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Antibacterial activity of human neutrophil peptide-1 against *Mycobacterium tuberculosis* $H_{37}Rv$: *in vitro* and *ex vivo* study

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Antibacterial activity of human neutrophil peptide-1 against Mycobacterium tuberculosis *$H_{37}Rv$:* in vitro *and* ex vivo *study. S. Sharma, I. Verma, G.K. Khuller. ©ERS Journals Ltd 2000.*

ABSTRACT: The aim of the study was to investigate the activity of human neutrophil peptide (HNP)-1 to kill *Mycobacterium tuberculosis* $H_{37}Rv$ *in vitro* and *ex vivo* in the murine macrophage cell line J744A.1 on the basis of colony forming units.

Macromolecular biosynthesis was studied by monitoring the incorporation of radioactive precursors into different macromolecules. The binding and localization studies were carried out with radioiodinated HNP-1 whereas the cytotoxicity of HNP-1 to macrophages was determined by trypan blue exclusion assay.

A concentration dependent inhibition in the growth of *M. tuberculosis* $H_{37}Rv$ was observed in the presence of HNP-1. The minimum inhibitory concentration and median inhibitory concentration of HNP-1 were found to be 2.5 $\mu g \cdot mL^{-1}$ and 0.8 $\mu g \cdot mL^{-1}$. Treatment of both *in vitro* grown and phagocytosed mycobacterial cells with HNP-1 resulted in generalized inhibition in the macromolecular biosynthesis with maximum inhibition in deoxyribonucleic acid and lipid biosynthesis. HNP-1 exhibited equilibrium binding with respect to time and two-thirds of bound radioactivity was shown to be present inside the macrophages. Approximately 50% and 98% killing of intracellular mycobacteria was observed after 3 days of treatment with 5 $\mu g \cdot mL^{-1}$ and 40 $\mu g \cdot mL^{-1}$ of HNP-1, respectively. HNP-1 exhibited low cytotoxicity towards the macrophage cell line at the bactericidal concentration to mycobacteria.

From the results of this study, it is concluded that human neutrophil peptide-1 possesses potent bactericidal activity against virulent mycobacteria *in vitro* as well as mycobacteria replicating within macrophages.
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Antimicrobial peptides are present in a wide range of species, from protozoa to man, as effector molecules of innate immunity. Most of these peptides are effective against a broad spectrum of micro-organisms including viruses [1], bacteria [2], fungi [3], spirochaetes [4] and mycobacterial species [5, 6]. The greater interest in these endogenous antimicrobial peptides in the recent past may lead to the development of new drugs for the treatment of infections [7]. Defensins constitute the most important and widely studied class of these peptides. They are a family of small antimicrobial peptides of mammals present in the neutrophil granules, macrophages and paneth cells. In human polymorphonuclear neutrophils, four types of these peptides are present, namely human neutrophil peptides (HNPs) 1–4.

In previous studies, it has been shown that HNP-1 kills *Mycobacterium tuberculosis* $H_{37}Ra$ *in vitro* by binding to the plasma membrane/cell wall, which is the primary target [8], deoxyribonucleic acid (DNA) appears to be the secondary target of HNP-1 action (unpublished data). However, no report is available regarding the activity of HNP-1 against virulent mycobacteria grown *in vitro* and intracellularly within macrophages. It has earlier been suggested that therapeutic strategies designed to increase the defensin content of macrophages could play a useful role in the therapy against intracellular pathogens [9]. Hence, this study was designed to assess the ability of HNP-1 to kill the virulent strain of mycobacteria growing *in vitro* or replicating within macrophages and to explore the potential of HNP-1 as an alternative chemotherapeutic agent against tuberculosis.

Materials and methods

Bacterial culture and growth conditions

M. tuberculosis $H_{37}Rv$ originally obtained from the National Collection of Type Cultures, London (UK) was maintained on Lowenstein Jensen medium [10] and was subsequently grown in Youman′s modified medium [11] containing 0.05% Tween 80 on an orbital shaker (100–120 revolutions per minute) at 37°C.

