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Review

Amphibian antimicrobial peptides and Protozoa: Lessons from parasites

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ABSTRACT

Antimicrobial peptides (AMPs) from amphibians and other eukaryotes recognize pathogenicity patterns mostly related to differences in membrane composition between the host and a variety of bacterial, fungal and protozoan pathogens. Compared to the other two groups, protozoa are fairly neglected targets in antimicrobial chemotherapy, despite their role as causative agents for scourges such as malaria, amoebiasis, Chagas' disease or leishmaniasis. Herein we review the scarce but growing body of knowledge addressing the use of amphibian AMPs on parasitic protozoa, the adaptations of the protozoan to AMP pressure and their impact on AMP efficacy and specificity, and the current and foreseeable strategies for developing AMPs into practical therapeutic alternatives against parasitic disease.

Contents

Abbreviations: AMP, antimicrobial peptide; ATR-FITR, attenuated total reflectance Fourier transform infrared; DOPS, dioleylphosphatidylserine; FPRL-1, formyl peptide receptor like-1; Gp63, major surface proteinase of *Leishmania* or leishmaniolysin; GPI, glycosylphosphatidylinositol; GUV, giant unilamellar vesicle; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; LC_{50}, lethal concentration 50; LPG, lipophosphoglycan; NK, natural killer cells; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipid; PM, plasma membrane; PS, phosphatidylserine; RNAi, interference RNA; ROS, reactive oxygen species; SAR, structure–activity relationship; WHO, World Health Organization

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1. Introduction: Protozoa as neglected target for AMPs

Antimicrobial peptides (AMPs) constitute a first barrier against pathogen dissemination in pluricellular organisms (see reviews [1–3]). As components of the innate immunity, they are able to act on a wide variety of pathogens, including Protozoa. Nevertheless, this group of microorganisms has received much less attention as a target for AMPs than pathogens such as bacteria or fungi. There are several causes for this relative neglect: i) the fact that pathogenic potential of Protozoa affects by and large the tropical and subtropical areas of the

world, where generally precarious economic conditions prevail, has given low priority to research investment by the pharmaceutical sector on devastating diseases such as malaria, leishmaniasis, or trypanosomiasis. While this situation has of late been partially ameliorated by partnerships among pharma industry, WHO, charities and academia [4], the development of anti-protozoan drugs still lags behind those addressing other infectious diseases [5]. ii) AMP production costs, about a log higher than for small molecule antibiotics, act as a brake on the development of clinical trials and ultimately therapeutic approaches. iii) While HIV-associated parasitic infections with worrisome mortality and morbidity rates (e.g., *Cryptosporidium* or *Leishmania*) have raised obvious concerns, they appear to be manageable by HAART therapy [6]. iv) A sizable gap in both basic knowledge and research tools penalizes Protozoa vs. other microbial or mammalian systems. For instance, techniques such as axenic culture methods have not been available for many parasitic protozoa until recently, substantially restricting the availability of microorganisms for systematic testing. v) The peculiarities of protozoan molecular biology preclude the use of otherwise highly effective approaches (e.g., RNAi in *Leishmania*) in the testing of different targets. vi) A further negative factor is the variety of protozoan groups, with complex life-cycles that often involve multiple stages with dramatic differences in metabolism, protein expression and, relevant for AMP, membrane composition. vii) Additionally, some of these stages entail intracellular hiding of the parasite, or at least impaired access of external AMPs. viii) Finally, the expression and action of autologous AMPs in either commensal or parasitic Protozoa is a completely unexplored field.

Despite these difficulties, Protozoa remain a highly appealing model for AMP research: a typical protozoan infection requires several weeks to reach its final stage, vs. the much faster pace of bacterial infections with generation times at least a log order lower than Protozoa. This explains why these microorganisms have been portrayed as master subverters of the host immune response, their subversion more pronounced at some stages in the invertebrate vector or the vertebrate host where AMP might be relevant.

2. Mechanism of action of AMPs

For the benefit of readers unfamiliar with the subject, this review leads off with an outline of the mechanism of action of AMPs. For a broader appreciation of the field, and to avoid constant reference to other reviews, AMPs other than amphibian are occasionally discussed, and partial overlap with other reviews in this issue is minimized but not totally avoided.

Antiprotozoal activities of natural amphibian AMPs are shown in Table 1. As different microbicidal parameters are used by different authors, the percent microbicidal activity at a given concentration is given. As usual with AMPs, most activities are in the micromolar range. As most AMPs hitherto described, amphibian AMPs are strongly cationic; of 210 entries in the AMSDb database (http://www.bbcm.univ.trieste.it/~tossi/pag2.htm) under the amphibian subcategory, only maximin H5 from *Bombina maxima* is anionic. While amphibian AMPs display a wide range of structural motifs (α-helical, Gly-rich with high conformational flexibility, e.g., plasticins; disulfide-bridged or even heteromeric), studies on protozoan targets focus mainly on dermaseptins, and to a lesser extent on magainins, phylloseptins and ranalexins, the latter an example of non-linear, internal disulfide-linked structure.

Broadly speaking, two major mechanisms of AMP action exist. The most general one refers to AMPs for which membrane permeabilization leads to pathogen death [7]. In an alternative mechanism, the AMP enters the cytoplasm as above, but killing relies on an intracellular target [8]. In either mechanism, two salient structural features of AMPs, cationic character and amphipathicity, underpin recognition of so called pathogen-associated molecular patterns (PAMPs), common to a wide set of pathogens, in the context of the innate immune response where AMPs belong. These features mainly refer to the ability of AMPs to exploit differences in lipid composition of the pathogen (vs. vertebrate host) plasma membrane (PM). The PM of prokaryotes and lower eukaryotes is characterized by the presence of anionic phospholipids (PLs) at the outer leaflet, by the content (or absence) of certain sterols, and to a lesser extent by a distinctive plasma membrane potential (see Section 3 for further details). Morphological (see Fig. 1) and functional assays confirm that membrane permeabilization is achieved by distortion of the PM structure, not by activation of a pre-existing pore or transporter. The ensuing effects, which depend on AMP and the severity of the damage, usually include dissipation of ionic gradients across the PM, leakage of nutrients and/or larger cytoplasmic components, and finally a collapse of the parasite bioenergetics and osmotic lysis. This killing mechanism, usually quite fast and causing reduction of log orders of pathogens in a few minutes, does not involve any chiral requirements, as *all*-D-enantiomers of AMPs perform similarly to the native versions [9]. Moreover, as it is based on simple physico-chemical rather than receptor-specific interactions, this mechanism does not favor the appearance of resistance, which requires substantial changes in phospholipid composition. Other resistance strategies, such as proteinase production, are equally thwarted due to the fast kinetics of the process. In fact, most resistance traits described for AMPs on Protozoa rely on the existence of extracellular barriers largely impervious to AMPs such as the wall structure of some cyst stages (see Section 3.3). Unfortunately, the current knowledge on overall composition and asymmetric distribution of phospholipids in Protozoa is very limited (see review in [10]). Most models accounting for AMP-induced membrane permeation are inferred from data obtained with model phospholipid mono- or bilayers. These simplified models entail substantial caveats, such as (i) the lack of proteins with important roles in molecular crowding or formation or maintenance of lipid domains, or (ii) the incomplete reproduction of natural bilayer characteristics not strictly related to membrane proteins, such as phospholipid asymmetry. Thus, extrapolation from an in vitro to an in vivo model of PM permeation mechanism is not straightforward.

