994a; Fig. 3.3b). In addition, chimeric peptides have been designed to generate molecules capable of modulating both the receptors of glucagon and GLP-1 (Pan et al. 2006; Claus et al. 2007). Since the first report of a non-peptide agonist in 1998 (Madsen et al. 1998), small molecule antagonists have arisen a high interest and several have been validated in preclinical models of type-2 diabetes (Shen et al. 2011). These compounds exhibit a variety of structural motifs that are reviewed in two recent review articles (Shen et al. 2011; Cho et al. 2012).

Potterat et al. (2004) reported that BI-32169 (Fig. 3.3c) exhibits a strong inhibitory activity against glucagon-induced cAMP elevation, with an IC_{50} value of 440 nM (Potterat et al. 2004). Its C-terminal methyl ester derivative also displayed antagonist activity (IC_{50} 320 nM). The inhibitory activity of BI-32169 and its derivative was assessed in a BHK-21 cell line stably transfected with a plasmid construct coding for the human glucagon receptor. Both compounds were found to be selective for the human glucagon antagonist versus the human GLP-1 receptor. BI-32169 is the first antagonist of peptidic nature having a sequence that is not derived from glucagon. Glucagon and BI-32169 are very different in terms of primary and secondary structures, and thus the mechanisms involved in the antagonist properties of BI-32169 are not understood and have not been investigated until now. Since peptide antagonists of the glucagon receptor appear less attractive than small molecules for therapeutic applications, due to a general lower stability, the lasso topology and its particular structural properties (see Chap. 2) could provide an attractive scaffold to develop new peptide antagonists with enhanced stability. Therefore, analyzing the pharmacokinetic and pharmacodynamic properties of BI-32169 together with its structure/activity relationships is of high interest for receptor antagonist drug design.

3.1.2 Enzyme Inhibitors

The lasso peptide MS-271 (formerly known as siamycin I; Tsunakawa et al., 1995), produced by *Streptomyces* sp., is an inhibitor of smooth muscle myosin light chain kinase (MLCK; Yano et al. 1996). MLCK is a Ca^{2+}/calmodulin-dependent kinase, distributed in higher vertebrates. In human, different isoforms derived from three different genes and resulting from alternative splicing or alternative initiation sites have been reported. These isoforms are named according to their pattern of expression. The skeletal and cardiac isoforms, mainly expressed in the skeletal and cardiac muscle, respectively, derive each from a single gene (*mylk*2 and *mylk*3, respectively). The smooth muscle isoform (or short isoform) and non-muscle isoform

(or long isoform), mainly expressed in the smooth muscle and non-muscle cells, respectively, derive from a single gene (*mylk*1) and result from alternative initiation sites (Hong et al. 2011). The smooth muscle isoform of MLCK is composed of an actin-binding domain, a proline-rich region and a fibronectin domain (whose functions are unknown), a kinase domain (the catalytic domain), a calmodulin-binding domain (the regulatory domain), an auto-inhibitory domain and a C-terminal immunoglobulin domain (Hong et al. 2011). MLCK is inactive when not bound to Ca^{2+}/calmodulin (auto-inhibited state). Upon binding to Ca^{2+}/calmodulin, the auto-inhibitory domain is displaced from the kinase domain, thereby allowing substrate access. The kinase domain binds to ATP and phosphorylates residue Ser19 (and subsequently Thr18) in the regulatory light chain of myosin II (Hirano et al. 2003). This phosphorylation increases the ATPase activity of myosin II and is thought to play major roles in a number of biological processes, including smooth muscle contraction, through the interaction of activated myosin II with actin filaments (Takashima 2009). MLCK (and in particular the non-muscle isoform) is also a key regulator of tight junction permeability (Turner et al. 1997; Shen et al. 2010; Cunningham and Turner 2012) and has revealed a role in barrier dysfunction, in response to inflammatory mediators (Rigor et al. 2013).

Inhibitors of MLCK have been proposed as therapeutics (1) acting as potential vasodilators for pathological conditions like vasospasm (Sasaki 1990; Kerendi et al. 2004), (2) decreasing the intestinal epithelial permeability (Feighery et al. 2008), for disorders such as ulcerative colitis (Liu et al. 2013), or (3) overcoming infectious agents such as herpes simplex virus type-1 (Antoine and Shukla 2013). Two MLCK inhibitors, the serine/threonine protein kinase inhibitors ML-7 and ML-9 (Fig. 3.4a), are used in most of the studies devoted to the physiological role of MLCK (Saitoh et al. 1987; Ishikawa et al. 1988). However, the therapeutic utility of these structurally related compounds is limited, since they also inhibit other kinases such as protein kinase A and protein kinase C (Saitoh et al. 1987). Peptidic antagonists have revealed a better specificity towards MLCK, such as the membrane-permeant inhibitor of MLCK (PIK, Fig. 3.4a), identified within a peptide library derived from the auto-inhibitory sequence of MLCK (IC_{50} 50 nM; Lukas et al. 1999; Owens et al. 2005). However, the low stability of the peptide in vivo has limited its applications, and analogues have been designed to enhance the resistance to protease while maintaining activity and selectivity, such as D-PIK and D-reverse PIK (Owens et al. 2005).

In 1996, in the course of a screening of microorganisms to identify MLCK inhibitors as potential vasodilators and bronchodilators, Yano et al. reported that MS-271 (i.e. siamycin I; Tsunakawa et al., 1995; Fig. 3.4b) inhibited the chicken gizzard MLCK with an IC_{50} of 8 μM (Yano et al. 1996). Chicken and turkey MLCKs, abundant and easily purified from the gizzard tissue, have been used extensively to study MLCK, although they lack a portion of the proline-rich region found in mammalian MLCKs (Olson et al. 1990; Hong et al. 2011). Propeptin did not inhibit cyclic AMP-dependent protein kinase, protein kinase C or calcium-/ calmodulin-dependent cyclic nucleotide phosphodiesterase at concentrations up to 400 μM (Yano et al. 1996). Non-peptidic inhibitors, such as dehydroaltenusin (IC_{50} 0.69 μM), were also reported by this group (Nakanishi et al. 1995).