Antibacterial activity of human neutrophil peptide-1 against Mycobacterium tuberculosis *$H_{37}Rv$*

Chemically synthesized HNP-1 with primary structure and disulphide linkages (*i.e.* between cysteine $(Cys)^2$ and

Cys^{30}, Cys^{4} and Cys^{19}, and Cys^{9} and Cys^{29}) as that of native HNP-1 was obtained from the Peptide Institute (Japan). It was dissolved in 0.01% acetic acid and stored as a stock solution of 100 $\mu g \cdot mL^{-1}$ at -20°C.

To study the effect of HNP-1 on the growth of *M. tuberculosis* $H_{37}Rv$, bacteria were grown in the presence of HNP-1 and the dry weight of cells was used as an index of growth [12]. HNP-1 was added to sterile Youman′s modified medium at an appropriate concentration in specially designed flat bottom tubes (to facilitate proper shaking of the medium) followed by the inoculation of 6.0×10^8 $cells \cdot mL^{-1}$. The tubes were incubated at 37°C on an orbital shaker until mid exponential phase was reached (7–8 days). Cells were fixed with formalin before harvesting on preweighed filter papers and dried at 56°C and the weight was recorded. The relative percentage of growth was calculated as:

Dry weight of cells grown in the presence of HNP-1/Dry weight of control cells (without HNP-1) × 100.

From this, minimum inhibitory concentration (MIC), *i.e.* the concentration at which there was more than 99% inhibition of growth, and median inhibitory concentration (IC_{50}) *i.e.* the concentration at which there was 50% inhibition of growth, were calculated. MIC and IC_{50} were confirmed by measuring the optical density at 580 nm. The bactericidal activity of HNP-1 at IC_{50}, was measured by colony forming unit (cfu) enumeration. Three independent experiments were performed and values were expres-sed as mean±SD.

Macromolecular biosynthesis

M. tuberculosis $H_{37}Rv$ cells grown in the presence of IC_{50} concentration of HNP-1 were harvested and resuspended in sterile Youman′s medium. The cells were then pulsed with various radioactive precursors ^{14}C-aspartic acid for proteins (37 κBq (1 μCi)), ^{3}H-thymidine for DNA (296 κBq (8 μCi)), ^{14}C-uracil for ribonucleic acid (RNA) (37 κBq), and ^{14}C-acetate for lipids (74 κBq 2 μCi)) for 90 min with constant shaking at 37°C. This was followed by the addition of 10% trichloroacetic acid and filtered on preweighed filter papers, which were then dried and weighed again. The radioactivity was measured by using Bray′s scintillation fluid in a 1210 Rack beta scintillation counter (Wallace, Turku, Finland). The results were calculated as counts per minute incorporated per milligram dry weight of cells and expressed as per cent inhibition of the incorporation of radioactive precursors into macromolecules in comparison to control. Three independent experiments were performed and values were expressed as mean±SD.

Maintenance of cell lines

The murine macrophage-like cell line (J744A.1) obtained from the National Centre for Cell Sciences, (Pune, India) was maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% heat inactivated foetal bovine serum (FBS) containing the antibiotics penicillin (100 $IU \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$).

Binding studies

Radioiodination of HNP-1 using $Na^{125}I$ was carried out by the chloramine T method [13]. The binding kinetics and localization of HNP-1 in macrophages was studied by using radioiodinated HNP-1. Briefly, 1.0×10^5 $macrophages \cdot mL^{-1}$ were treated with ^{125}I -HNP-1 for different time points, *i.e.* from 1 min to 60 min. At each time point, 1 mL of this suspension was overlayered on 0.4 mL of silicone oil in a microcentrifuge tube, immediately centrifuged at $12{,}000 \times g$ for 3 min. Upper aqueous and silicone layers were removed and radioactivity in the cellular pellet was measured. Localization of HNP-1 inside the macrophages was studied by performing the binding for 45 min at 37°C. Macrophages were centrifuged through silicone oil and the cellular pellet was lysed with 0.25% sodium dodecylsulphate (SDS), followed by centrifugation at $12{,}000 \times g$ for 3 min and then the radioactivity was measured in the lysate. Two independent experiments were performed in triplicate.