The main models advanced for PM permeation range from a canonical trans-membrane pore (barrel-stave), to solubilization of the membrane by a detergent-like action, based on the amphipathic character of the AMPs and their massive accumulation into the membrane (carpet-like model) (Fig. 2) [7,11,12]. The barrel-stave model of PM permeation predicts membrane permeation at very low peptide:phospholipid ratio, assuming that peptide–peptide interaction is stronger than peptide–phospholipid. The model also entails, first, a scarce modification of the overall physical parameters of the bilayer, and secondly, poor selectivity of pore formation with respect to membrane composition. Moreover, if a strict peptide stoichiometry is required for pore formation, an upper size limit for the leakiness of molecules is imposed, at least in the first steps of the mechanism prior to osmotic lysis. In the opposite carpet-like model the AMP, due to its cationic character, accumulates massively in the in-plane interfacial region of the outer leaflet of the PM, where monomer–monomer electrostatic repulsion is quenched by the anionic phospholipids. A calculation of this model based on the insect AMP cecropin A, taking into account peptide dimension and bacterial size, concluded that the AMPs practically must cover the whole organism in order to kill it [13] (Fig. 2B). Once a threshold accumulation is reached, the membrane is solubilized [14]. The intermediate worm-hole or two-state model proposed independently by Matsuzaki and Huang (reviewed in [7]) tries to reconcile three experimental observations: i) the change in orientation undergone by a fraction of membrane-bound peptide once a threshold is reached, ii) peptide-induced phospholipid flip-flop, and iii) peptide translocation into the cytoplasm, a fact ignored by the two previous models. In this model the massive union of the AMPs into the external monolayer of the PM leads to its expansion, causing a mechanical stress. Once a threshold is reached, a fraction of the

Table 1
Activity of amphibian antimicrobial peptides on Protozoa

Peptide[a]	Sequence	Protozoan specie (stage)[b]	Microbicidal effect		Comments	References
			Inhibition (%)	[Peptide] (μM)		
PGLa	GMASKAGAIAGKIAKVALKA-NH_2	*Plasmodium falciparum* (tph + sch)[c]	50	40	Inhibition of RBC reinvasion	[138]
		Paramecium caudatum	100	2.7	Cell disruption,	[139]
		Tetrahymena pyriformis	100	10.8	"	[139]
		Acanthamoeba castellanii (tph)	100	1.0	"	[139]
Xenopsin	GWASKIGQTLGKIAKVGLKELIQPK	*P. caudatum*	100	3.8	"	[139]
		T. pyriformis	100	7.6	"	[139]
		A. castellanii (tph)	100	0.75	"	[139]
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	*P. caudatum*	100	4.1	Cell disruption,	[139,140]
		T. pyriformis	100	8.1	"	[139]
		A. castellanii	100	0.9	"	[139]
		P. falciparum (tph + sch)	90	100	Inhibition of RBC reinvasion	[138]
		Cryptosporidium parvum (spo + ooc)[c]	39.2	50		[34]
Magainin 2-amidated	GIGKFLHSAKKFGKAFVGEIMNS-NH_2	*Plasmodium cynomolgi*	94	0.1 nmol/mosquito	Inhibition of oocyst formation in *Anopheles* after peptide inoculation	[136]
		Plasmodium knowlesi	95	"		
		P. falciparum	86	"		
Buforin 2	TRSSRAGLQFPVGRVHRLLRK	*C. parvum* (spo)	100	4	After 3 h incubation	[34]
		(ooc)	29	40	"	
		C. parvum (mer + gam)[c]	55.7	20	After 48 h incubation	[60]
Ranalexin-1CB	FLGGLIKIVPAMICAVTKKC-NH_2	*C. parvum* (mer + gam)[c]	42	50	Inhibition of intracellular growth in A549 cells	[61]
Dermaseptin-S1 (DRS-S1)	ALWKTMLKKLGTMALHAGKAALGA-AADTISQGTQ	*Leishmania major* (pro)	50	4.5	After 3 h incubation	[141]
		Leishmania mexicana (pro)	50	1.5	MIC after 48h	[142,144]
			100	4.3	"	
Dermaseptin-S3 (DRS-S3)	ALWKNMLKGIGKLAGKAALGAVKKLVGAES	*P. falciparum* (i. f.)	50	0.3–1.5	Range of LC_{50} depending on the strain tested	[47]
Dermaseptin-S4 (DRS-S4)	ALWMTLLKKVLKAAAKALNAVLVGANA	*P. falciparum* (tph)	50	1.2	50% hemolysis at 1.4 μM	[46]
		L. major (pro)	50	1.5–2.0		[9,79,141]
Dermaseptin-O1 DRS-O1	GLWSTIKQKGKEAAIAAAKAAGQAALGAL-NH_2	*Trypanosoma cruzi* (try)	100	2.8	After 2 h incubation	[143]
		Leishmania amazonensis (pro)	20	11.6	Biphasic killing	[145]
			100	23.4	"	
Dermaseptin-H3 (DRS-H3)	GLWSTIKNVGKEAAIAAGKAALGAL-NH_2	*L. amazonensis* (pro)	78	13.5	Biphasic killing	[145]
			100	27.0		
Dermaseptin-DI2 (DRS-DI2)	ALWKTLLKNVGKAAGKAALNAVTDMVNQ	*T. cruzi* (try)	100	2.7	After 2 h incubation	[143]
Dermaseptin-DI1 (DRS-DI1)	GLWSKIKAAGKEAAKAAAKAAGKAALN-AVSEAV	*T. cruzi* (try)	100	2.5	"	[143]
Bombinin H2	IIGPVLGLVGSALGGLLKKI-NH_2	*Leishmania donovani* (pro)	50	7.3	Inhibition of cell proliferation	[41]
		Leishmania pifanoi (ama)	50	11.0	"	[41]
Bombinin H4	IiGPVLGLVGSALGGLLKKI-NH_2[d]	*L. donovani* (pro)	50	1.7	"	[41]
		L. pifanoi (ama)	50	5.6	"	[41]
Temporin A	FLPLIGRVLSGIL-NH_2	*L. donovani* (pro)	50	8.4	"	[44]
		L. pifanoi (ama)	50	14.6	"	[44]
Temporin B	LLPIVGNLLKSLL-NH_2	*L. donovani* (pro)	50	8.6	"	[44]
		L. pifanoi (ama)	50	7.1	"	[44]
Temporin-1Sa	FLSGIVGMLGKLF-NH_2	*Leishmania infantum* (pro)	50	18.1	Inhibition of cell proliferation	[146]
		(ama)	50	22.8		
Phylloseptin O1 (PLS-O1)	FLSLIPHAINAVSTLVHHSG-NH_2	*T. cruzi* (try)	50	5.1	"	[147]
Phylloseptin O2 (PLS-O2)	FLSLIPHAINAVSAIAKHS-NH_2	*T. cruzi* (try)	50	4.9	"	[147]
Skin peptide YY	YPPKPESPGEDASPEEMNKYLTALRHYINL-VTRQRY-NH_2	*L. major* (pro)	100	5.9	Faster killing of promastigotes	[148]
		(ama)	100	6.2		

