

Original Article

Antiprotozoan and Antiviral Activities of Non-cytotoxic Truncated and Variant Analogues of Mussel Defensin

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We previously reported the crucial role displayed by loop 3 of defensin isolated from the Mediterranean mussel, *Mytilus galloprovincialis*, in antibacterial and antifungal activities. We now investigated antiprotozoan and antiviral activities of some previously reported fragments B, D, E, P and Q. Two fragments (D and P) efficiently killed *Trypanosoma brucei* (ID₅₀ 4–12 µM) and *Leishmania major* (ID₅₀ 12–45 µM) in a time/dose-dependent manner. Killing of *T. brucei* started as early as 1 h after initiation of contact with fragment D and reached 55% mortality after 6 h. Killing was temperature dependent and a temperature of 4°C efficiently impaired the ability to kill *T. brucei*. Fragments bound to the entire external epithelium of *T. brucei*. Prevention of HIV-1 infestation was obtained only with fragments P and Q at 20 µM. Even if fragment P was active on both targets, the specificity of fragments D and Q suggest that antiprotozoan and antiviral activities are mediated by different mechanisms. Truncated sequences of mussel defensin, including amino acid replacement to maintain 3D structure and increased positive net charge, also possess antiprotozoan and antiviral capabilities. New alternative and/or complementary antibiotics can be derived from the vast reservoir of natural antimicrobial peptides (AMPs) contained in marine invertebrates.

Keywords: *Trypanosoma* – *Leishmania* – HIV – antibiotics – protozoa – virus – defensin – mussel

Introduction

One of the characteristics of animal life is mobility, offering multiple advantages among which are escape, food gathering and partner encounter for reproduction. Most marine animal life is fixed to a substratum. As a consequence, animals must adapt to this unusual situation. Obviously they cannot escape, they must protect themselves against deadly radiation, especially UVA, they compete for available space, they must avoid predation and they are exposed to colonization. According to phyla, different strategies evolved leading to various visible morphologies and

behaviors. Millions of invertebrate species exist in contrast to only 45 000 vertebrate species. Among such an enormous variety of animal species one can expect to find original systems/mechanisms with putative benefits for human health.

It is the immune system that plays a major role in determining host fitness in the wild, i.e. under the constraints imposed by ecology and life history. But immune responses are costly and theory predicts when a host should switch between constitutive (e.g. pre-existing phenoloxidase cascade) and inducible (e.g. antimicrobial peptides or AMPs) defenses, or when they should combine those defenses (1). In addition, genetic variations for resistance to pathogens are high and immune genes appear to evolve relatively rapidly (2). Most of our knowledge on the functionality of invertebrate immune systems has been obtained through laboratory-based observations, most of the time in the absence of true pathogens. The link with natural forces that drive evolution of those genes in natural

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populations is still missing and it constitutes the rapidly expanding field of ecological immunology (3).

Permanent conflict interactions with the environment are the natural situation for a living creature. To partially resolve this, the immune system evolved and is characterized by an enormous variety of mechanisms and effectors, including the AMPs, although their specificity is poor. In fact, AMPs are universal and extremely successful in dealing with a huge range of pathogens, including bacteria, fungi, protozoa and viruses (4). However, AMPs differ widely in amino acid sequence and 3D structures. Classically, they are arranged in three to five classes (5,6). Activity of AMPs is generally restricted to one type of target, i.e. insect defensins, dipterin, drosocin and attacin (see 7 for review), drosomycin (8), mollusc mytimycin (9) and myticin (10). Some of them are multipotent, i.e. ceratotoxins (11), mytilins (12), metchnikowin (13) and cecropins (14). All the previous peptides possess a positive surface charge, i.e. they are cationic. Some others are anionic, i.e. lung lavage fluid (15) and ovine pulmonary surfactant (16) with still unknown modes of action. Some others are released from the cleavage of macromolecules, i.e. lactoferrin (17), hen ovotransferrin (18) and crustacean hemocyanin (19).