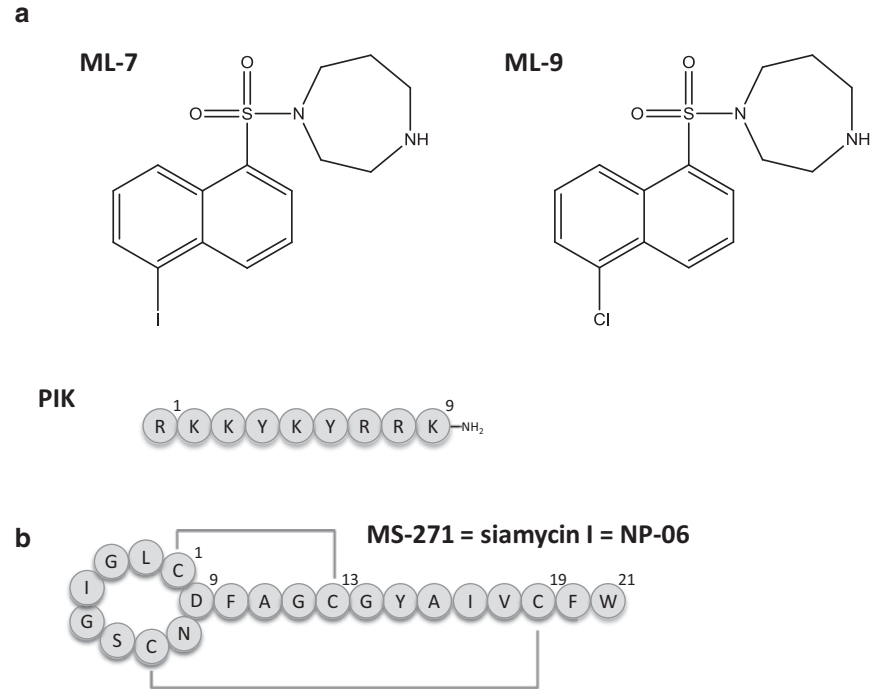


Fig. 3.4 Selected inhibitors of muscle myosin light chain kinase (MLCK). **a** Non-peptidic ML-7 and ML-9 (Saitoh et al. 1987; Ishikawa et al. 1988) and PIK peptide (Lukas et al. 1999; Owens et al. 2005). **b** Primary structure of MS-271 (also known as siamycin I or NP-06)

The lasso peptide propeptin, produced by *Microbispora* sp., is an inhibitor of prolylendopeptidase (Kimura et al. 1997a). Prolylendopeptidase (rather termed today prolyloligopeptidase; NC-IUBMB 1992) is a serine protease that cleaves small peptides (up to 30 amino acid long) at the carboxyl site of internal proline residues (Polgar 2002; Garcia-Horsman et al. 2007; Gass and Khosla 2007). It is found in archaea, bacteria and eukaryotic organisms (Venalainen et al. 2004). In humans, it is broadly distributed in all tissues, with a high activity detected in the brain (Goossens et al. 1996). Human prolyloligopeptidase digests proline-containing biologically active peptides such as substance P, angiotensins and bradykinin (Fig. 3.5a; Garcia-Horsman et al. 2007). It is therefore thought to regulate neuropeptide and peptide hormone levels, and has been proposed to be involved in different physiological functions, such as cell division and differentiation, learning and memory and signal transduction (Garcia-Horsman et al. 2007; Szeltner and Polgar 2008). However, the mechanisms subtending these activities are not clearly understood. Prolyloligopeptidase has been associated to different neurodegenerative and psychiatric disorders (Brandt et al. 2007). Its activity appears to be altered for patients with neurodegenerative diseases such as Alzheimer's disease, Lewy body dementia, Parkinson's disease or Huntington's disease (Mantle et al. 1996). In addition, a decrease in serum

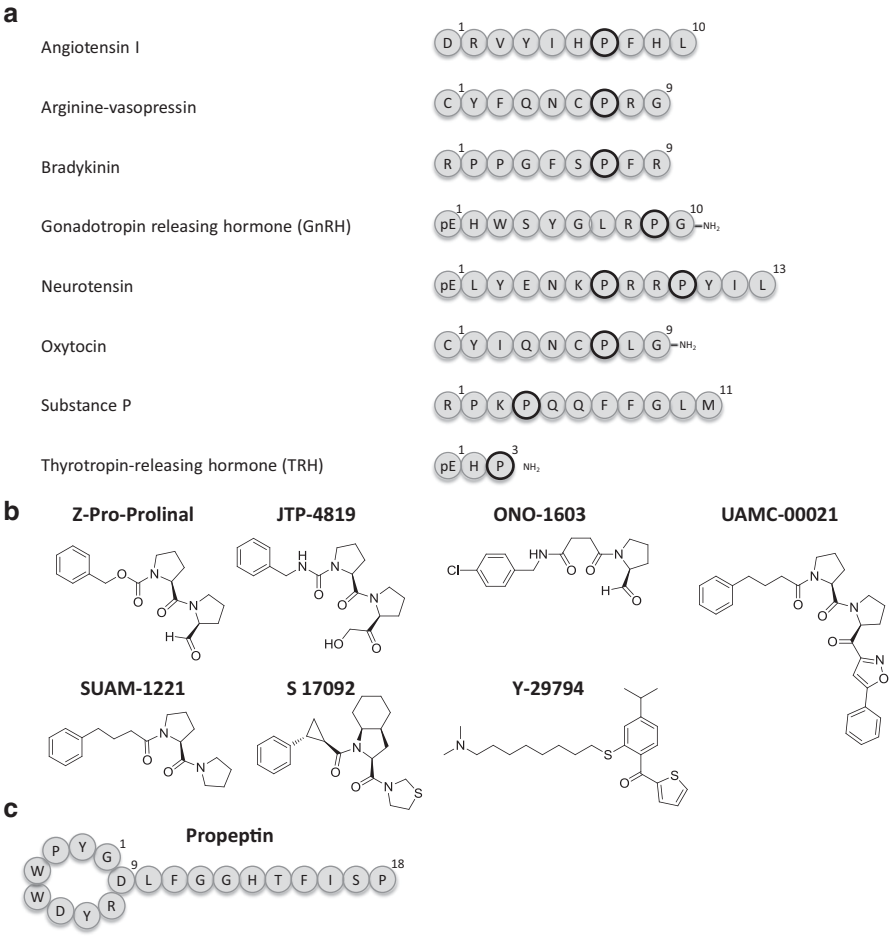


Fig. 3.5 Prolyloligopeptidase and main substrates and inhibitors. **a** Selected substrates of prolyloligopeptidase (Garcia-Horsman et al. 2007; Lawandi et al. 2010). pE: pyroglutamate. **b** Main inhibitors of prolyloligopeptidase: Z-Pro-Prolinal, JTP-4819, ONO-1603, SUAM-1221, S 17092, Y-29794, UAMC-00021 (Garcia-Horsman et al. 2007; Lawandi et al. 2010). **c** Primary structure of propeptin

prolyloligopeptidase activity has been observed in patients suffering from different stages of depression, while an increased activity has been detected for patients with mania and schizophrenia (Maes et al. 1994, 1995). The activity of prolyloligopeptidase in relation with mood stabilization, learning and memory has been related to the control of inositol, which is an important cellular second messenger (Williams et al. 1999, 2005; Schulz et al. 2002).

The three-dimensional structure of prolyloligopeptidase (Fulop et al. 1998) solved for the porcine homologue, which is more than 97% identical in sequence to the human protein (Lawandi et al. 2010), shows a two-domain structure, with a

peptidase domain arranged in a α/β-hydrolase fold, and a seven-blade β-propeller domain. The latter is proposed to act as a gating filter that excludes large peptides and proteins from the catalytic site, and thus restricts the activity of the peptidase towards small peptides (Kaszuba et al. 2012; Kaushik et al. 2014). The catalytic triad (Ser 554, Asp 680, His680 in the porcine sequence) is located in a large cavity at the interface of the two domains. The enzyme interacts with six amino acids of the substrate peptide: those in positions P4, P3 and P2 from the N-side, and those in positions P1′ and P2′ from the C-side of the proline that occupies the P1 position (Fulop et al. 1998; Garcia-Horsman et al. 2007).