[a] The new peptide nomenclature proposed in Refs. [149,150] was adopted.
[b] Abbreviations: ama, amastigote; epi, epimastigote; gam: gamont; i.f., intracellular forms; mer, meront; ooc: oocyst; pro, promastigote; RBC, erythrocytes (red blood cell); sch: schyzont; spo: sporozoite; tph, trophozoite; try, trypomastigote.
[c] No differentiation between both stages was carried out.
[d] - *i* .- Stands for D-*allo*-isoleucine.

peptides lying parallel to the plane of the membrane change their orientation from parallel to transversal, promoting a positive curvature of the membrane and forming a mixed phospholipid–peptide toroidal pore where the hydrophobic lining is provided both by the polar heads of the phospholipids plus the hydrophilic face of the peptides. This pore also acts as catalyst for phospholipid

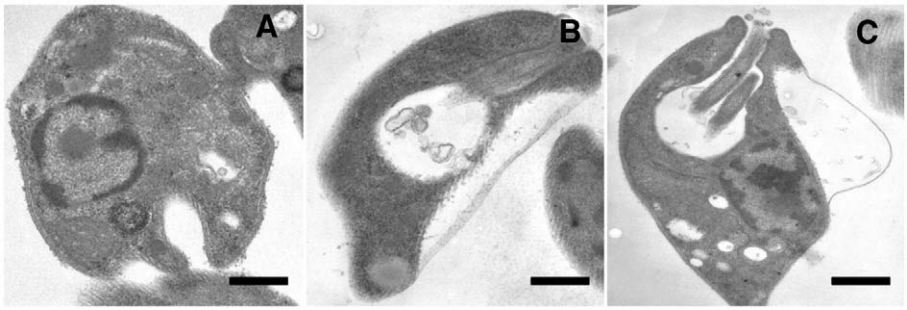

Fig. 1. Electron microscopy micrographs of *Leishmania pifanoi* amastigotes treated with amphibian antimicrobial peptides. Panels (A) control parasites; (B) 5 μM bombinin H4; (C) 7.5 μM temporin B. Magnification bar = 0.5 μm. (Photo J.M. Saugar).

interchange between the two leaflets. This pore is transitory and, when it disappears, stochastically sends its forming monomers to either side of the membrane. This more comprehensive model represents a subtle tour de force refinement over the detergent carpet-like model and may account for the step-wise increase in conductivity observed for several AMPs. Still a fourth mechanism, the "aggregate model" [7], relaxes the structural requirements intrinsic to the toroidal model, mostly applicable to α-helical peptides, to accommodate peptides not adopting this prototypical cylindrical shape. Finally a fifth model, the so called "Droste mechanism", has been proposed for melittin [15], where the toroidal lumen adopts a poor orientation and hydrophilic lining is mostly provided by the positive curvature of the phospholipids, with scarce protagonism of the peptide, which accumulates at the rim of the pore and stabilizes it.

3. Defense systems by the parasite

Although Protozoa share most traits of AMP susceptibility and resistance with microorganisms such as fungi or bacteria, there are some specific aspects that deserve commentary.

3.1. Phospholipid composition

Like other lower eukaryotes, Protozoa expose acidic PLs at the outer leaflet of the membrane, a feature that becomes a main specificity factor for preferential lysis by AMPs. Model membrane studies have brought forth a growing awareness that the specific composition of the external phospholipid monolayer, either anionic or zwitterionic, can modulate to a significant extent the final outcome of the permeation event for a given peptide. As the AMP inserts into the external monolayer, the specific type and abundance of the outer PLs imposes charge density and geometrical constraints that influence the formation of the lytic structure. For instance, many AMPs promote a positive curvature of the membrane so as to form a mixed phospholipid–peptide pore, hence PLs with a tendency to promote a negative curvature such as phosphatidylserine (PS) or phosphatidic acid (PA), require a much higher peptide:lipid ratio than phosphatidylglycerol (PG) for permeabilization by magainin 2 [16].

Despite the scarcity of data on protozoan membrane PL composition and membrane asymmetry (see above), the presence of anionic PLs may at times be inferred by the binding of cationic molecules. For

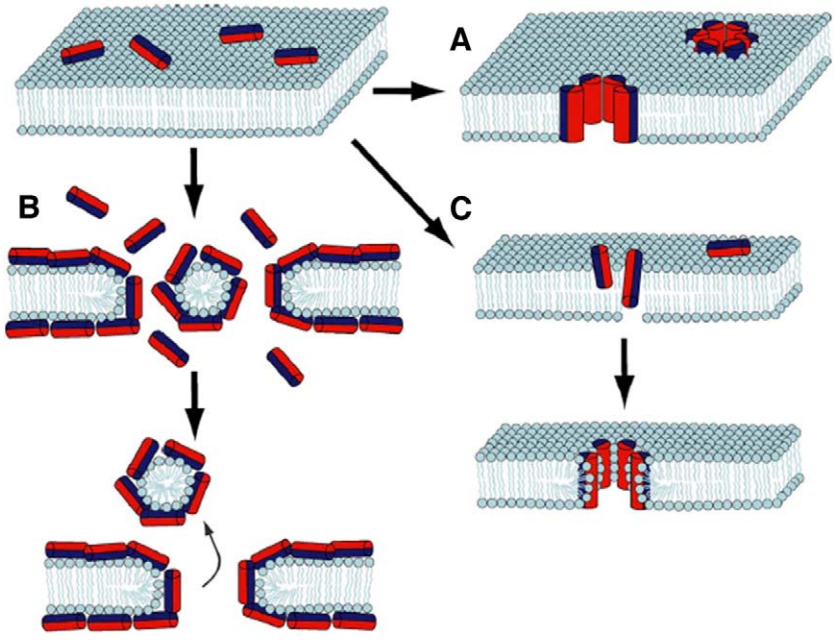

Fig. 2. Schematic representation of the three basic models of interaction of antimicrobial peptides (AMPs) with cellular membranes. All models share the initial binding of the AMP to the membrane, but differ in the following steps. The barrel-stave model (A) forms a classical pore lined with the polar face (red) of the peptide whereas the hydrophobic one (blue) is in contact with the acyl chains of phospholipids. In the carpet like model (B), the peptide accumulates massively at the membrane interphase, with final disruption of the membrane. The two-states or toroidal model (C) also starts as the carpet-like with massive peptide accumulation, but the mechanical tension created by the peptide accumulation was relieved by forcing some of the peptides to adopt a transmembrane orientation, forming a mixed phospholipid–peptide pore spanning the membrane; in a further step, the pore undergoes a stochastical disruption, with relocation of the monomers at both sides of the membrane. (Reprinted from Ref. [7] with permission, Copyright 2006, Elsevier).

instance, in the metacyclogenesis of *Leishmania tropica*, a process involved in the acquisition of virulence by promastigotes, an enhancement of the external exposure of PS, a PL usually confined to the inner leaflet of the PM in eukaryotes, has been documented [17]. More importantly, the pathological form of *Leishmania*, i.e., the amastigote, also exposes PS as part of a macrophage-deceiving strategy which nonetheless might be exploited by leishmanicidal AMPs [18].