Infection of macrophages with Mycobacterium tuberculosis *$H_{37}Rv$*

To standardize the optimum ratio of macrophage: mycobacteria, phagocytosis was performed by the radiometeric method of BAND *et al.* [14]. Briefly, *M. tuberculosis* $H_{37}Rv$ was radiolabelled by incubating the cells with 925 κBq (25 μCi) of ^{14}C-acetate at 37°C in Kreb′s Ringer′s buffer (KRB) pH 7.4 for 4 h. Cells were harvested and washed three times with KRB to remove the free radioactive precursor. Radioactivity was counted and cells were stored at -20°C until use.

Monolayers of a macrophage cell line were prepared in 24-well tissue culture plates (Costar Corning, Cambridge, MA, USA) and non-adherent cells were removed by washing with plain RPMI-1640 medium. Fresh RPMI-1640 medium with 2% FBS and without antibiotics was added to monolayers. It was followed by infection with radiolabelled *M. tuberculosis* $H_{37}Rv$ suspended in plain RPMI-1640 medium at different ratios of macrophages: mycobacteria. Plates were kept at 37°C in 5% CO_2 for 2 h and the nonphagocytosed mycobacteria were removed by washing the monolayers three times with plain RPMI-1640 medium. The monolayers were lysed with 1.0 mL of cold 0.25% SDS and the radioactivity was measured in the lysate using Bray′s scintillation fluid in a 1210 Rack Beta scintillation counter. It was observed that with an increase in the ratio of macrophages:mycobacteria from 1:1 to 1:100, there was an increase in the percentage of phagocytosis from 14.44±0.32 to 31.01± 0.38 respectively. The optimum ratio was found to be 1:10 resulting in 23.68± 0.11% phagocytosis. Acid fast staining revealed that ~60–80% macrophages contained 2–3 bacilli per macrophage.

Intracellular killing of mycobacteria by human neutrophil peptide-1 inside the macrophages

Monolayers were infected with *M. tuberculosis* $H_{37}Rv$ as described above at a 1:10 ratio of macrophages:mycobacteria for 2 h. Infected monolayers were washed three times with plain RPMI-1640 medium to remove nonphagocytosed mycobacteria, followed by addition of RPMI-1640 medium with 2% FBS without antibiotics in each well. HNP-1 at different concentrations was added to test wells whereas 0.01% acetic acid was added to control

wells. After 3 days, the supernatants were removed, monolayers were washed gently with plain RPMI-1640 medium and lysed with cold 0.25% SDS. Lysates were appropriately diluted and plated on solid Youman′s modified medium supplemented with 1% bovine serum albumin. Plates were incubated at 37°C for 4–6 weeks. Colonies were counted in control and test plates and the results were expressed as $\log_{10}$cfu. Three independent experiments were performed and their values were expressed as mean±SD.

Macromolecular biosynthesis of phagocytosed mycobacteria

Macromolecular biosynthesis of intracellular *M. tuberculosis* $H_{37}Rv$ was studied by the method of CHITAMBER *et al.* [15]. Monolayers of macrophage cell line were infected with *M. tuberculosis* $H_{37}Rv$ at a 1:10 ratio of macrophages:mycobacteria for 2 h. Monolayers were washed three times to remove nonphagocytosed mycobacteria and RPMI-1640 medium with 2% FBS without antibiotics was added to each well. HNP-1 at different concentrations was added to test wells, whereas 0.01% acetic acid was added to the control wells. After 3 days, supernatants were removed, monolayers were washed gently with plain RPMI-1640 medium and lysed with the addition of 0.5 ml of cold distilled water and to the lysate 0.5 mL of 5 × Youman′s modified medium was added. The radioactive precursors ^{14}C-acetate and ^{3}H-thymidine were then added to each well. Plates were kept at 37°C for 12 h under constant shaking. Mycobacteria were fixed with formalin, and harvested on filter papers, dried and the radioactivity was measured in the scintillation counter.