Whatever their commercial names, all the antibiotics we used have been derived from a restricted number of proteins with only slight differences introduced into the frameworks. Systematic use and abuse of antibiotics have selected for multi-drug resistant bacteria. The number and danger of such adapted bacteria are already a major public health problem. We rapidly need new antibiotic molecules possessing original modes of action that pathogens cannot adapt to. It is the purpose of this report to present one solution derived from marine invertebrate AMPs. We previously reported antibacterial and antifungal activities (20) of fragments derived from mussel defensin structure (21). It is the purpose of this report to extend such activities to protozoa and virus. In addition, cytotoxicity towards mammal cells was evaluated.

Subjects and Methods

Truncated and Variant Fragments Defensin

Among the 19 fragments previously reported by Romestand *et al.* (20) for their antibacterial and antifungal activities, we selected three fragments of nine amino acids (B, D and E) and two fragments of 19 amino acids (P and Q). Fragments D and P were the counterparts of fragments B and Q including two more positive charges each (Fig. 1). Fragment E was an open fragment B.

Cytotoxic Assay

Human HeLa MAGIC-5 cells were incubated with fragments at 100 μ M. Toxicity was evaluated after 48 h at 37°C by measuring the optical density of the culture at 570/690 nm using the In Vitro Toxicology Assay Kit (Sigma), based on reduction of the

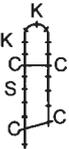
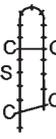
One-letter names of the fragments				
B	D	E	P	Q
Net positive charges				
2	4	2	5	3
				

Figure 1. Structure of fragments and variant analogues with special mention of disulfide bond(s) and replacements by lysines. Amino acids are expressed with one-letter codes: C, cysteine; S, serine; K, lysine. Drawings are from the sequences reported by Romestand *et al.* (20).

yellow tetrazolium salt MTT into purple formazan crystals by mitochondria of active cells, as reported by Hansen *et al.* (23).

Antiprotozoan Assay

Frozen stabilates of *Trypanosoma brucei* Antat1.1 were expanded in Balb/c mice (IFFA Credo). Four to five days post-infection, animals were exsanguinated on heparin, and parasites were purified by DEAE-cellulose (DE52, Whatman) chromatography in PSG buffer (8.45 g/l Na₂HPO₄, 0.43 g/l NaH₂PO₄, 2.13 g/l NaCl, 8 g/l glucose, pH 8.0). *Trypanosoma brucei* samples (2×10^5 in 100 μ l PSG) were dispensed, in triplicate, in 96-well plates containing 100 μ l of serial dilutions of mussel peptides and incubated at 29°C. Living parasites were counted microscopically under contrast phase at indicated times.

Frozen stabilates of *Leishmania major* were injected in the footpad of Balb/c mice. Four days later, popliteal lymph nodes were collected, gently scratched and put in culture in RPMI 1640 containing 10% fetal calf serum. Trypomastigotes were allowed to grow *in vitro* at 29°C, regularly refreshing the medium. *Leishmania major* were resuspended in RPMI-10% fetal calf serum, and the killing assay was performed as described for *T. brucei*, with the exception that the experiments were performed in RPMI-10% fetal calf serum.

Antiviral Assay

We used a simple phenotypic assay for drug susceptibility of human immunodeficiency virus type-1 (HIV-1) using a CCR5 and CXCR4-expressing HeLa/CD4⁺ cell clone, named MAGIC-5B (24). One day before the assay, 50 000 MAGIC-5B cells in 880 μ l Dulbecco's Modified Eagles' Medium