The efficacy of AMPs depends not only on the presence of specific types of acidic PLs but also on a favorable overall composition, with low levels of specific PLs detrimental to AMP action. For instance, phosphatidylethanolamine (PE) is a well-known inhibitor of positive curvature and as such severely impairs pore formation by magainin 2 [16], or suppresses buforin II membrane translocation [19]. An inversion in the phosphatidylcholine (PC) PC:PE ratio upon treatment with several antiparasitic drugs has been described for Trypanosomatidae, together with an increased PS exposure and with a possible ensuing loss in the efficacy of an AMP-based treatment (see Section 5). Another insight into the modification of AMP performance comes from recent results on the possible role of oxidized PLs as preferential targets for AMPs. Thus, incorporation of the oxidized PC analog 1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine (PoxnoPC) into lipid monolayers increases temporin B and L insertion, possibly by formation of a Schiff's base between the aldehyde group of the oxidized PL and an amino group of the AMP [20]. This may be quite relevant for parasites under oxidative stress, either from chemotherapy or from the host immune response, such as *Leishmania* [21], *Trypanosoma* [22], or the intraerythrocytic *Plasmodium* [23].

3.2. Sterols of the plasma membrane

The preferential activity of AMPs on prokaryotes vs. eukaryotes is known to be partially dependent on the type and relative amount of sterols in the PM [24]. In model membranes, cholesterol impairs permeabilization by amphibian AMP magainin 2 [25]. It also decreases temporin L binding to liposomes [26], as well as the depth of membrane penetration by AMPs [27] or GUV vesiculation [28]. At a functional level, cholesterol depletion by cyclodextrin treatment increases the toxicity of AMPs against mammalian cells [29].

Plasmodium and *Cryptosporidium*, which do not biosynthesize sterols, totally depend on intake from the host to maintain sterol levels in their membranes. In contrast, Trypanosomatidae can produce ergosterol by a biosynthetic pathway similar to that of fungi [30]. Interestingly, the bulkier side chain of ergosterol relative to cholesterol makes for poorer membrane packing and thereby weaker inhibition of vesicle permeabilization [26]. It has also been reported that ergosterol sensitizes DOPS membranes to magainin 2 permeabilization [31]; a specific binding of the peptide to ergosterol has been advanced, also proposed for dermaseptins [32].

3.3. Glycocalix and other external barriers to AMP action

Contrary to fungi or yeasts, Protozoa do not have a permanent cell wall throughout their life cycle. For parasites with environmental rather than vectorial transmission (e.g., *Entamoeba*, *Giardia*, *Acanthamoeba*, *Toxoplasma*, *Cryptosporidium*), encystment is a practical option to survive in harsh environments (e.g., nutritional deficits) until a suitable host for completing the cycle is available. Encystment is a substantial hurdle to AMP action on Protozoa. For instance, the oocyst of *Cryptosporidium parvum* [33], a parasite of significant concern in drink water treatment due to its low permeability to water-soluble agents, is highly resistant to AMP action (Table 1) [34,35]. This imperviousness to AMP activity is mostly achieved by an oocyst wall that contains the extensively cross-linked *Cryptosporidium* outer wall protein (COWP), a 165–175 kDa protein with cysteine-rich C- and N-termini that generate a disulfide mesh in the wall [36].

Other parasites such as Trypanosomatidae possess a well-developed glycocalix rich in GPI-anchored elements that can equally hamper AMP access to the PM and decrease peptide efficiency. This abundance of GPI-anchored components is imposed by the subpellicular microtubule layer that runs beneath the PM and impairs integral protein diffusion in the membrane plane other than at the apical end (flagellar pocket), where all membrane traffic takes place [37].

In *Leishmania* promastigotes two major glycocalix components, both anchored through GPI structures, can be differentiated: (i) the lipophosphoglycan (LPG), an anionic oligosaccharide, and (ii) Gp63 (also named major surface proteinase or leishmaniolysin), a metalloproteinase of broad specificity. The strong anionic character of LPG results from the so called repeat region, formed (in *L. donovani*) by the disaccharide repeat $[Gal(\beta\ 1,4)Man(\alpha 1\text{-}PO_4\rightarrow 6)]_n$ ($n = 16$–30). A calculation [38] established that 6×10^6 copies of LPG/cell covered about 40% of the promastigote surface. LPG has been shown to provide partial protection for *L. donovani* promastigotes against AMPs such as magainin 2 and analogs; from the activity ratio vs. the R2D2 strain, deficient in the repeat region, a protection factor of 2 has been assigned to LPG [39]. On the other hand, temporins A or B, having smaller size (13 vs. 23 residues) and less cationic character ($+2$ vs. $+4.5$ charge at neutral pH) than magainin 2, are equally active against either strain, and likewise unaffected by the presence of other oligosaccharides with a higher anionic character as heparin [40]. Similarly, bombinins H2 and H4, showed a similar activity towards R2D2 and a poor inhibition by heparin [41].

The other *Leishmania* glycocalix component, metalloprotease Gp63, with ca. 5×10^5 copies/cell in the promastigote [42], also exerts a protective effect against AMPs, as shown by the fact that Gp63-deficient *L. major* promastigotes are twice more susceptible than the wild type to pexiganan, an optimized magainin 2 analog [43].

The above observations aside, the protection afforded by the two glycocalix components must not be viewed as a key factor in the resistance strategies of *Leishmania* against AMPs. This is borne out by the fact that axenic strains of the amastigote (intracellular) form of the parasite are consistently more resistant than promastigotes [41,44], despite their practically nil expression of LPG or Gp63 [45].

3.4. Challenges of intracellular parasitism

For *Plasmodium*, *Leishmania*, *Trypanosoma cruzi*, *Toxoplasma* or *Cryptosporidium*, to cite the most relevant parasites in terms of human health impact, pathogenesis in the vertebrate host is due to the intracellular stage of the life cycle. This has obvious implications for any potential application of AMPs in clinics, namely that evidence of lethal activity on the intracellular stages is mandatory. While the requirement for intracellular activity appears to limit the applicability of AMPs as antiparasitic agents, at the same time it opens the possibility of AMP cooperation with the defensive mechanisms of the infected cell in order to eliminate the pathogen. Despite the relative paucity of research on AMPs in parasite–host cell systems, the substantial variety of the data at hand makes a case-by-case approach advisable.