Cytotoxicity assay

Cytotoxicity of HNP-1 to the macrophage cell lines was studied by the dye exclusion method [16]. Monolayers of macrophage cell lines were treated with HNP-1 at 5–40 μg·mL^{-1} in RPMI-1640 containing 2% FBS without antibiotics and in control wells 0.01% acetic acid was added. Plates were kept at 37°C in 5% CO_2 for 24, 48 and 72 h and after each time point trypan blue exclusion was performed to calculate the per cent cytotoxicity:

$$\frac{\text{MVM in control} - \text{MVM in test}}{\text{MVM in control}} \times 100$$

Two independent experiments were carried out in triplicate and values were expressed as the mean of the two experiments.

Results

Antibacterial activity against Mycobacterium tuberculosis *$H_{37}Rv$*

The effect of HNP-1 against *M. tuberculosis* $H_{37}Rv$ was examined by growing the cells in the presence of HNP-1 at concentrations ranging 0.5–2.5 μg·mL^{-1}. HNP-1 resulted in decreased growth, which was concentration dependent, when the relative percentage of growth (as compared to that of control) was plotted against the concentration of HNP-1. The IC50 and MIC were found to be 0.8 μg·mL^{-1} and 2.5 μg·mL^{-1} respectively (fig. 1). HNP-1 resulted in a 50% reduction in the cfu (6.66×10^7) of *M. tuberculosis* $H_{37}Rv$ cells grown at 0.8 μg·mL^{-1} of HNP-1 as compared to controls (1.31×10^8).

Macromolecular biosynthesis of Mycobacterium tuberculosis *$H_{37}Rv$ grown in the presence of human neutrophil peptide-1 and phagocytosed mycobacteria*

When *M. tuberculosis* $H_{37}Rv$ was grown in the presence of HNP-1 at IC50, there was a generalized inhibition in the incorporation of all the radioactive precursors into their respective macromolecules. Maximum inhibition was observed with the incorporation of ^{3}H-thymidine into DNA (66.70±6.07) and ^{14}C-acetate into lipids (63.45±3.95). There was 47.20±2.19 and 51.55±4.79% inhibition with the incorporation of ^{14}C-uracil and ^{14}C-aspartic acid into the RNA and proteins respectively (fig. 2a). Similar inhibition of protein biosynthesis was observed with other radioactive precursors *i.e.* ^{14}C-serine and ^{14}C-lysine (data not shown).

The incorporation of radioactive precursors into macromolecules of mycobacteria released from HNP-1 treated infected macrophages was found to be inhibited significantly as compared to controls, *i.e.* without HNP-1. At 5.0–40 μg·mL^{-1} concentration of HNP-1, there was ~49–99% inhibition respectively with the incorporation of ^{3}H-thymidine into DNA. Similar inhibition with the incorporation of ^{14}C-acetate into lipids was observed. However, the inhibition in the lipid biosynthesis was less (89%) as compared to inhibition in DNA biosynthesis (99%) at 40 μg·mL^{-1} concentration of HNP-1 (fig. 2b).

Binding and localization of human neutrophil peptide-1 inside the macrophages

Binding studies performed with radiolabelled peptide revealed that ^{125}I-HNP-1 binds to macrophage cell lines showing equilibrium kinetics with respect to time. As

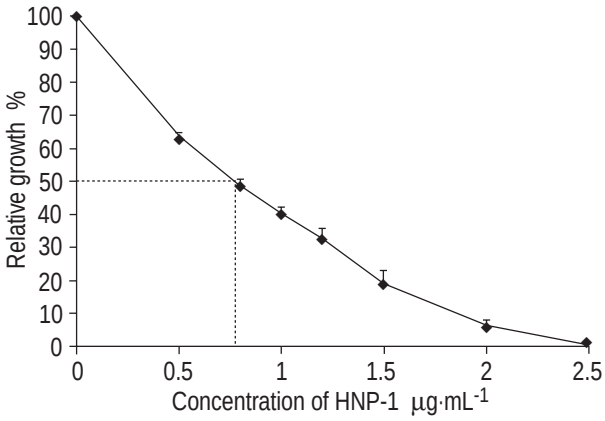

Fig. 1. – Effect of human neutrophil peptide (HNP)-1 on the growth of *Mycobacterium tuberculosis* $H_{37}Rv$. Cells were grown in the presence of HNP-1 at different concentrations until mid exponential phase was reached (7–8 days). Values are mean±SD of three independent experiments. The dashed lines from 50% relative growth represent the median inhibitory concentration (0.8 μg·mL^{-1}) of HNP-1.