(DMEM, Gibco) containing 10% fetal calf serum, were added to each well of a 24-well microtest plate and incubated at 37°C under 95% air–5% CO₂. For the assay, 100 µl of a 10× stock solution of the fragment in DMEM were added to the cells and incubated for 15 min at 37°C. After gentle washing to remove excess of fragment, 20 µl aliquots of HIV-1 solution, corresponding to 80 000 c.p.m., were added. Alternatively, fragment solutions were incubated for 15 min with HIV-1 before addition to the cells, without washing of fragment excess. β-Galactosidase enzyme assay with Reporter lysis buffer (Promega, France) was used according to the manufacturer's instructions. Briefly, MAGIC-5B cells were incubated for 72 h at 37°C, lysed by addition of 1 ml assay 2× buffer which contained the substrate ONPG (*o*-nitrophenyl-β-D-galactopyranoside). Samples were incubated for 30 min to allow β-galactosidase to hydrolyze the colorless substrate to yellow *o*-nitrophenyl. Reaction was stopped by addition of 50 µl of 1 M Na₂CO₃. Absorbance at 420 nm was measured in an enzyme-linked immunosorbent assay reader (Multiskan Labsystems Bichromatic). Incubations with untreated HIV-1 (maximum infestation) in the presence of 10 µM AZT (used as inhibitor of infection) were used to verify the functionality of the assay. Except when indicated, combinations were carried out in triplicate and entire experiments were performed twice.

Statistics

Results are expressed as arithmetical mean ± SEM of at least triplicates from one experiment (see Fig. 3) or from between two and five independent experiments. Statistical significance of differences was established by Student's *t*-test with $P < 0.015$.

Fluorescence Detection of Fragments B and D

Biotin tagged fragments B and D were from Romestand *et al.* (20). Purified *T. brucei* were maintained in Eppendorf tubes in an ice bath throughout the procedure. Parasites (10⁶ in 40 µl) were incubated for 30 min in an ice bath in PSG containing 10% normal mouse serum prior to adding biotinylated fragment B or D (10 µM final, in 40 µl). After 40 min, the parasites were washed twice in large volumes of PSG containing 10% normal mice serum (1400 r.p.m., 3 min) and fixed for 1 h in PBS containing 3% formaldehyde, 0.5% glutaraldehyde. After two washes in PSG containing 1% bovine serum albumin (BSA), streptavidin-FITC (7.5 µM final, Sigma) was added for 1 h. Observations were done after three washes in PSG containing 1% BSA with a Nikon ECLIPSE E600 fluorescence microscope equipped with phase contrast.

Results

Antiprotozoan activity

Among the tested defensin fragments, only fragments D and P were able to kill *in vitro* *T. brucei* (Fig. 2). As little as 1.5 µM of fragment D killed 17.1 ± 0.9% of the parasites after 2 h of contact at 29°C. Furthermore, 89.3 ± 1.9% of the parasites were killed by 25 µM of fragment D. Fragment P was also toxic, but to a lesser extent, as only 1.6 ± 0.6% of *T. brucei* were killed by 1.5 µM ($P < 0.0001$) and 70.3 ± 1.4% by 25 µM ($P < 0.0001$). Such differences in the killing capacity was statistically highly significant at all concentrations, except the lowest values for which killing was non-existent (0.4 µM) or marginal (0.8 µM). The killing capacity was reflected by the