Given its multiple physiological and physiopathological activities, prolyloligopeptidase has been considered as a potential therapeutic target as well as a therapeutic agent (Gass and Khosla 2007). On the one hand, prolyloligopeptidase from bacteria or fungi, administrated orally, revealed efficient to enhance gluten digestion in the gastrointestinal tract for patients with celiac sprue, a high-prevalence heritable pathology characterized by an inflammatory response to gluten (Schuppan et al. 2009). On the second hand, prolyloligopeptidase inhibitors have shown neuroprotective, anti-amnesic and cognition-enhancing properties in animal models, resulting in a high interest to treat neurodegenerative and psychiatric disorders (Männistö et al. 2007; Lawandi et al. 2010; López et al. 2011). Most inhibitors are pseudopeptidic and peptidomimetic inhibitors, containing a pyrrolidinyl moiety reminiscent of the proline residue of the substrate (Fig. 3.5b). Covalent inhibitors containing a reactive functional group (such as an aldehyde for Z-Pro-prolinal) that covalently binds to the catalytic serine residue of the enzyme (Wilk and Orlowski 1983) have revealed a potent inhibitory effect, as compared to competitive inhibitors (Garcia-Horsman et al. 2007; Lawandi et al. 2010). Three levels of selectivity have to be considered in the inhibition of prolyloligopeptidase: (1) the selectivity over all other proteases and peptidases, (2) that over other enzymes that cleave at sites adjacent to proline residues and (3) that over prolyloligopeptidase from other species.

In 1996, Kimura et al. reported that propeptin (Fig. 3.5c) is a competitive inhibitor of prolyloligopeptidase of the genus *Flavobacterium,* with an IC_{50} value of 1.1 μM (Kimura et al. 1997a). The activities were measured using Z-Gly-Pro-*para*-nitroanilide as substrate. Propeptin also inhibited mammalian prolyloligopeptidase from human placenta and bovine brain at equivalent concentrations. By contrast, propeptin did not inhibit other serine proteases such as trypsin, chymotrypsin, plasmin, pancreatic kallikrein, thrombin and elastase at 10 μM. Propeptin contains two proline residues, at positions 3 and 19, which could be involved in the binding to the enzyme. Propeptin T, obtained by trypsin digestion of propeptin (cleaved in the macrolactam ring between Arg8 and Asp9), showed a similar activity (Kimura et al. 1997b; Esumi et al. 2002). This indicates that the macrolactam ring is not important for the enzyme inhibition activity. The lasso topology has not been established for propeptin, but it is most probable that propeptin T, hydrolyzed within the ring, is a non-lasso peptide, suggesting that the lasso fold is not important for this activity. Finally, propeptin-2, missing the two C-terminal residues from propeptin, showed a similar enzyme inhibition activity (Kimura et al. 2007), indicating that the C-terminal Pro19 residue of propeptin in not involved in the inhibition.

MccJ25 and capistruin are two antimicrobial lasso peptides produced by proteobacteria that inhibit bacterial RNA polymerase (RNAP; Delgado et al. 2001; Mukhopadhyay et al. 2004; Kuznedelov et al. 2011). RNAP is a nucleotidyl transferase enzyme involved in the transcription of the genetic information, i.e. RNA synthesis from a DNA template, in all living cells (Cramer 2002; Borukhov and Nudler 2008). While eukaryotes have three RNAPs involved in the synthesis of ribosomal RNA, pre-messenger RNA and small RNAs (including transfer RNAs), respectively, bacteria and archaea have one RNAP only. Bacterial RNAP is a large protein (about 400 kDa). The core enzyme is constituted of five subunits ($\alpha_2\beta\beta'\omega$; Borukhov and Nudler 2008). Its three-dimensional structure, obtained for the bacteria *Thermus aquaticus* (Zhang et al. 1999), resembles a "crab claw". Its active centre is located in the cleft between the two "pincers of the claw", constituted by the β and β′ subunits. It contains a Mg^{2+} ion coordinated through three conserved aspartate residues. The nucleoside triphosphate (NTP) substrates access the active centre through the secondary channel (Vassylyev et al. 2007), and nascent RNA goes out through the RNA exit channel. The core enzyme binds to one of a variety of initiation factors (σ), involved in the recognition of promoter regions of DNA, to form the RNAP holoenzyme (Vassylyev et al. 2002). The mechanism of transcription consists of several key stages: (1) RNAP binding to the promoter to yield an RNAP/promoter closed complex; (2) melting of a segment of promoter DNA next to the transcription start site to yield the RNAP/promoter open complex; (3) abortive initiation, which consists of multiple rounds of synthesis and release of short (< 10 nt) RNA products; (4) from 9- to 11-nt incorporation, release of the initiation factor and processive elongation, through translocation of RNAP along the DNA template; (5) termination: dissociation of the transcribing complex, when a termination factor or signal is encountered. These steps rely on a complex set of interactions, conformational changes and movements that are reviewed in Borukhov and Nudler (2008) and Svetlov and Nudler (2009). Bacterial RNAP constitutes an important target for antibiotics, because it is essential for bacterial growth and survival, is well conserved within bacteria and possesses particular features that permit targeting it selectively without affecting eukaryotic RNAPs (Artsimovitch and Vassylyev 2006; Chopra 2007; Mariani and Maffioli 2009; Srivastava et al. 2011).

Several potent broad-spectrum antibiotics target bacterial RNAP (Artsimovitch and Vassylyev 2006; Mariani and Maffioli 2009; Srivastava et al. 2011; Fig. 3.6a). The best known are rifamycins and derivatives (Floss and Yu 2005), which belong to the family of ansamycin antibiotics, characterized by an aromatic moiety bridged at nonadjacent positions by an aliphatic chain. The rifamycins, isolated from an actinomycete, display a broad-spectrum antibiotic activity against Gram-positive and, to a lesser extent, Gram-negative bacteria. A rifamycin analogue, rifampicin, is one of the main molecules used clinically for the treatment of tuberculosis, leprosy and AIDS-associated mycobacterial infections (Floss and Yu 2005). The structure of the *Thermus aquaticus* core enzyme, in complex with rifampicin (Campbell et al. 2001), has permitted to show that the antibiotic binds to a site of the β subunit located in the path of nascent RNA. The different inhibitors of bacterial RNAP show a wide diversity of structures, binding sites and mechanism of action (Fig. 3.6a).

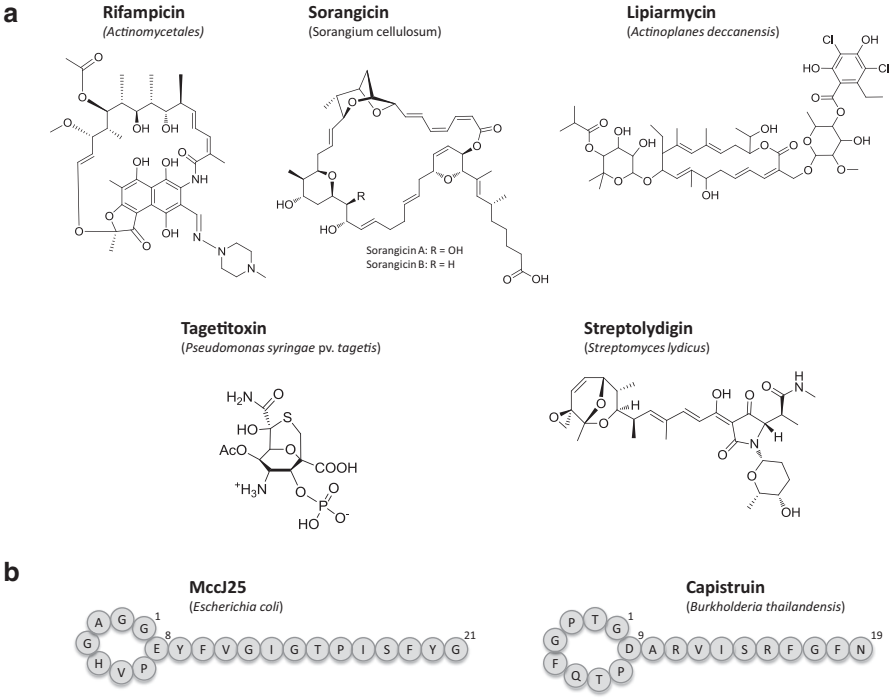


Fig. 3.6 Structures of antibiotics targeting RNAP. **a** Small-molecule antibiotics (Mariani and Maffioli 2009). **b** Primary structure of MccJ25 and capistruin (see secondary structure in Fig. 3.2)