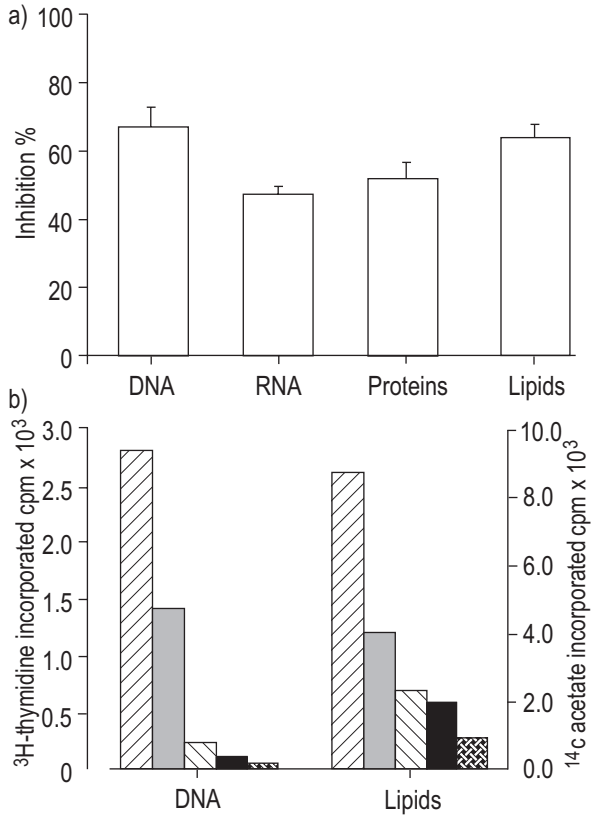

Fig. 2. – Macromolecular biosynthesis of *Mycobacterium tuberculosis* $H_{37}Rv$ grown in the presence of human neutrophil peptide (HNP)-1 as well as of phagocytosed mycobacteria. *M. tuberculosis* $H_{37}Rv$ cells were grown in the presence of HNP-1 at median inhibitory concentration until mid-exponential phase was reached. Cells were harvested and pulsed with ^{3}H-thymidine, ^{14}C-uracil, ^{14}C-aspartic acid and ^{14}C-acetate for 90 min. The results were calculated as counts per minute (cpm) incorporated per milligram dry weight of cells and expressed as per cent inhibition of the incorporation of precursors into the macromolecules as compared to control (a). Macromolecular biosynthesis of phagocytosed mycobacteria was studied by the incorporation of precursors in the mycobacteria released from the HNP-1 treated macrophages. Results were expressed as cpm of radioactive precursors incorporated in the macromolecules (b). In a values are mean±SD of three independent experiments, and in b, values are means of two independent experiments. DNA: deoxyribonucleic acid; RNA; ribonucleic acid. ▨: control; ▢: 5 μg·mL^{-1}; ▧: 10 μg·mL^{-1}; ■: 20 μg·mL^{-1}; ▩: 40 μg·mL^{-1}.

shown in figure 3, the binding curve approaches equilibrium after 45 min of incubation, with ~20% of the added radioactivity bound to macrophages.

To study the localization of HNP-1 inside the macrophages, binding was performed for 45 min corresponding to equilibrium stage. It was observed that two-thirds (66.3%) of the bound radioactivity was present inside the macrophages.