For malaria, the most extensive work on amphibian AMPs with antiplasmodial activities has focused on the dermaseptins. On these, two major classes with therapeutic potential and specificity have been described: (i) those causing preferential lysis of infected over non-infected erythrocytes, such as K_4K_{20}-S4 and K_4S4(1–13)a [46], and (ii) peptides with highly lytic activity on the parasite, regardless of the parasitation stage, at concentrations innocuous for the erythrocyte, such as the natural dermaseptin-S3 (DRS-S3) [47] or the N-terminally acylated C3-K_4S4(1–13)a and iC4-K_4S4(1–13)a [48], and the aminoacylated NC7-K_4S4(1–13)a [49] (Table 2). For lytic peptides of this kind, the question remains of how the peptide can reach the intracellular parasite.

Some hallmarks of *Plasmodium* parasitism may provide clues on how to achieve the required AMP intracellular targeting. In order to

Table 2
Synthetic analogs of amphibian peptides with antiprotozoal activity

Peptide	Sequence[a]	Protozoan specie (stage)[b]	Microbicidal effect		Comments	References
			Inhibition (%)	[Peptide] (μM)		
MSI-94	GIGKFLKKAKKFGKAFVKMKK-NH_2	*T. cruzi* (epi)	50	100		[151]
		(try)	50	64.8		
		Leishmania braziliensis (pro)	20	100		[151]
		Acanthamoeba polyphaga (tph)	100	16	As minimal amoebicidal concentration	[75]
MSI-103	KIAGKIAKIAGKIAKIAGKIA-NH_2	*A. polyphaga* (tph)	100	12.1	"	[75]
F5WMagainin2	GIGKWLHSAKKFGKAFVGEIMNS	*L. donovani* (pro)	50	6.1		[39]
			90	9.2		
Magainin B	GIGKFLHAAKKFAKAFVAEIMNS-NH_2	*Blastocystis hominis*	100	200		[82]
		Entamoeba histolytica (tph)	90	20.2		[82]
		T. cruzi (stage unknown)	100	40.4		[82]
MGH1	GIKKFLHIIWKFIKAFVGEIMNS	*L. donovani* (pro)	50	2.4		[39]
			90	4.3		
MGH2	IIKKFLHSIWKFGKAFVGEIMNI	*L. donovani* (pro)	50	0.9		[39]
			90	1.0		
Peptide Z [D-Lys^{4,10,11,14}, DPhe^{5,12,16}]	GIGkfLHSAkkfGkAfVGEIMNS-NH_2	*Paramecium caudatum*		>100	As minimal disruptive concentration	[139]
		T. pyriformis		>100		[139]
		A. castellanii	100	12.2		[139]
Magainin H	GIGKFLHSaKKFaKAFVaEIIMNS-NH_2	*B. hominis*	0	200		[82]
		E. histolytica (trp)	0	200		[82]
		T. cruzi (stage unknown)	Slight damage	200		[82]
Pexiganan	GIGKFLKKAKKFGKAFVKILKK-NH_2	*L. amazonensis* (pro)	30	100		[43]
Magainin G	βAGIGKFLHSAKKFAKAFVAEIMNS-NH_2	*B. hominis*	50	195		[82]
		E. histolytica (tph)	90	20.2		[82]
DS1(1-29)-NH_2	ALWKTMLKKLGTMALHAGKAALGAAADTI-NH_2	*L. major* (pro)	50	2.3		[152]
$K_4K_{20}S4$	ALWKTLLKKVLKAAAKAAL*K*AVLVGANA	*P. falciparum* (tph)	50	0.2	Preferential lysis of infected RBC	[46]
		L. major (pro)	50	1.5		[9,79,141]
$K_4S4(1–13)a$	ALWKTLLKKVLKA-NH_2	*P. falciparum* (tph)	50	3.3	Preferential lysis of infected RBC	[46,48,49]
		(rng)	50	7.6		
		L. major (pro)	50	9		[79]
C3-$K_4S4(1–13)a$	Propionyl-ALWKTLLKKVLKA-NH_2	*P. falciparum* (trp)	50	4.3	Improved therapeutic index. No discrimination between infected and uninfected human RBC	[48]
iC4-$K_4S4(1–13)a$	Isobutyryl-ALWKTLLKKVLKA-NH_2	*P. falciparum* (tph)	50	3.8	"	[48]
NC7-$K_4S4(1–13)a$	Aminoheptanoyl-ALWKTLLKKVLKA-NH_2	*P. falciparum* (rng)	50	14.2		[49]
		L. major (pro)	100	6.2		[94]
NC4-$K_4S4(1–13)a$	Aminobutyryl-ALWKTLLKKVLKA-NH_2	*L. major* (pro)	100	6.2		[94]

[a] Small capitals stand for D-amino acids.
[b] Abbreviations: ama, amastigote; epi, epimastigote; gam, gamont; i.f., intracellular forms; mer, meront; ooc, oocyst; pro, promastigote; RBC, erythrocytes (red blood cell); sch: schyzont; spo: sporozoite; rng, ring; tph, trophozoite; try, trypomastigote.

thrive inside a cell such as the erythrocyte, devoid of membrane traffic and of transcriptional and translational activities, *Plasmodium* undertakes extensive remodeling of the host cell. This involves a considerable increment in the membrane content of the infected erythrocyte, with a five-fold rise in PL content over a non-infected cell; also the formation of an intra-erythrocyte parasitophorous vacuole where the parasite dwells, plus an extensive system of trans-membrane communication between vacuole, cytoplasm and external medium that includes the tubovesicular network, Maurer's clefts, and other not yet fully unveiled transport systems [50,51]. A fluorescent analog of NC7-$K_4S4(1–13)a$ with antiplasmodial but no haemolytic activity does not stain the infected erythrocyte but localizes on the PM of *Plasmodium* and, more importantly, in the tubovesicular system of the infected erythrocyte, suggesting a possible intra-erythrocytic pathway for the peptide to reach the parasite [49].

Another parasite-induced modification, partly related to the one above, is a substantial modification of the PM of the infected erythrocyte. This affects, first, the PM protein pattern; a secretome of over 300 proteins, including transporters encoded by the parasite, has been reported for the adaptation of the erythrocyte to *P. falciparum* [52]. Second, and more relevant from the AMP perspective, adaptation involves changes in PM physical structure. Among PL constituents, the most significant changes are in fatty acid composition [53] and asymmetry; in the latter case, an increase in amino phospholipid exposure in the outer leaflet of the infected erythrocyte can be detected by chemical labeling [54]. *Plasmodium* also induces a drop in the cholesterol: PL ratio of the PM [55]; streptolysin O, a cholesterol-specific toxin, lyses preferentially non-infected vs. infected erythrocytes [56]. An increase in erythrocyte PM fluidity has also been described [57].

Altogether, these changes plus the in vitro data on liposome lysis by AMPs, may presumably account for the enhanced susceptibility of infected erythrocytes towards AMPs. Although conclusive proof of their involvement in AMP-mediated erythrocyte lysis is still missing, for AMPs such as the NK-lysin analog NK-2, the higher (infected vs. non-infected) erythrocyte lysis rate is abrogated by preincubation with annexin-V, a specific PS-binding protein, thus suggesting that preferential exposure of this PL is a main factor in PM remodeling [58].