Delgado et al. (2001) showed that RNA polymerase is the target of the antibacterial peptide MccJ25 (Fig. 3.6b), a lasso peptide produced by *Escherichia coli* AY25 (Delgado et al. 2001). This target was identified from an *E. coli* MccJ25-resistant mutant, revealing a single substitution on the *rpoC* gene encoding the β′ subunit of bacterial RNAP (resulting in the substitution of Thr931 to Ile). Thr931 is part of segment G, whose sequence is well conserved in the largest (β′-like) RNAP subunits from bacteria to eukaryotic organisms. The inhibition of RNA synthesis by MccJ25 was confirmed in vivo and in vitro (Delgado et al. 2001). Yuzenkova et al. (2002) then identified six additional single substitutions in the gene *rpoC* leading to resistance to MccJ25 by random mutagenesis. These mutations were positioned in the evolutionarily conserved segments G, G′ and F of RNAP, exposed in the inside surface of RNAP secondary channel. Therefore, the authors proposed that MccJ25 inhibits transcription by binding to the RNAP secondary channel and blocking substrate access to the active centre. This mechanism of action was confirmed and clearly shown in 2004 by Mukhopadhyay et al. (2004). This study showed that MccJ25 does not affect the formation of the RNAP/promoter open complex, but inhibits abortive initiation and elongation. Saturation mutagenesis of the *rpoC* gene permitted to identify 106 single-substitution mutants resistant to MccJ25, corresponding to 47 different sites within the subunit β′ and 4 different sites within β.

These positions correspond to a nearly continuous surface in the RNAP secondary channel. In addition, the association between MccJ25 and RNAP was shown by fluorescence energy transfer (FRET)-binding experiments (K_d 1 μM). The whole data reported permitted to show that the transcription inhibition by MccJ25 relies on binding within and obstructing the RNAP secondary channel, generating interference with NTP uptake and/or binding by RNAP. This mechanism was further supported by Adelman et al. (2004) from in vitro studies using biochemical and single-molecule biophysical approaches. This represents a unique mechanism of inhibition of RNAP. Kuznedelov et al. (2011) showed that capistruin (Fig. 3.6b), a lasso peptide produced by *Burkholderia thailandensis* E264, also inhibited *E. coli* RNAP but not mutant, MccJ25-resistant *E. coli* RNAP (Kuznedelov et al. 2011). This suggests that RNAP would be a target common to antimicrobial lasso peptides. The antimicrobial activities of lasso peptides and structure–activity relationships will be discussed in Sects. 3.1.4 and 3.2, respectively.

3.1.3 HIV Inhibitors

Despite the advances made in the antiretroviral treatment of human immunodeficiency virus (HIV), permitting to halt the replication of HIV and ease AIDS symptoms, HIV remains a major public health challenge. HIV replication cycle contains different key stages that have been targeted by antiretroviral drugs (Richman et al. 2009; Moss 2013). HIV initiates infection by fusing its envelope membrane with the host cell membrane (Wilen et al. 2012b, a; Melikyan 2014). The fusion process is triggered through sequential interactions between the virus envelope glycoprotein gp120 with the host cell protein CD4 and the chemokine receptors CCR5 or CXCR4. The formation of the ternary complex gp120-CD4-CCR5 (orCXCR4) leads to a conformational change in gp120 and to dissociation from the transmembrane segment gp41, which inserts into the host cell membrane leading to fusion. These early steps of the viral replication constitute an attractive target for anti-HIV therapy (Kazmierski et al. 2006; Garg et al. 2011).

The 21-residue lasso peptides siamycin I (also named NP-06 or MS-271), siamycin II and RP 71955 (also named aborycin; see siamycin I primary structure in Fig. 3.4b), isolated from *Streptomyces* sp., inhibit HIV fusion and viral replication in cell culture. These peptides were discovered in the context of screening microbial extracts for anti-HIV activities. Helynck et al. (1993) discovered RP 71955 through a fluorescent assay that aimed at finding inhibitors of the HIV protease (Helynck et al. 1993). In 1995, two independent studies reported the discovery of siamycin I (or NP-06), one through a tetrazolium-based colorimetric assay (MTT) using MT-4 cells, for the detection of anti-HIV compounds (Chokekijchai et al. 1995), and the other through a syncytia inhibition assay, for the detection of HIV fusion inhibition (Tsunakawa et al. 1995). The latter study also reported the discovery of siamycin II. The three peptides only differ at position 4 or 17 (Val or Ile residue in each case, see Chap. 2). Chokekijchai et al. confirmed that siamycin I inhibits the formation of

syncytia and did not observe significant activity of this peptide against the reverse transcriptase enzyme, the integrase and the HIV protease (Chokekijchai et al. 1995). This further supports that HIV fusion is the main event inhibited by siamycin I and analogues.

Siamycins and RP 71955 show a wedge-shaped structure, one face being predominantly hydrophobic and the other predominantly hydrophilic (Frechet et al. 1994; Constantine et al. 1995; see Fig. 2.2 in Chap. 2). From their sequences and three-dimensional structures, they have been proposed to inhibit HIV fusion through an effect on gp41 or gp120 (Frechet et al. 1994; Constantine et al. 1995). The linear 21-residue peptide corresponding to the sequence of siamycin I did not show anti-HIV activity at concentrations up to 23 μM (Chokekijchai et al. 1995), suggesting that the disulfide bridges and/or the interlocked topology of the peptides play a role in the antiviral activity.

Lin et al. further elucidated the mechanism of action of siamycin I (Lin et al. 1996). Siamycin I was shown to inhibit acute HIV infection, with effective doses (ED_{50}s) ranging from 0.05 to 0.6 μM for laboratory strains of HIV-1 and HIV-2 and 0.89 to 5.7 μM for clinical isolates. Interestingly, siamycin I was effective against HIV clinical isolates and laboratory mutants resistant to other inhibitors affecting the reverse transcriptase or the protease of HIV. Finally, siamycin I inhibited the infection of mononuclear cells by syncytium-inducing and non-syncytium-inducing clinical isolates of HIV.

The activity of siamycin I revealed specific towards human (HIV) and simian immunodeficiency virus (SIV) infections (Lin et al. 1996). The peptide displayed an ED_{50} of 3.2 μM against SIV and had significantly less activity against herpes simplex virus 1 (HSV-1) and influenza virus, with ED_{50}s of 60 μM in both cases, in agreement with the first results published for siamycin I and II against HSV (Tsunakawa et al. 1995). Finally, siamycin I inhibited HIV-induced fusion between C8166 cells and CEM-SS cells chronically infected with HIV (ED_{50} 0.08 μM), but had no significant effect on Sendai virus-induced fusion or murine myoblast fusion (Lin et al. 1996).