Intracellular killing of Mycobacterium tuberculosis *$H_{37}Rv$ by human neutrophil peptide-1*

The ability of HNP-1 to kill *M. tuberculosis* $H_{37}Rv$ inside the macrophages was investigated by treating the infected macrophages with increasing concentrations of HNP-1. It was observed that HNP-1 resulted in ~47% killing of *M. tuberculosis* $H_{37}Rv$ at 5 μg·mL^{-1} concentration after 3 days of treatment. However, >98% killing was observed at 40 μg·mL^{-1} concentration, resulting in decrease in ~2 log cfu (fig. 4).

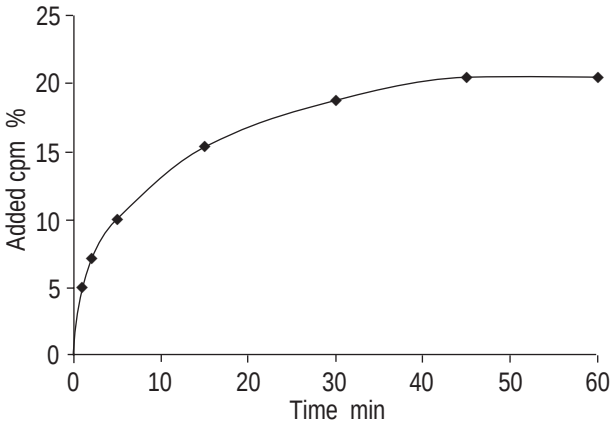

Fig. 3. – Bindings kinetics of human neutrophil peptide (HNP)-1 to macrophage cell lines. 1×10^5 cells per millilitre of murine macrophage cell line J744A.1 were incubated with ^{125}I HNP-1 in Roswell Park Memorial Institute-1640 medium for 60 min at 37°C. Values are means of two independent experiments. cpm: counts per minute.

Cytotoxicity of human neutrophil peptide-1 to macrophages

Cytotoxicity of HNP-1 to macrophage cell lines was determined by a dye exclusion assay in RPMI-1640 medium supplemented with 2% FBS. It was seen that with an increase in HNP-1 concentration from 5–40 μg·mL^{-1}, there was a slight increase in the percentage cytotoxicity from 2.8% to 10.5% after 24 h of incubation. However, after 72 h of incubation, 23.9% cytotoxicity was observed at 40 μg·mL^{-1} of HNP-1 (table 1).

Discussion

Antimicrobial peptides are an important component of the innate defences. These peptides offer exciting treatment options in the face of the declining efficacy of conventional antibiotics owing to the rise of antibiotic-resistant organisms. This is the first report of the activity of HNP-1 against virulent strains of mycobacteria *in vitro* as well as mycobacteria replicating within the macrophages.

In the present study, HNP-1 was observed to possess potent antimycobacterial activity *in vitro* as well as against intracellular mycobacteria. It kills the mycobacteria by inhibiting the biosynthesis of macromolecules. HNP-1 resulted in a low cytotoxicity towards macrophages at concentrations bactericidal to mycobacteria.

The study of *in vitro* antimicrobial activity of HNP-1 against *M. tuberculosis* $H_{37}Rv$ demonstrated its MIC much lower as compared to that reported by MIYAKAWA *et al.* [6] against *M. tuberculosis* $H_{37}Ra$. This discrepancy could be due to different experimental design as these authors calculated the MIC by treating the already grown cells with HNP-1 for 24–48 h. However, similar results were obtained in a previous study for *M. tuberculosis* $H_{37}Ra$ (unpublished data) when the MIC was calculated according to the method of MIYAKAWA *et al.* [6].