Cryptosporidium and *Leishmania*, the other two parasites with a significant number of studies with amphibian AMPs, infect metabolically active cells with functional membrane traffic. Some natural amphibian AMPs reduce to various degrees the burden of intracellular

forms of *Cryptosporidium* (see Table 1) [34,59–64]. *Cryptosporidium* develops a special type of relationship with the host cell, called epicellular (i.e., intracellular but extra-cytoplasmic) parasitism, characterized by the formation of a parasitophorous vacuole at the apical end of epithelial intestinal cells, separated from the external medium by a thin rim of plasma membrane, and feeding on the cytoplasm of the host cell by formation of a feeder organelle [65]. Furthermore it has the capability to complete the full cycle in extracellular manner, thus it is not an obligatory intracellular parasite. How peptides are capable to affect the viability of such intracellular parasites is not known.

Leishmania is the other paradigmatic model of intracellular parasite, with a preference for macrophages, i.e., terminally differentiated cells whose professional phagocytic and antigen-presenting roles involves high metabolic activity and extensive membrane traffic. Among natural AMPs of amphibian origin, temporins A and B are known to curb proliferation of the intracellular amastigote [44], which dwells inside a parasitophorus vacuole with late endosome–lysosome features [66].

While genomic data reveal extensive transcriptional reprogramming of the macrophage on account of *Leishmania* infection [67,68], in contrast to *Plasmodium* (see above), no conclusive evidence on how parasite infection affects AMP killing of intracellular parasites has been found. An increase in PM fluidity for macrophages infected with *L. donovani*, which perhaps may influence AMP lysis, has been reported; interestingly, it is reverted by incubation with cholesterol-containing liposomes [69], suggesting changes in its content.

Other parasiticidal effects of AMPs on intracellular protozoa would appear to derive not from a direct microbicidal effect, but from peptide-induced immunomodulatory activities on the macrophage. For instance, temporin A and some analogs are chemotactic for human monocytes, macrophages and neutrophils through the FPRL-1 receptor [70] and dermaseptin-S9 (DRS-S9) also causes chemotaxis although in this case the microbicidal and chemotactic activities are dependent on the aggregational state of the peptide [71]. For its part, dermaseptin-S1 (DRS-S1) is able to induce in human neutrophils production of reactive oxygen species (ROS), toxic for many intracellular parasites [72].

3.5. Synergy of amphibian AMPs with other chemotherapeutic compounds

The ability of AMPs to synergize with other AMPs or other antibiotic agents is well established for bacteria. Synergism among AMPs for the same organism and anatomical location underlines the efficacy of innate immunity in controlling pathogen dissemination, as demonstrated for dermaseptins, temporin B and L [73], or by the synergism between PGLa and magainin 2 either as an heterodimer artificial presentation or formed spontaneously when inserted into the membrane [74].

Cryptosporidium has been widely used as a target for diverse amphibian AMP combinations (Table 3). Thus, ranalexin shows an additive outcome with Lasocid, a polyether ionophore that causes PM depolarization [64], with azithromycin [61,64] or clarithromycin [61], macrolide antibiotics inhibiting protein synthesis. Ranalexin also synergizes with rifanbutin and rifampin [61], two antibiotics of the ansa family that in bacteria act as transcription inhibitors but whose targets in *Cryptosporidium* remain unknown, and with magainin 2 and amiloride, a diuretic that acts by blocking the epithelial sodium channel [62]. Interestingly, ranalexin-1CB shows additive though not synergic behavior when given together with other membrane-active AMPs such as indolicidin or magainin 2 [61].

For its part, magainin 2 in combination with some acanthamoebicidal agents (silver nitrate, ketoconazole, propamidine isethionate, gramicidin S or neomycin sulfate) showed enhanced therapeutic outcome against the amoeba form, whereas the silver nitrate combination was the only one active against the cyst form [75] (Table 3).

4. Optimization of the antiprotozoal activity of amphibian AMPs

Since the very discovery of amphibian AMPs, SAR studies have pursued the double goal of unraveling the structural basis of their activity and specificity [76] and developing novel and attractive clinical chemotherapeutic alternatives [77]. The most paradigmatic example in this respect is pexiganan acetate, a magainin analog that reached phase III trial. As with AMPs from other biological sources, linear peptides have received special attention due to their easier synthetic accessibility [1,2,78]. This section will focus on the main structural features used to modulate the anti-protozoan activities of amphibian AMPs.

4.1. Cationic character

A minimal cationic character is widely assumed to be a requirement for recognition of the different charge densities between protozoan parasites and their hosts. From this it follows that an increase in the overall positive charge should improve the microbicidal activity of AMPs on parasites. For instance, a dual replacement (M4K and N20K) in dermaseptin-S4 (DRS-S4), with a gain of two positive charges, translated into a gain of leishmanicidal specificity [9]. A similar approach enhanced the antiplasmodial [46] activities of the parental peptide, together with a significant decrease in hemolytic activity [46,79]. It has also been found, however, that once an upper threshold of cationic charge is reached, the peptide can become irreversibly stuck to the membrane or to the anionic glycocalix of the pathogen, preventing access to the membrane or adoption of an active conformation [80], and causing a loss of specificity due to enhanced cytotoxicity [81]. A molecular interpretation of these adverse results is that repulsion between peptide monomers (see Section 2) shortens the half-life of the forming pore [80]. Indeed, the cationicity requirement would appear to be not simply quantitative but topological. Thus for buforin 2, an α-helical peptide active on *Cryptosporidium* [34,59,60], the local accumulation of positive charges required for membrane translocation induces

Table 3
Antiprotozoal activity of amphibian AMPs in combination with other antibiotics

Combination (A + B)	Protozoa (stage)[a]	Microbicidal effect (%)		Comments[a]	Reference
		(A, B)	(A + B)		
Ranalexin (50 μM) + magainin II (50 μM)	*C. parvum* (mer + gam)	42; 35	93.6	Additive effects	[61]
Ranalexin (50 μM) + Azithromycin (8 μg/mL)		42; 16	74.4	"	[64]
Ranalexin (30 μM) + lasalocid (2 μg/mL)	*C. parvum* (mer + gam)	33.8; 70.3	100	Additive effect	[64]
Buforin 20 μM + Azithromycin 8 μg/mL	*C. parvum* (mer + gam)	55.7; 32.1	94.4	"	[60]
Buforin 20 μM + Minocycline 8 μg/mL	*C. parvum* (mer + gam)	54.4; 24.6	90.5	"	[60]
MSI-94 10 μM + 200 μg/mL $AgNO_3$	*A. polyphaga* (tph)	92; 80	100	Combination showed full amoebicidal effect	[75]
MSI-94 10 μM + 25 μg/mL Ketoconazol	*A. polyphaga* (tph)	92; 100	100	"	[75]
MSI-103 9.7 μM + 200 μg/mL $AgNO_3$	*A. polyphaga* (tph)	100; 91.6	100	tph and cyt elimination	[75]

[a] Abbreviations: cyt, cyst; gam, gamont; mer, meront; tph, trophozoite.

repulsion among monomers, hence pore destabilization and impaired peptide uptake [19].