Enzyme-linked immunosorbent assays (ELISA) showed that siamycin I does not inhibit the interaction between gp120 and CD4 (Lin et al. 1996). In addition, the analysis of a mutant resistant to siamycin I permitted to show that the resistance maps to the gene *env* encoding gp160 (the precursor of gp120 and gp41) and is associated with six amino acid changes spanning both the gp120 and gp41 regions: Asn188Lys, Gly332Glu, Asn351Asp, Ala550Thr, Asn663Asp and Leu762Ser (the amino acid numbering refers to gp120; the gp41 sequence starts at position 520).

3.1.4 Antimicrobials

Antibacterial activities have been reported for different lasso peptides (Table 3.2), indicating that these peptides can play a role in microbial competitions. Interestingly, the spectrum of activity is strongly dependent on the producing bacteria.

Table 3.2 Antimicrobial activities reported for lasso peptides[a]

Peptides	Producing bacteria	Sensible bacteria (MIC if known, in μM)[b]	Insensible microorganisms[c]	References
Lasso peptides fromActinobacteria				
Siamycin I /MS 271/NP06	*Streptomyces* sp.	*Bacillus subtillis* (0.7-2.4)[S] *Enterococcus faecium* (2.4,)[S] *Enteroccocus faecalis* (5)[L] *Micrococcus luteus* (0.7)[S] *Staphylococcus aureus* (1.4-2.8)[S]	*Citrobacter freundii* *Escherichia coli* *Klebsiella pneumonia* *Proteus vulgaris* *Pseudomonas aeruginosa* *Salmonella typhi* *Salmonella typhosa* *Shigella sonnei* *Candida albicans*	(Tsunakawa et al. 1995; Yano et al. 1996; Nakayama et al. 2007)
Siamycin II	*Streptomyces* sp.	*Bacillus subtillis* (0.7)[S] *Micrococcus luteus* (0.7)[S] *Staphylococcus aureus* (1.4-2.8)[S]	*Citrobacter freundii* *Escherichia coli* *Klebsiella pneumonia* *Pseudomonas aeruginosa* *Salmonella typhi*	(Tsunakawa et al. 1995)
Aborycin/ RP 71955	*Streptomyces* sp.	*Bacilus brevis* (11.5)[L] *Bacillus subtilis* (9.2)[L] *Staphylococcus aureus* (6.9)[L] *Streptomyces viridochromogenes* (0.9)[L] *Pseudomonas saccharophila* (6.9)[L]	*Escherichia coli* *Salmonella typhimurium* *Mucor hiemalis* *Mucor michi* *Yarrowia lipolytica*	(Potterat et al. 1994)
Anantin	*Streptomyces coerulescens*	No activity detected[c]	Broad variety of bacteria and fungi (list not reported)	(Weber et al. 1991)
Propeptin	*Microbispora* sp.	Weak activity[c] *Mycobacterium phlei* *Xanthomonas oryzae* *Pseudomonas aeruginosa*	n.d.	(Kimura et al. 1997a)
Lariatin[d]	*Rhodococcusjostii*	*Mycobacterium smegmatis* (2.8, 1.5)[S] *Mycobacterium tuberculosis* (n.d., 0.2)[L]	*Bacillus subtilis* *Micrococcus luteus* *Staphylococcus aureus* *Escherichia coli* *Pseudomonas aeruginosa* *Xanthomonas campestris* *Bacteroides fragilis* *Acholeplasma laidlawii* *Pyricularia oryzae* *Aspergillus niger* *Mucor racemosus* *Candida albicans* *Saccharomyces cerevisiae*	(Iwatsuki et al. 2007)

Table 3.2 (continued)

Peptides	Producing bacteria	Sensible bacteria (MIC if known, in μM)[b]	Insensible microorganisms[c]	References
Lasso peptides fromProteobacteria				
Microcin J25 (MccJ25)	*Escherichia coli*	*Escherichia coli* (0.05-1)[L] *Shigella flexneri* *Salmonella enteritidis* (2.10^{-3})[L] *Salmonella newport* (5.10^{-3})[L] *Salmonella heidelberg* *Salmonella paratyphi B* (4.10^{-3})[L]	*Bacillus subtilis* *Klebsiella pneumoniae* *Proteus* sp. *Pseudomonas mendocina* *Salmonella derby* *Salmonella typhimurium* *Salmonella typhi* *Lactobacillus acidophilus* *Saccharomyces cerevisiae*	(Salomón and Farías 1992; Blond et al. 1999; Blond et al. 2002; Vincent et al. 2004)
Capistruin	*Burkholderiathailandensis*	*Burkholderia caledonica* (12)[L] *Burkholderia caribensis* (150)[L] *Burkholderia ubonensis* (150)[L] *Burkholderia vietnamiensis* (100)[L] *Escherichia coli* 363 (25)[L] *Pseudomonas aeruginosa* (50)[L]	*Pseudomonas azotoformans* *Pseudomonas cremoricolorata* *Pseudomonas oryzihabitans* *Pseudomonas fulva* *Pseudomonas parafulva* *Pseudomonas straminea* *Escherichia coli* *Klebsiella pneumoniae* *Salmonella enterica* *Enterobacter cloacae* Erwinia carotovora *Aerococcus viridans* *Bacillus megaterium* *Staphylococcus aureus*	(Knappe et al. 2008)
Astexin-1	*Asticcacaulis excentricus*	Weak activity[c] *Caulobacter crescentus*	*Escherichia coli* *Vibrio harveyi* *Vibrio fischeri* *Burkholderia thailandensis* *Salmonella newport* *Caulobacter crescentus*	(Maksimov et al. 2012)
Caulosegnins I–III	*Caulobacter segnis*	No activity detected[c]	*Asticaccaulis excentricus* *Burkholderia thailendensis* *Burkholderia rhizoxinica*	(Hegemann et al. 2013)

Table 3.2 (continued)

Peptides	Producing bacteria	Sensible bacteria (MIC if known, in μM)[b]	Insensible microorganisms[c]	References
			Caulobacter crescentus *Caulobacter* sp. *Caulobacter segnis* *Sphingobium japonicum*, *Sphingopyxis alaskensis* *Xanthomonas gardneri* *Bacillus subtilis* *Micrococcus flavus*	
Xanthomonins I and II	*Xanthomonas gardneri*	No activity detected[c]	*Asticaccaulis excentricus* *Burkholderia thailendensis* *Burkholderia rhizoxinica* *Caulobacter crescentus* *Caulobacter* sp. *Caulobactersegnis* *Sphingobium japonicum* *Sphingopyxis alaskensis* *Xanthomonas gardneri* *Bacillus subtilis* *Micrococcus flavus*	(Hegemann et al. 2014)

[a] Gram-positive bacteria, Gram-negative bacteria, and fungi are indicated in *blue, red* and *green,* respectively. n.d.: not reported.

[b] Antibacterial assays and MIC measurements were performed from series dilutions, using either the agar diffusion method (S) or liquid cultures in microplates (L).

[c] As revealed by radial diffusion assay.