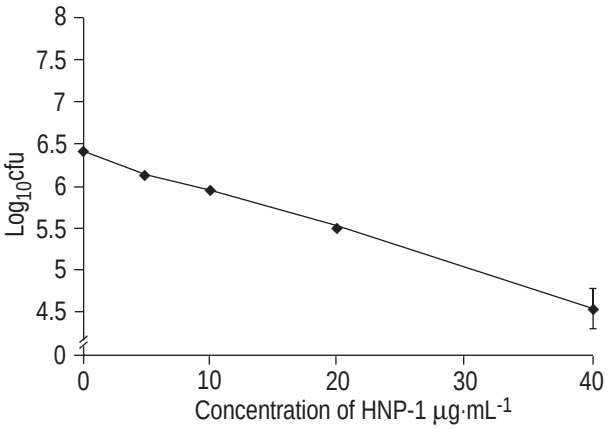

Fig. 4. – Bactericidal activity of human neutrophil peptide (HNP)-1 against intracellular *Mycobacterium tuberculosis* $H_{37}Rv$ replicating inside the macrophages. Macrophage cell lines were infected with *M. tuberculosis* $H_{37}Rv$ and were treated with HNP-1 at different concentrations for 3 days. Colony forming unit (cfu) enumeration was performed and results were expressed as $\log_{10}$cfu. Values are mean±SD of three independent experiments.

Biosynthesis of major macromolecules was inhibited by HNP-1 similar to the previous observations in avirulent mycobacteria [8] as well as the earlier findings of LEHRER *et al.* [2] with *Escherichia coli*. The maximum inhibition in DNA biosynthesis suggests that HNP-1 kills the mycobacteria by interacting with DNA metabolism, as inhibition of this macromolecule leads to the generalized inhibition in the biosynthesis of other major macromolecules. This is in accordance with earlier findings that showed DNA to be the secondary target of HNP-1 action (unpublished data).

The preformed peptides and proteins are released into the extracellular environment by neutrophils and are taken up by macrophages to kill the intracellular pathogens. Ingestion of granulocyte material is known to enhance the activity of peritoneal macrophages against *Mycobacterium avium* and *Mycobacterium microti* [17]. Likewise, in the present study, HNP-1 was found to bind to the macrophages and to be taken up inside the cytosol, which is a prerequisite for the intracellular killing of mycobacteria.

Although defensins are effective microbicidal agents *in vitro*, their antimicrobial activity within intact macrophages has not been established. However, inhibition in the replication of intracellular *Histoplasma capsulatum* was observed in the murine macrophage cell lines RAW 264.7, which were transduced with complementary DNA encoding for HNP-1 [9]. In the present study, it was demonstrated that HNP-1 kills the mycobacteria inside the macrophages probably by direct interaction of HNP-1 with *M. tuberculosis*, resulting in the inhibition of macromolecular biosynthesis.

Antimicrobial peptides of the cathelicidin family are cytotoxic towards human erythrocytes and polymorphonuclear cells, although at much higher concentrations than those required for microbicidal activity (30 µM *versus* 1–4 µM) [18]. Similarly, HNP-1 has also been shown to exhibit significant cytotoxicity to a number of murine cell lines [16]. However, in the current study, a relatively low level of cytotoxicity was observed even after 72 h of treatment of murine cell line J744 A.1 at microbicidal concentrations of HNP-1. The observed low cytotoxicity

Table 1. – Cytotoxicity of human neutrophil peptide (HNP)-1 to murine macrophage cell line

Concentration of HNP-1 µg·mL^{-1}	Cytotoxicity after %		
	24 h	48 h	72 h
5	2.8	6.5	9.8
10	7.7	10.7	13.4
20	8.4	14.1	17.6
40	10.5	17.7	23.9

Values are the mean of two independent experiments performed in triplicate.

of HNP-1 could be due to the presence of FBS in the culture media suggesting that HNP-1 mediated cytolysis might not occur *in vivo* in the presence of serum proteins [19].

From the results of this study, it is concluded that human neutrophil peptide-1 possesses potent antibacterial activity against virulent mycobacteria *in vitro*. Human neutrophil peptide-1 kills *Mycobacterium tuberculosis* $H_{37}Rv$ inside the macrophages, a prerequisite of antitubercular drugs. The presence of human neutrophil peptide-1 inside the macrophages, its bactericidal activity against intracellular mycobacteria and low cytotoxicity to the macrophages makes it a potential candidate in the development of an alternative therapeutic agent against tuberculosis. Further studies are in progress to study the *in vivo* effect of these peptides against experimental tuberculosis in an animal model.

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