4.2. Minimization of active structures

Sequence shortening of AMPs while retaining significant activity and specificity is one of the obviously desirable goals in the chemotherapeutic development of AMPs, with a view to reducing production costs and minimizing the risks of a large peptide inducing an unwanted immune response. A representative example in this direction is the systematic truncation of $K_4K_{20}S4$, a "cationized" version of dermaseptin-S4 with lower tendency to aggregate than the native peptide. From the initial 27-mer, a tridecapeptide $K_4S4(1–13)a$ with an above 30-fold decrease in cytotoxicity was derived, while leishmanicidal [79] and antiplasmodial activities [46] decreased less than one log order.

4.3. Overall peptide structure

A high α-helix content for peptide binding to the membrane has also been assumed to be an essential requirement for AMP activity on most targets, including parasitic Protozoa [82]. Thus, short dermaseptin-S4 analogs active on *Leishmania* and *Plasmodium* such as $K_4S4(1–13)a$ or $K_4S4(1–16)a$ were designed so as to preserve a strong α-helical conformation [79]. However, the finding that pathogen permeabilization can be achieved by poorly structured AMPs has challenged this hypothesis [11]. For instance, dermaseptin-S3 (DRS-S3), selective on *Plasmodium* over erythrocytes [47], is found by NMR to be unstructured, thus questioning the α-helix requirement as essential for strong parasiticidal effect [83].

A further concern in the design of optimized versions of AMPs is the control of peptide aggregation in solution, a somehow intrinsic trend of amphipathic structures with fairly extensive hydrophobic surfaces that favor intermolecular association. While aggregation has been reported to hinder effective membrane binding and eventually impair the killing of bacteria with intact external barriers, for protozoan targets the evidence in this respect is a bit ambiguous. Thus, on the one hand, magainin analogs suspected of aggregation, such as MG-H1, show diminished leishmanicidal activity relative to monomeric analogs [39]. On the other hand, aggregation-prone dermaseptin-S4 analog $K_4K_{20}S4$ appears to enjoy unhindered access to the membrane despite the compact glycocalix of *Leishmania*. Also, the higher antiplasmodial activity of dermaseptin-S4 (aggregated) vs. dermaseptin-S3 (monomeric) has been accounted by the binding of peptide aggregates causing a substantial concentration at the membrane insertion point [47]. Along similar lines, preformed aggregates of the synthetic KL diastereomeric peptide substantially increased the poor fungicidal activity of the monomeric form [84], and suprastructures mimicking α-helical bundles showed enhanced hemolytic activity over the monomer version [85]. In conclusion, it would appear that the issue of peptide self-association does not lend itself to a uniform, simplistic approach, but is best approached on a case-by-case basis, where the different variables (peptide aggregation state, membrane structure and superstructure) are addressed.

A minor but often not trivial contribution to peptide overall structure is the nature of the C-terminal group. A substantial fraction of the natural AMPs are C-terminally amidated, as a result of post-translational decarboxylation of a precursor with an additional Gly residue. Many AMP analogs are similarly C-terminally amidated, often for additional synthetic considerations. Amidation affords partial protection against endopeptidases, increases the positive charge of the peptide (vs. a free carboxyl version), and enhances the α-helical intrinsic dipole, thereby stabilizing any potential helical conformation. These factors, whatever their respective contribution, usually bear favorably on AMP activity. For instance, amidation of short dermaseptin-S4 analogs $K_4K_{20}S4$ and $K_4S4(1–16)$ increases the activity on *Plasmodium* by one log order [79,86] vs. a 5-fold increase in hemolytic activity, thus achieving a moderate increase in selectivity. For dermaseptin-S3, amidation induces a substantial increase in peptide structuration [87] and a concomitant increase in microbicidal activity on bacteria and fungi [88].

Several natural AMPs contain a D-amino acid residue at a single position [89], including the bombinins H4 and H7 from *Bombina* species, in which D-*allo*-isoleucine and D-leucine, respectively, are incorporated at position 2 [90]. Comparison of bombinin H4 with its epimer bombinin H2 (l-Ile at position 2) shows better leishmanicidal activity for the former [41], which in a molecular dynamics study appears to possess a broader conformational repertoire in solution than H2. Analysis of the membrane-bound peptides by ATR-FTIR shows H4 to be slightly more α-helical than H2, regardless of membrane composition, while in an NMR study the differences between the two epimers are confined to the first four residues, postulated to determine the aggregation propensity. In model membranes mimicking the *Leishmania* PM composition, partial aggregation of H2 but not of H4 has been detected, presumably impairing pore formation and hence *Leishmania* killing (for a detailed discussion see [91]). A similar amyloidogenic behavior on the membrane has also been recently described for dermaseptin-S9 (DRS-S9) [71].

Synthetic diastereomers, in which D-amino acids replace native L-residues at designed positions, have been promoted by the group of Shai as a tool to assess the role of conformation in the activity of α-helical peptides. The impact of this modification on the global peptide conformation depends on the hydrophobicity of the region where the substitutions take place [11]. An evenly and extensive substitution on magainin leads to a substantial loss of activity of magainin 2 on *T. cruzi* [82].

4.4. Acylation

Most SAR studies on AMPs routinely include residue replacements altering the charge or hydrophobicity at a given position or region of the sequence. As such changes need involve modification in side chain structure, they may undesirably impinge on other peptide parameters as well. An alternative approach to increasing the hydrophobicity of an AMP is conjugation to a hydrophobic moiety (usually a fatty acid) at the amino end (see Ref. [92] for acylation in internal positions of an AMP active on *Leishmania*). In this way the primary structure and its associated properties other than hydrophobicity are largely preserved. Among amphibian AMPs, acylation has been studied on magainin 2 [93] and dermaseptin-S4 (DRS-S4) analogs [46,48,83,94], although only the latter have been tested on Protozoa. Acylation neutralizes the positive charge at the α-amino group (an exception being the acylation with a lipophilic aminoacyl moiety [83,94]), and partially protects against proteolytic attack, as observed for the dermaseptin-S4 analogs NC_{12}-$K_4S4(1–13)a$ and C_{12}-$K_4S4(1–13)a$ [94] and for palmitoyl-magainin 2 [93]. The acyl chain also constitutes a hydrophobic template which may favor either structuration or aggregation of the peptide. The effect of acyl chain length on these two parameters has been studied in detail for magainin 2 and the dermaseptin-S4 analog $K_4S4(1–13)a$. While in the first case, a bell-shaped effect for aggregation vs. chain length was observed [93], for $K_4S4(1–13)a$ the monomeric status was maintained up to the miristoyl (C_{12}) [83,94], further lengthening promoting peptide aggregation. The aggregation propensity may be partly reverted by aminoacylation: the NC_{12}-$K_4S4(1–13)a$ analog [94] shows the same aggregation state as the unacylated parental peptide, even higher than that produced with the equivalent acylation [83]. In the NC_{12} analog these modifications bring about an increase in structuration and amphipaticity of the N-terminal region [83]. Interestingly, for magainin 2 acylation does not change the parallel

orientation of the monomer in the PM plane, therefore the acyl chain must be inserted perpendicular to the bilayer plane [93].