[d] The MIC values indicated correspond to lariatin (termed initially lariatin B) and its two amino acid truncated variant (termed lariatin A), respectively.

Lasso peptides produced by actinobacteria are generally active against Gram-positive bacteria, while those produced by proteobacteria show a narrow spectrum of activity directed against bacteria closely related to the producing strain. Propeptin and aborycin constitute exceptions to this trend, being active on both Gram-positive and Gram-negative bacteria such as *Pseudomonas* (Potterat et al. 1994; Kimura et al. 1997a). Antimicrobial assays showed that propeptin (Kimura et al. 1997a), capistruin (Knappe et al. 2008) and astexin-1 (Maksimov et al. 2012) have only a weak activity and no significant activity is noticed for anantin (Weber et al. 1991), sungsanpin (Um et al. 2013), caulosegnins (Hegemann et al. 2013) and xanthomonins (Hegemann et al. 2014). This suggests either that the most sensible bacteria to these lasso peptides have not been identified or that the antibacterial activity is in fact a secondary function for lasso peptides, which could play another ecological role.

MccJ25 has the most potent antibacterial activity among lasso peptides (Vincent and Morero 2009). It is active against bacteria phylogenetically related to the producing strain (*Enterobacteriaceae* such as certain *Escherichia, Salmonella* and *Shigella* species) and shows minimal inhibitory concentrations (MICs) in the nanomolar range against *Salmonella* (Table 3.2). It is the lasso peptide that is best characterized in terms of mechanism of action. Its antibacterial activity relies on (1) uptake by the target bacteria, which involves the outer membrane iron-siderophore receptor FhuA (Salomón and Farías 1993; Destoumieux-Garzón et al. 2005; Mathavan et al. 2014), the inner-membrane energy transduction complex TonB–ExbB–ExbD and the inner-membrane protein SbmA (Salomón and Farías 1995; de Cristóbal et al. 2006), followed by (2) inhibition of the bacterial RNAP (Delgado et al. 2001; Yuzenkova et al. 2002; Adelman et al. 2004; Mukhopadhyay et al. 2004; see Sect. 3.1.2).

FhuA is a 79-kDa outer-membrane siderophore receptor, which transports Fe(III) chelated to the hydroxamate siderophore ferrichrome in *E. coli* (Chakraborty et al. 2007). It is a monomeric β-barrel protein consisting of 22 antiparallel β-strands. Its N-terminus folds inside the β-barrel from the periplasmic side, forming the cork domain (residues 20–160), and a large extracellular ligand-binding pocket open to the external medium (Locher et al. 1998). Following recognition, transport by FhuA uses energy that is provided by the proton motive force and transduced by the TonB/ExbB/ExbD complex (called the Ton system), located at the inner membrane (Braun and Endriss 2007; Postle and Larsen 2007). Energy transduction from the inner membrane to FhuA involves contacts established in the periplasm between a TonB region called the TonB box and FhuA (Killmann et al. 2002; Carter et al. 2006). Besides its essential role in iron uptake, FhuA can be hijacked for uptake by the siderophore-conjugated antibiotic albomycin (Braun 1999; Ferguson et al. 2000), a structural analogue of ferrichrome, but also by antibiotics and antimicrobial peptides with no structural similarity with ferrichrome, such as rifamycin, CGP 4832 (Pugsley et al. 1987; Ferguson et al. 2001) and colicin M (Killmann et al. 1995). It is also the receptor for phages T1, T5 and Φ80 (Killmann et al. 1995; Bonhivers et al. 1998). The interaction between the viral receptor-binding protein (rbp) and FhuA results ultimately in the phage DNA release in the host cytoplasm (Flayhan et al. 2012). As for its conventional role in iron uptake, the hijacked activity of FhuA requires the Ton system, except for phage T5 (Braun et al. 2002a, b).

Mutants of *E. coli* resistant to MccJ25 permitted to propose that FhuA and the Ton system are involved in the uptake of the peptide in the target bacteria (Salomón and Farías 1993, 1995). The role of FhuA in MccJ25 uptake was confirmed and further characterized in 2005 (Destoumieux-Garzón et al. 2005). MccJ25 binding to FhuA was shown by size exclusion chromatography and isothermal titration calorimetry (K_d 1.2 μM, 2:1 stoichiometry). MccJ25 inhibited phage infection by phage T5 in *E. coli,* suggesting that MccJ25 and the viral rbp5 (Flayhan et al. 2012) compete for FhuA binding. Binding to FhuA was altered and antibacterial activity was significantly lowered for MccJ25 cleaved within the Val11-Pro16 region by thermolysin (Rosengren et al. 2004; Destoumieux-Garzón et al. 2005), indicating that the loop region of MccJ25 is required for recognition by FhuA. The structure of FhuA

in complex with MccJ25, recently published (Mathavan et al. 2014), permitted to delineate the recognition mechanism. Comparison of the MccJ25- and ferrichrome-bound FhuA structures revealed that MccJ25 and ferrichrome bind at a very similar location. MccJ25 completely occupies and occludes the FhuA channel. The loop region of Mcc25 (residues 9–18) shows significant conformational changes upon FhuA binding, as compared to the NMR structure of the peptide alone (Bayro et al. 2003; Rosengren et al. 2003; Wilson et al. 2003). This further supports that the integrity of the loop is essential for binding to FhuA. FhuA/MccJ25 complex is stabilized by hydrogen bonds involving residues Ala3 and His5 from MccJ25.

SbmA is a homodimeric inner-membrane protein of Gram-negative bacteria, with seven predicted transmembrane domains (Corbalan et al. 2013; Runti et al. 2013). It is supposed to be a secondary transporter, although its physiological substrates are not known. SbmA has been involved in the uptake of diverse antibiotic agents active on bacteria through an intracellular target: bleomycin (Yorgey et al. 1994) and MccB17 (Lavina et al. 1986), both containing thiazole and oxazole moieties, proline-rich antimicrobial peptides (Mattiuzzo et al. 2007) and peptide nucleic acid–peptide conjugates (Ghosal et al. 2013). Mutants of *E. coli* resistant to MccJ25 have permitted proposing that SbmA is involved in the uptake of MccJ25 (Salomón and Farías 1995), and residue His5 of MccJ25 has revealed important for SbmA-mediated uptake (de Cristóbal et al. 2006).

The knowledge on the function of SbmA has recently been broadened, providing new leads to understand how MccJ25 crosses the inner membrane of Gram-negative bacteria. SbmA is homologous and exchangeable with BacA, a bacterial protein required for bacteria/eukaryotic host chronic relationships. BacA plays an essential role in *Rhizobium* spp. symbiosis with leguminous plants (Glazebrook et al. 1993; Ichige and Walker 1997) and in *Brucella abortus* pathogenesis of mammals, which involves bacteria replication in the host macrophages (LeVier et al. 2000). The role of BacA in the rhizobial association relies on lipopolysaccharide synthesis and peptide transport (Ardissone et al. 2011). Furthermore, the gene *sbmA* is adjacent to a recently found gene *yaiW,* and the two genes are co-transcribed in *E. coli* and *Salmonella* species (Arnold et al. 2014). YaiW is a surface-exposed outer-membrane lipoprotein, which positively affects the uptake of proline-rich peptides (like SbmA), and a connection between the cellular functions of SbmA and YaiW has been suggested. Thus, the role of YaiW in MccJ25 uptake remains to be investigated.