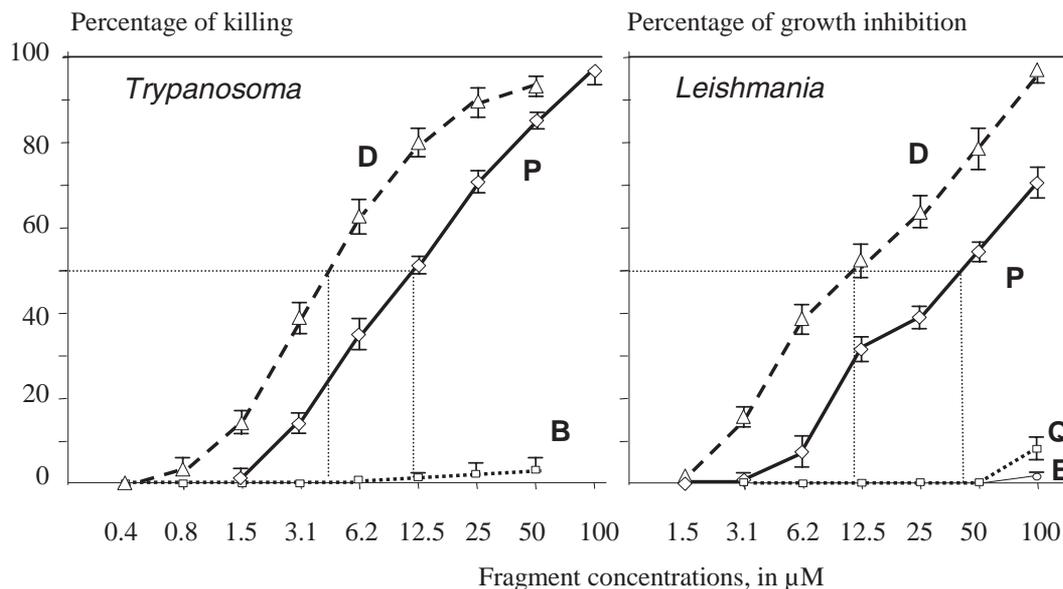


Figure 2. Antiprotozoan activity of truncated defensins. *Trypanosoma brucei* were incubated *in vitro* for 2 h at 29°C in survival medium in the presence of various fragment concentrations. *Leishmania major* were incubated for 24 h at 29°C in growing medium containing various fragment concentrations. Percentages of killing or percentages of growth inhibition were determined upon microscopic examination. Values are arithmetic means of three replicates of at least three independent experiments ± SEM (bars). For *T. brucei*, the difference between D and P values are statistically extremely significant ($P < 0.0001$) except for 0.4 and 0.8 µM. For *L. major*, the difference between D and P values are at least significant ($P = 0.01$ for 50 µM) except for 1.5 µM ($P = 0.37$). Dotted lines are for ID₅₀.

respective ID_{50} values: 4 μM for fragment D compared to 12 μM for fragment P. All the other tested fragments were not toxic for *T. brucei*, with the exception of fragment B at the highest concentration tested: $3.3 \pm 1.6\%$ killed at 50 μM .

When tested against *L. major* *in vitro*, again fragment D appeared as the most active. After 24 h of contact with parasites at 29°C, 6.2 μM of fragment D induced $39.0 \pm 2.3\%$ of killing whereas 100 μM induced $97.6 \pm 1.4\%$ of killing (ID_{50} 12 μM). Concomitantly, $8.6 \pm 2.9\%$ ($P = 0.0012$) of killing was observed with 6.2 μM of peptide P and $70.0 \pm 4.5\%$ ($P = 0.0045$) with 100 μM (ID_{50} 45 μM) (Fig. 2). No activity was noted with fragment B, even at 100 μM . Only marginal mortality was observed with 100 μM of fragments E ($3.6 \pm 0.6\%$) and Q ($9.0 \pm 4.5\%$).

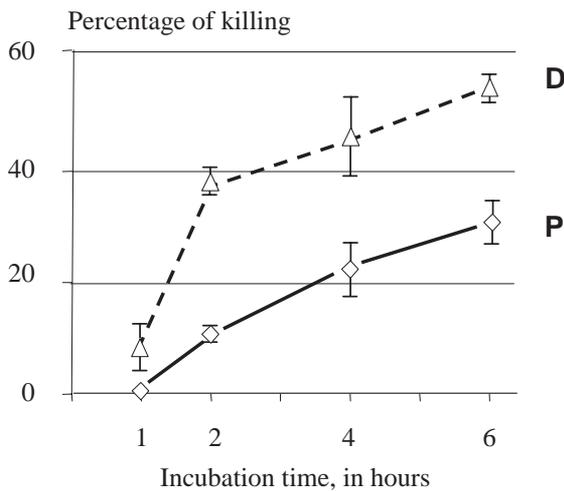


Figure 3. Kinetics of *in vitro* killing of *T. brucei* at 29°C in the presence of 3.1 μM of defensin fragment D and P. Values are arithmetic means of at least three replicates \pm SEM (bars). Difference between D and P values are statistically extremely significant ($P < 0.0001$) after 2 h and significant ($P = 0.016$) after 4 and 6 h.