The acylated analogs of K_4S4(1–13)a showed an increase in hemolysis for acyl group length over five carbons [48,94], whereas the leishmanicidal and antiplasmodial activities levelled off at N-pentanoyl- and N-hexanoyl-analogs respectively [48,94]. For the later activity, the best analogs correspond to propionyl-K_4S4(1–13)a and isobutiryl-K_4S4(1–13)a, not only because of their high selectivity index, but for their selective lysis of the intracellular *Plasmodium* but not of the PM of the infected erythrocyte [48]. Interestingly, the inclusion of an ω-amino group in the acyl group, causes a lower hydrophobicity, an additional positive charge and reduction of aggregation; furthermore aminoacylated analogs were less hemolytic than the parent up to NC_7. NC_4-K_4S4(1–13)a, the least toxic analog, has an LC_{50} for hemolysis higher than 100 μM whereas for the parent K_4S4(1–13)a is 50 μM [94]; furthermore this analog possesses the higher selectivity index for *Leishmania* [94].

5. Perspectives on amphibian AMPs and Protozoa

As all pluricellular organisms, Amphibia endure parasitism and commensalism by a wide variety of Protozoa (reviewed by [95]). For the approximately 4000 amphibians described by 1998, over 200 species of parasites and microbiota were reported, including human parasites such as kinetoplastids [96], sporozoans [95] or amoebazoans [97]. Information on amphibian pathologies is mostly limited to those in amphibian husbandry [98] rather than in the natural habitat. Even less is known about the protection accorded to Amphibia by AMPs active on protozoan, nor about the potential role of those pathogens as AMP inducers [99]. A recent study, spurred by the global decline of Amphibia, has focused on chytridiomycosis, an infection by the fungus *Batrachochytrium dendrobatidis*, and on the role of AMPs in such a context [100].

As it is, the biodiversity of Amphibia, along with the notion that each species may possess a distinct set of AMPs [101] whose even minute structural variations may modulate activity on different microorganisms [88], make this animal class a virtually inexhaustible source of AMP leads. The generalized application of proteomics [102,103] and transcriptomics [104,105] technologies is likely to expand even further the set of natural AMPs to be tested. In fact, the current number of amphibian AMPs is arguably underestimated, for at least two reasons: (i) the quest for amphibian AMPs has focused almost exclusively on cutaneous secretions, mostly ignoring other anatomical locations [106,107]; (ii) the increasing evidence of intracellular targets, a step ahead of relatively unspecific membrane permeation [8], is likely to broaden the roster of amphibian AMPs. Within this latter group, buforin 2 has acquired notoriety by its DNA-binding properties, both in bacteria [108] and in tumor cells [109]. Other amphibian AMPs formerly assumed to act by mere membrane permeation have on closer inspection seen to induce apoptosis (i.e., a necrotic rather than an apoptotic scenario); these include brevinin-2R on tumoral cells [110], dermaseptin-S3(1–16) on yeast [111,112], magainin 2 [112] on yeast, or pexiganan on *Leishmania* [43].

As for protozoan targets, the feasibility of AMPs as antiparasitic chemotherapeutical alternative has been dealt with in previous sections, noting as well their efficacy in situations where specificity is at a premium, such as intracellular *Plasmodium*, *Cryptosporidium* or *Leishmania*, or cloroquine-resistant *Plasmodium* strains [46]. Other protozoans acting as extracellular parasites in vertebrate hosts, such as *Trichomonas vaginalis*, *Giardia lamblia*, or African trypanosomes, have been shown to be susceptible to at least one type of AMP.

Most if not all of the structural modulation strategies used in the therapeutic optimization of amphibian AMPs against non-protozoan pathogens [76,113,114], including cyclization [115], dimerization [116], hybridization with other AMP sequences (amphibian or not) [117–119], use of non-proteinogenic amino acids [113], or various modes of stereoisomeric intervention [116,120] can also be tested on protozoan targets. The availability of fast systematic AMP screens on parasites [121] is a valuable tool in the development of anti-protozoan candidates.

It may be argued that the main caveat for a future extensive use of AMPs in anti-parasite or, in general, anti-infectious chemotherapy possibly lies in the production costs. Though accurate if a strict comparison between conventional small molecule drugs and AMPs is enforced, the above notion needs to be properly qualified. For small size AMP candidates preferably accessible by chemical synthesis, the SAR strategies discussed in Section 4 above, aimed at size reduction and proteolytic stabilization, can have a favorable impact on cost reduction. For longer sequences, recombinant and other (e.g., plant) production alternatives are advisable, if the foreseeable toxicity of the end product can somehow be averted. A number of examples of recombinant production of amphibian AMPs have been described and reviewed [122].

AMP combination therapy with classical antiparasitic drugs is a further option. In this regard, a seemingly attractive approach is one that exploits “crisis solutions” for lipid biosynthesis undertaken by protozoa in the face of drug pressure. Those include changes in PL composition and orientation, or in type and content of sterol [10,123–125]. Evidently, drugs affecting those systems are likely to modulate the outcome of AMP action. Selected findings supporting this approach include the shifts (e.g., reversion of the PC:PE ratio) in *T. cruzi* membrane PL composition caused by ajoene [126]; also on *T. cruzi*, the effect on sterol biosynthesis of inhibitors such as azasterols [127], the triazole UR-9825 [128], or miltefosine (hexadecylphosphocholine) [129], which causes similar effects on *Leishmania donovani* [130]. For Trypanosomatidae, inhibition of ergosterol biosynthesis can backfire on AMP-based treatments, since the natural sterol is at least partially replaced by cholesterol from the host [131], or by accumulation of ergosterol precursors such as lanosterol, as found for *L. mexicana* promastigotes [132]. Either solution turns out to be detrimental for the activity of AMPs such as temporin L, for which a marked inhibition of membrane insertion is noted [26]. For its part, miltefosine resistance in *L. donovani* promastigotes results in PLs with fewer unsaturations and shorter chains, altogether leading to a decrease in PM fluidity [133]; a similar increase in saturated fatty acid chains was observed for *T. cruzi* upon treatment with ketoconazole [127] or ajoene [126].

Finally, other effects include an increase in the PS content of *T. cruzi* brought about by the aforesaid RU-9825 [128], or the simultaneous decrease in PS and increase in PI achieved by the 22, 26-azasterol [134]; the same drug on *Plasmodium* causes an increase in PS content [135].

Related applications of amphibian and other AMPs vs. Protozoa include the elimination of *T. cruzi* contamination in blood transfusion, a serious concern in blood banks, or the development of transgenic arthropod vectors able to impair parasite transmission, as shown by the direct inoculation of magainin in *Anopheles* [136].

It will be clear from the previous exposition that, in the realm of parasite–peptide relationships, the questions still far outnumber the answers, to a significant extent because of the neglected target condition of parasites, and ensuing reasons already alluded to in the Introduction. Even so, it is likely that novel AMPs for parasites will be developed, benefiting from cost reductions achieved in the pharmacological development against more economically valuable pathogens, a situation not unlike that observed in recent years for drugs such as miltefosine or amphotericin, initially developed for antitumoral and antifungal applications [137].

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