Finally, once in the cytoplasm of target bacteria, MccJ25 inhibits RNAP through obstructing the RNAP secondary channel, generating interference with NTP uptake and/or binding by RNAP (Delgado et al. 2001; Yuzenkova et al. 2002; Adelman et al. 2004; Mukhopadhyay et al. 2004; see Sect. 3.1.2).

An alternative target of MccJ25 is the membrane respiratory chain, through the production of reactive oxygen species (Rintoul et al. 2001; Bellomio et al. 2007; Chalon et al. 2011; Vincent and Morero 2009). The respiratory chain of *E. coli* contains different dehydrogenases and terminal reductases (or oxidases), which are linked by quinones (Unden and Bongaerts 1997). These proteins generate the proton motive force. O_2 is the preferred final electron acceptor and represses the terminal reductases of anaerobic respiration. The inhibitory effect of MccJ25 on

the respiratory chain was first described in *Salmonella* (Rintoul et al. 2001), and later in *E. coli* (Bellomio et al. 2007). MccJ25 was shown to disrupt the membrane potential, thus inhibiting oxygen consumption. This activity was supported by the observations on MccJ25 interaction with liposomes and membranes (Rintoul et al. 2000; Dupuy and Morero 2011). Chemical amidation of the C-terminal glycine of MccJ25 specifically blocks the capacity to inhibit RNAP, but not cell respiration, or peptide uptake, in *Salmonella enterica* serovar Newport (Vincent et al. 2005). This discriminant property permitted to show that RNAP inhibition and cell respiration inhibition are independent, and to analyze the two processes separately (Bellomio et al. 2007). A strain carrying a mutation in the gene encoding SbmA, associated to a resistance to MccJ25, was still resistant when overexpressing FhuA. This showed that import in the cytoplasm is required for inhibition of both RNAP and cell respiration. The MIC of amidated MccJ25 revealed 100–1000 higher values than those of MccJ25, suggesting that inhibition of cell respiration is a secondary mechanism of action as compared to RNAP inhibition. The activity of MccJ25 on *E. coli* strains harbouring MccJ25-resistant RNAP confirmed this trend (Bellomio et al. 2007). However, when overproducing FhuA, the strains harbouring wild-type RNAP and MccJ25-resistant RNAP revealed similar sensibility. Therefore, the inhibitory effect of MccJ25 on cell respiration strongly depends on the expression and/or activity of the outer-membrane receptor FhuA, and thus on the peptide concentration in the cytoplasm. The inhibitory effect of MccJ25 on the membrane respiratory chain was related to the production of reactive oxygen species such as the superoxide (O_2^-) in bacterial cells (Bellomio et al. 2007; Dupuy et al. 2009). Tyr9 has been identified as a key residue in this process (Chalon et al. 2009, 2011). Production of oxygen reactive species has been involved in the activity of different antibiotics such as ciprofloxacin (Becerra and Albesa 2002; Albesa et al. 2004; Akhova and Tkachenko 2014). MccJ25-induced superoxide production has also been related to mitochondrial transition pore and cytochrome c release in rat heart mitochondria, leading to antimitochondrial activity (Niklison Chirou et al. 2004, 2008, 2011).

The antibacterial activity of MccJ25 was maintained in complex fluid biomatrices and in a mouse model of *Salmonella* infection (Lopez et al. 2007). The infection was induced by intraperitoneal inoculation of *Salmonella newport,* followed after 2 h of treatment with MccJ25 (intraperitoneal injection). This good efficacy in vivo suggests that the interlocked topology of MccJ25 provides enhanced pharmacokinetic properties as compared to conventional peptides.

Capistruin, a lasso peptide produced by *Burkholderia thailandensis,* shows a weak antibacterial activity against strains closely related to the producing strain and against a hyper-permeable *E. coli* strain *E. coli* 363 (Table 3.2; Knappe et al. 2008). Its internalization process is not known. Capistruin does not protect *E. coli* from phage T5 infection (Mathavan et al. 2014) and is inactive against *E. coli* (with the exception of a hyper-permeable strain, *E. coli* 363; Knappe et al. 2008). Nonetheless, it is an inhibitor of *E. coli* RNAP, which shows an inhibition efficiency equal to that of MccJ25 (Kuznedelov et al. 2011). The amino acid sequences of MccJ25 and capistruin are very different, which suggests that the topology is a key recognition element for binding to RNAP secondary channel, independently of the amino acid sequence. This common intracellular target between two antibacterial peptides

with a different spectrum of activity supports the idea that the spectrum of activity of lasso peptides is mainly governed by the uptake process.

A very different mechanism of action has recently been reported for siamycin I (Nakayama et al. 2007; Ma et al. 2011). Siamycin I (also named MS-271 and NP-06) is a class I lasso peptide produced by *Streptomyces*. It exerts antibacterial activity against Gram-positive bacteria, including the hospital-acquired infection agent *Enterococcus faecalis* (Tsunakawa et al. 1995; Yano et al. 1996; Nakayama et al. 2007; Table 3.2). Siamycin I has been shown to attenuate quorum-sensing-mediated virulence in *E. faecalis* (Nakayama et al. 2007; Ma et al. 2011). Gelatinase is a major virulence factor in *E. faecalis,* being involved in the formation of biofilms, and thus adherence and pathogenicity (Su et al. 1991). Its expression is regulated by the FsrABCD two-component regulation system. The kinase sensor FsrC sensor histidine kinase, upon activation by the gelatinase biosynthesis-activating pheromone (GBAP) peptide encoded by the *fsrBD* genes, phosphorylates the FsrA response regulator (Qin et al. 2001; Hancock and Perego 2004; Del Papa and Perego 2011), thus activating the transcription of different genes, including *fsrBCD*. In 2007, the lasso peptide siamycin I was isolated during the screening of actinomycete culture supernatants for inhibition of quorum-sensing-mediated gelatinase activity (Nakayama et al. 2007). In 2011, Ma et al. showed that siamycin I inhibits FsrC sensor kinase activity (Ma et al. 2011). A study of the interaction between siamycin I with FsrC by synchrotron radiation circular dichroism spectroscopy (SRCD) indicated that the peptide binding occurs at a different, nonoverlapping site to the native ligand, GBAP (Phillips-Jones et al. 2013). However, this inhibition was not specific to FsrC, since siamycin I also inhibited several ATP-binding enzymes, including nine membrane sensor kinases from *E. faecalis* (Ma et al. 2011). This observation raises questions on the real origin of the antibacterial activity, and on the role of lasso peptides in bacterial communication.

3.2 Structure–Activity Relationship

Extensive structure–activity relationship studies, involving chemical modifications, enzymatic hydrolysis and saturation or site-directed mutagenesis, have permitted to delineate the residues involved in the key stages of MccJ25 mechanism of action. In addition, the comparison of the lasso peptide sequences, producing strains and spectrum of activity, has revealed general tendencies to account for the selectivity of the antibacterial activity, at least for peptides active against Gram-negative bacteria.