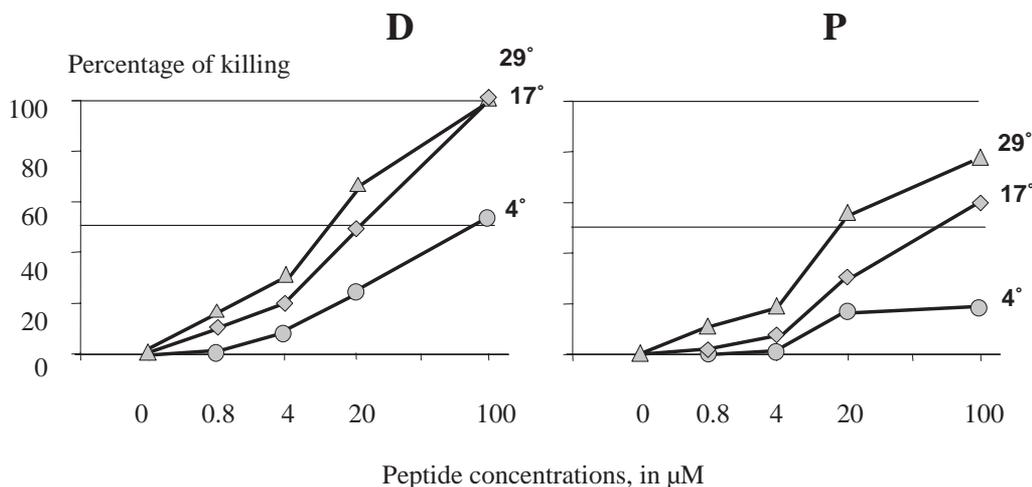


Figure 4. Effect of temperature on *in vitro* killing of *T. brucei* after 2 h incubation with defensin fragment D and P. Results are arithmetic means of three replicates of one experiment. SEM is not indicated as contained within marks. Differences between temperatures are at least statistically significant ($P < 0.014$) for fragment D, and at least statistically very significant ($P < 0.0048$) for fragment P. Only the killing percentages observed with 100 μM of fragment D at 17 and 29°C and with 0.8 μM of fragment P at 4 and 17°C were not statistically different.

Kinetics of *T. brucei* Killing

The kinetics of *in vitro* killing of *T. brucei* were investigated in the presence of several molarities of fragments D and P. At all concentrations, the difference in killing activity of fragments D and P could not be overcome by increasing incubation times up to 6 h. This is illustrated in Fig. 3 which shows killing activity in the presence of 3.1 μM of fragments D and P. As early as 1 h after contact with fragment D, killing was $5.5 \pm 2.5\%$, reaching $39.5 \pm 1.5\%$ after only 2 h. At that time, fragment P induced $14.1 \pm 1.6\%$ of mortality, a highly significant difference ($P < 0.0001$). Between 2 and 6 h, the slopes of the two killing curves appeared parallel, reaching $53.0 \pm 2.0\%$ for fragment D at 6 h and $31.0 \pm 2.0\%$ for fragment P ($P = 0.016$).

Temperature Effect

To investigate whether the defensin fragments needed to be internalized by *T. brucei* in order to exert their activity, killing was measured after 2 h incubations at 4, 17 and 29°C in the presence of fragments D and P at a range of concentrations. At temperature below 17°C, the membrane of trypanosomes adopts a rigid conformation that impairs phagocytic processes including endosome/lysosome fusion (25). Results indicated that incubation at 4°C efficiently impaired the ability of fragments D and P to kill the parasites (Fig. 4), suggesting that membrane fluidity is required for peptides to be active and that general cytotoxicity is highly improbable. The difference between 4 and 17°C was statistically significant at 0.8 μM of fragment D ($P < 0.0001$) and 4 μM of fragment P ($P = 0.0015$). With the exception of 100 μM of fragment D, killing observed at 29°C was always statistically higher than at 17°C. At all tested temperatures and with all concentrations, fragment D was more toxic than fragment P, confirming the data in Figs 2 and 3.

Membrane Binding

Possible interaction between defensin fragments and *T. brucei* membranes was visualized by fluorescence microscopy. We controlled that antiprotozoan capacity of biotinylated fragments B and D were not significantly different from untagged fragments, revealing that biotinylation did not alter such capacities. When parasites were incubated with biotinylated fragments, the entire surface of *T. brucei* was labeled so that peptides did not bind to a specific location, such as the flagellar pocket (Fig. 5). Surprisingly, labeling observed with both fragments B and D were similar, even though only fragment D killed the parasites. We controlled so that the FITC-labeled streptavidin used to detect the bound defensin fragments did not bind to *T. brucei* (Fig. 5) and had no toxic effect on viability of parasites at least over 2 h at 29°C (not shown).

In Vitro Prevention of Viral Infection

In addition to antiprotozoan activity, we were interested in putative interference of defensin fragments with virus infestation. When

infected by untreated HIV-1, MAGIC-5B cells were able to metabolize the ONPG substrate, giving measurable absorption at 420 nm. Such absorption was dramatically reduced by ~70% when viruses were pre-incubated with 20 µM of fragment Q or P (Fig. 6). No significant inhibition was observed with 10 µM of fragment Q or P. In addition, fragment B, D and E were not inhibitory, even when tested at 20 µM. Interestingly, when the MAGIC-5B cells were incubated with the same fragments, washed, then submitted to HIV-1 infection, no inhibition was observed (Fig. 6). We controlled such that 20 µM of peptide was not toxic for MAGIC-5B cells, at least up to 100 µM of fragments B, D, E, P and Q over 48 h at 37°C. In addition, the well-known inhibitor AZT used at 10 µM totally suppressed HIV-1 infection.

Discussion

Antiprotozoan activity of defensin fragments was evaluated on the African trypanosome *T. brucei* which belongs to the genus that causes sleeping sickness, and on *L. major* that causes cutaneous leishmaniasis. Our data show that two of the five

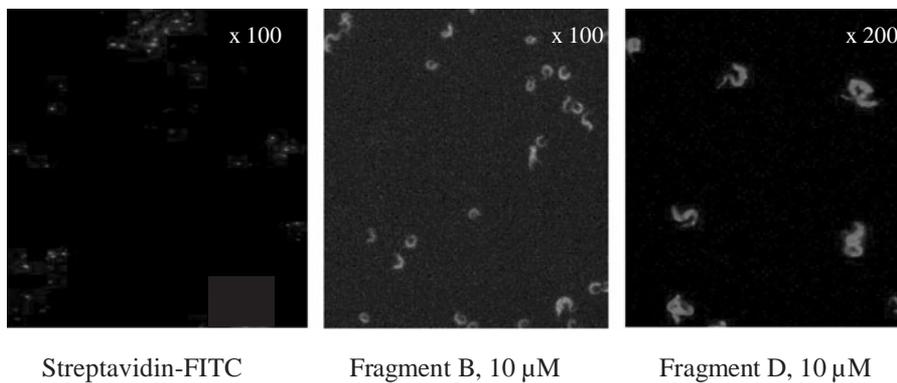


Figure 5. Binding of biotinylated defensin fragment B and D (10 µM) on *T. brucei*. Note that FITC-labeled streptavidin did not bind to the targets.