3.2.1 MccJ25

The evaluation of the antibacterial activities and the ability of variants to inhibit RNAP of respiratory chain permitted to identify the key elements involved in MccJ25 mechanism of action (Fig. 3.7). First of all, the branched-cyclic peptide

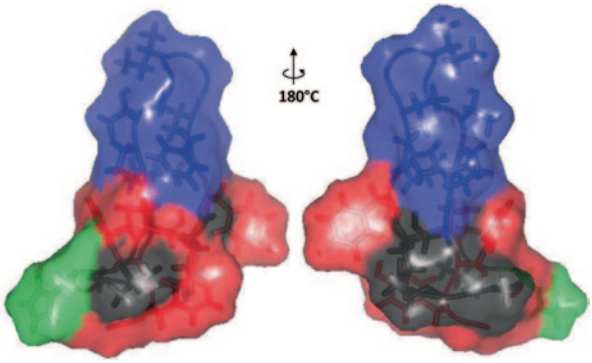


Fig. 3.7 Structure–activity relationship of MccJ25. Stick and surface representation of the three-dimensional structure of MccJ25 (from Rosengren et al. 2003), showing the residues involved in the FhuA-mediated uptake (in *blue*), histidine involved in both FhuA and SbmA-mediated uptake (in *green*) and RNAP inhibition (in *red*)

topoisomer of MccJ25 (containing the macrolactam ring but without interlocked topology) revealed no antibacterial activity (Ducasse et al. 2012b). This illustrates that the lasso scaffold is a prerequisite for the activity. The lasso fold is maintained thanks to optimized size of the ring and stabilization of the tail within the ring by bulky amino acids and disulfide bridges (see Chap. 2). MccJ25 cleaved by thermolysin in the loop region (Rosengren et al. 2004) did not bind FhuA and revealed much less activity than the native peptide, but showed unaltered propensity to inhibit RNAP (Destoumieux-Garzón et al. 2005; Semenova et al. 2005). Therefore, the loop region was identified as the key region for FhuA binding, and distinct regions were proposed to be involved in peptide uptake and RNAP inhibition. The amidation of the C-terminus of MccJ25 reduced importantly the antibacterial activity and RNAP inhibition (Bellomio et al. 2003; Vincent et al. 2005), showing that this part of MccJ25 is a key element for RNAP binding.

In 2008, a systematic structure–activity relationship study of MccJ25 has been performed by Pavlova et al. (Pavlova et al. 2008). Three hundred and eighty one singly substituted variants generated by saturation mutagenesis permitted delineating the positions critical for the biosynthesis and antibacterial properties of MccJ25. Of the 242 variants successfully biosynthesized and exported, 155 were competent for RNAP inhibition in vitro, 70 of which revealed antibacterial activity. This permitted to decipher the residues involved in MccJ25 uptake and RNAP inhibition activity, respectively. Residues Tyr9 (located upstream the macrolactam ring), Gly4 and Pro 7 (within the ring) and Phe19 and Tyr 20 (plug residues straddling the ring) revealed particularly important for RNAP inhibition. These residues form a continuous surface on one face on the three-dimensional structure of MccJ25, suggesting that they constitute the RNAP binding site (Fig. 3.7). Multiple-site mutagenesis in the loop region permitted to obtain variants with enhanced antibacterial activity (such as MccJ25 [Gly12His, Ile13Phe, Thr15Ile]; Pan and Link 2011). In the latter study, an elegant strategy permitted to screen the active/inactive character of MccJ25 variants.

This method is based on an orthogonal control of the expression of *mcjA* and *mcjD,* permitting independent control of MccJ25 production and export/immunity. Site-directed mutagenesis studies have been performed in our group to generate a series of variants specifically designed with varying sizes of macrolactam ring, loop and C-terminal tail below the ring, aiming at deciphering the residues that are critical for both the lasso fold and the antibacterial activity (Ducasse et al. 2012b). This study was completed by a characterization of the topology of the variants generated, which permitted to discriminate lasso and branched-cyclic peptides. The size of the loop revealed critical for preserving the antibacterial activity, due to its role in the interaction with FhuA (Ducasse et al. 2012b). The C-terminal tail could be extended while preserving antibacterial activity, but for the normal length peptide, the nature of the C-terminal residue appeared essential for the antimicrobial activity: Asp or Asn residues allow maintaining a weak activity, while Arg, Lys, Glu or Tyr residues result in a total loss of activity. Finally, the His5 residue has revealed critical for MccJ25 uptake, being involved in both FhuA binding (Mathavan et al. 2014) and SbmA-mediated entry (de Cristóbal et al. 2006). Synthetic peptides derived from the sequence of MccJ25, designed to form a compact conformation maintained by disulfide bridges, showed a weak antibacterial activity against *Salmonella* strains for one peptide, through inhibition of cell respiration (Soudy et al. 2012). This suggests that inhibition of the membrane respiratory chain, which constitutes a secondary mechanism of MccJ25 activity against *Salmonella* and *Escherichia* that requires higher concentration of MccJ25, does not necessitate the lasso topology.

3.2.2 Parameters Governing the Activity Spectrum

MccJ25 does not induce inhibition of yeast RNAP II and RNAP III, nor of RNAP from the Gram-positive bacteria *Bacillus subtilis* and the thermophilic Gram-negative *Thermus aquaticus* (Yuzenkova et al. 2002). This trend suggests selectivity in the activity of MccJ25, in accordance with its narrow spectrum of antibacterial activity. However, the main factor governing the activity spectrum of antibacterial lasso peptides is most probably the uptake in target cell. Differences in FhuA sequence within Gram-negative bacteria may account for the narrow spectrum of activity of MccJ25. Indeed, *Salmonella typhimurium,* which is totally resistant to MccJ25, becomes highly sensitive when expressing *E. coli* FhuA (Vincent et al. 2004), while a FhuA-defective *E. coli* expressing wild-type FhuA of *Salmonella typhimurium* became resistant to MccJ25 (Killmann et al. 2001). In addition, the combination of MccJ25 to a membrane-permeabilizing peptide $(KFF)_3K$ allowed MccJ25 penetration in an FhuA and SbmA-independent manner, extending the spectrum of activity towards pathogenic *Salmonella* strains such as *Salmonella typhimurium* (Pomares et al. 2010). The fact that RNAP is a common intracellular target for MccJ25 and capistruin, two lasso peptides that exhibit a different spectrum of activity (Kuznedelov et al. 2011), also supports this idea. All these elements indicate that the narrow spectrum of activity of lasso peptides is due to specific

interaction of the lasso peptides with the outer membrane receptors (Mathavan et al. 2014). A remaining question to elucidate is "how are antibacterial lasso peptides internalized in Gram-positive bacteria."

Conclusion

Lasso peptides exhibit a wide range of biological activities and the lasso scaffold enhances the pharmacokinetic features of peptides. These characteristics make these peptides very attractive for drug design. The highly restrained structures of lasso peptides generate stabilized loops potentially important for the binding to the membrane proteins or cytoplasmic targets. Siamycins have been discovered through bioactivity screening in five independent studies, as an antimicrobial, anti-HIV agent and MLCK (Chokekijchai et al. 1995; Constantine et al. 1995; Tsunakawa et al. 1995; Yano et al. 1996; Nakayama et al. 2007). This suggests that these peptides are widely distributed within *Streptomyces,* and makes them a very interesting scaffold for biotechnological applications (see Chap. 5). The activities of lasso peptides as human receptor antagonists and activities on bacteria (mainly antimicrobial) may not be totally disconnected, since human natriuretic peptides exhibit antimicrobial activity (Xing et al. 1985; Krause et al. 2001) and modulate quorum sensing and toxin production in bacteria (Blier et al. 2011).

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