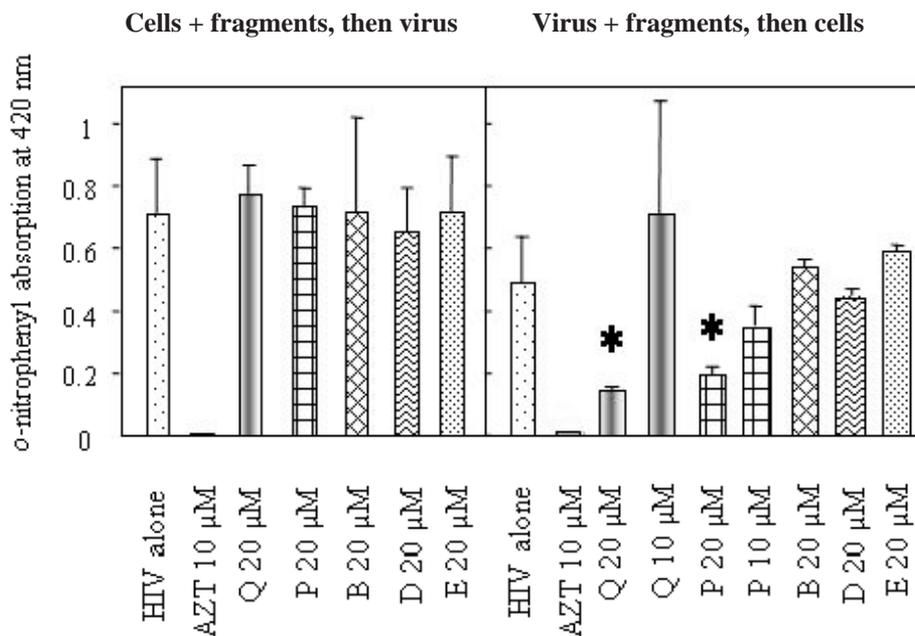


Figure 6. Interference of defensin fragments Q, P, B, D and E with infection of MAGIC-5B cells by HIV-1 compared to AZT inhibitory effect. Results are from one of two independent experiments and presented as arithmetic mean values \pm SEM (bars) of triplicates. Stars indicate extremely significant difference ($P < 0.0001$) with infection by HIV-1 alone. Note that fragment P was tested three times but only in mixture with cells and virus, without washing of excess fragment.

tested fragments derived from mussel defensin efficiently killed *T. brucei* and *L. major* in a time/dose-dependent manner. Differences in ID_{50} (4–12 μM for *T. brucei* versus 12–45 μM for *L. major*) can be due to the fact that experiments were performed in a survival medium for trypanosomes and in a growing medium for *L. major*. Dose–response curves of fragments D and P were parallel, with stronger killing capacity of fragment D. This stronger and faster activity of fragment D was confirmed by kinetic curves and by the effects of temperature during incubation. As revealed by observation of fluorescence, fragments bind to parasite membranes and fluidity of these membranes appears to be crucial as incubation at 4°C significantly impaired the ability to kill trypanosomes. In addition, and with the exception of the 100 μM concentration, killing was higher at 29°C compared to 17°C, suggesting that fusion endosome/lysosome is required to display killing.

Fragments D and P are the counterparts of fragments B and Q, with the addition of two positive charges located at loop 3. The presence of these extra charges appears to be fundamental for both antiprotozoan and antiviral activities as has been reported for antibacterial activity (20). Both fragments B and D bound to parasite membranes, suggests that to be positively charged is a condition sufficient to bind to the membrane. Activity appeared to be related to peptide size as fragment D (nine amino acids) is much more efficient than fragment P (19 amino acids) on protozoa. It is well known that AMPs act at the membrane level, by inserting into the phospholipid bilayer. The better activity observed with fragment D might be due to its small size, favoring penetration into the parasite membrane. Concerning antiviral activity, correlation between size and activity seems to exist as fragment Q was active in contrast to fragment B. Increasing the net positive charge (from Q to P and from B to D) did not result in better activity. In all cases, activity of fragments B and E was marginal and not sufficient to confirm the importance of folding as demonstrated with bacteria (20).

The present paper provides evidence that truncated AMPs act on structurally different targets: protozoa and viruses. Compared to numerous reports on antibacterial activity of AMPs, including truncated mussel defensin (20), there are few reports of antiviral or antiprotozoan activity. Recently, another mussel AMP, mytilin, has been reported as preventing *in vivo* viral infection due to interference between the virus (White Spot Syndrome Virus) and the peptide (26). Generally, antiviral activity is at the very early stages of viral multiplication as for melittin (27,28), synthetic magainin derivatives (29) and dermaseptins (30). Putative mode of action is by suppressing viral gene expression as for melittin and cecropin (31) or by binding to gp120 and CD4 as for retrocyclin (32).

Different effector structures are active on various target structures. We report here that a sequence of only nine amino acids can kill both *Trypanosoma* and *Leishmania*. Longer sequences of 18 amino acids (protegrin-1), 29 amino acids (cathelicidins) or 30–50 amino acids (α -defensins) have been reported as killing both insect and bloodstream forms of *T. brucei* (33). Action of

cathelicidins involves disruption of parasite membrane integrity, which is in agreement with our observation of parasite labeling. Meanwhile, effects were observed with 5 μM (on procyclics) to 25 μM (on bloodstream forms) and routine experiments engaged 25–50 μM , compared to 3 μM in our experiments with truncated nine-amino-acid defensin. Concerning the antiviral activity, a longer sequence of 19 amino acids seemed necessary to prevent infection. This is in agreement with Dupuy *et al.* (26) who reported antiviral activity of mytilin (39 amino acids) but not of a 13 amino acid folded fragment. Minidefensins of 16–18 residues (out of 30–45) with net positive charge, β -sheet structure and intramolecular disulfide bonds have also been reported as capable of preventing *in vitro* HIV-1 infection (33). The question is, how can a simple structure be active on multiple target structures? Several models have been constructed to explain modes of action at the cell membrane level (35,36). But some authors have suggested that AMPs penetrate cells and also act inside the cytoplasm (37,38). Adding the antiviral activity made a unique mode of action highly improbable.

Even if short amino acid sequences were reported for potent activity towards various targets, the definition of a unique motif to be employed as universal killer is probably too optimistic. In contrast, more sophisticated peptides that combine the products of two genes have been reported (39) and one can think about chimeric peptides constructed by association of several active sites from different peptides, thus increasing the target spectrum. We must also consider the possible *in vivo* increase of *in vitro* activity by synergistic effects with other innate immune mechanisms such as phagocytosis, self antimicrobial peptides and free radicals. It is most probable that, in the near future, new antibiotic generations will be used to complement an association with classical antibiotic molecules.

Introducing a single bacteria species into *Drosophila* resulted in the up-regulation of more than 350 genes, most of them being unknown or having no immune-related functions (40). Constructions of expressed sequence tag (EST) libraries following challenges, released panels of immune-related genes, the vast majority with only potential immune function in shrimp (41), oysters (42,43) and mussels (44). Consequently (i) most of the mechanisms triggered by a microorganism are unknown, (ii) the immune system is not the only one responding to introduction of foreign material and (iii) complex interactions between several physiological functions must occur.

Immune research focuses on polypeptide structures, relying on genetic sequences. However, recognition is also mediated by carbohydrate moieties, sometimes combined with lipids, which constitute the usual external barriers. This is particularly the case for protozoan parasites and bacteria with the crucial role of LPS. In addition, an enormous variety of molecular structures, mostly cyclic, isolated from marine environments have been reported to exert anti-infectious activity (45) and to affect the cardiovascular, immune and nervous systems (46). Some active compounds are as simple as iron (47) or free radicals (48). Consequently, the ribosomally synthesized

peptides involved in immune mechanisms must not be considered separately, but combined with all the other molecular moieties employed by living creatures to ensure a more sophisticated picture.

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