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Antimicrobial Peptides and Their Anti-*Leishmanial* Efficacies on *Leishmania tropica* Promastigotes *In vitro*

Antimikrobiyal Peptitler ve Leishmania tropica Promastigotları ile In vitro Çalışma

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ABSTRACT

Objective: Antimicrobial resistance is a real threat to humanity. Pentavalent antimonials are reported non-effective in leishmaniasis treatment today, in countries like India. New treatment options have been assessed worldwide lately. Antimicrobial peptides (AMP) are the leading antibiotic candidates due to their large spectrum, fast efficacy, and low resistance risks. Cathelicidins are the AMP with well-documented antimicrobial activities against bacteria, fungi, and protozoa, over their positively charged membranes. Here, we aim to design cathelicidine-like helical peptides (CLHP), and compare their anti-*Leishmanial* efficacies *in vitro*, with meglumine antimoniate (MA) on *Leishmania tropica*.

Methods: A total of five study [TN-1-5] and two control (MA and non-drug) groups were formed. Cryopreserved *L. tropica* isolate was thawed and cultivated in Novy-MacNeal-Nicolle medium and then in RPMI. Five different CLHPs (TN1-5) were diluted in dimethyl sulphoxide. A total of 150 uL of CLHPs and MA were added into the first wells of the test plaques, followed by serial dilutions that revealed doses within 4 and 512 ug/mL. Then, 100 uL of cultures including $1x10^8$/mL of *L. tropica* promastigotes were added into each well. Viability of promastigotes was checked with XTT, while the parasite count was assessed at 24th and 48th hours.

Results: TN3 was effective at 32 ug/mL. All tested CLHPs exhibited varying degrees of anti-*Leishmanial* activities, except TN5, even at its highest dose.

Conclusion: TN3 showed a particular efficacy against *L. tropica in vitro*. Further studies including *in vivo* testing of the candidate's both efficacy and toxicity are essential.

Keywords: *Leishmania*, antimicrobial peptide, cathelicidin, treatment, Türkiye

ÖZ

Amaç: Antimikrobiyal direnç insanlık için gerçek bir tehdittir. Beş değerlikli antimon bileşiklerinin günümüzde Hindistan gibi ülkelerde leishmaniasis tedavisinde etkili olmadığı bildirilmektedir. Son zamanlarda dünya çapında yeni tedavi seçenekleri değerlendirilmektedir. Antimikrobiyal peptitler (AMP) geniş spektrumları, hızlı etkinlikleri ve düşük direnç riskleri nedeniyle önde gelen antibiyotik adaylarıdır. Katelisidinler, pozitif yüklü membranları üzerinden bakteri, mantar ve protozoonlara karşı iyi belgelenmiş antimikrobiyal aktivitelere sahip AMP'lerdir. Burada, "katelisidin benzeri helikal peptidler" (CLHP) tasarlamayı ve bunların *Leishmania tropica* üzerindeki anti-leishmanial etkinliklerini *in vitro* olarak meglumin antimoniate (MA) ile karşılaştırmayı amaçladık.

Yöntemler: Toplam beş çalışma [TN-1-5] ve iki kontrol antimoniat (MA) ve ilaçsız) grubu oluşturuldu. Kriyoprezerve edilmiş *L. tropica* izolatı çözüldü ve Novy-MacNeal-Nicolle ve ardından RPMI besiyerlerinde kültüre edildi. Beş farklı CLHP (TN1-5) dimetil

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Türkiye PARAZİTOLOJİ Dergisi

sülfoksit içinde seyreltildi. Test plaklarının ilk kuyucuklarına toplam 150 uL CLHP ve MA eklendi ve ardından 4 ve 512 ug/mL arasında dozlar ortaya çıkaran seri seyreltmeler yapıldı. Daha sonra, her bir kuyucuğa 1x10^8/mL *L. tropica* promastigotları içeren 100 uL kültür eklendi. Promastigotların canlılığı XTT ile kontrol edilirken, parazit sayımı 24. ve 48. saatlerde yapıldı.

Bulgular: TN3'ün 32 ug/mL'de etkili olduğu gözlenmiştir. TN5 dışında test edilen tüm CLHP'ler değişen derecelerde anti-leishmanial aktivite sergilerken, TN5'in en yüksek dozunda bile etkisiz kaldığı gözlenmiştir.

Sonuç: TN3'ün *L. tropica*'ya karşı *in vitro* etkinlik gösterdiği belirlenmiştir. Adayın hem etkinliğinin hem de toksisitesinin *in vivo* testleri içeren daha ileri çalışmalarla araştırılması gereklidir.

Anahtar Kelimeler: *Leishmania*, antimikrobiyal peptid, katelisidin, tedavi, Türkiye

INTRODUCTION

Antimicrobial resistance is an urgent global public health problem since it may affect people from all ages or races, as well as the healthcare, veterinary, and agriculture industries. It is estimated that more than 2.8 million antimicrobial-resistant infections occur annually, and 35,000 people die in the United States of America, due to antibiotic resistance in 2019 (1). Main reasons of emerging antimicrobial resistance are inappropriate prescription of antibiotics, their overuse worldwide, both for humans and in agriculture, and availability of few new antibiotics in the market (2). Antimicrobial resistance is not limited to bacterial infections and is documented as an emerging issue for the treatment of parasitic infections as well (3).

Antimicrobial resistance is also an emerging problem for leishmaniasis, as well. Leishmaniasis is a neglected, vector-borne parasitic infection common in tropical and subtropical regions of the world. It is caused by a flagellated protozoan, *Leishmania* sp., and transmitted to humans via the bite of a sandfly (*Phlebotomus* sp. in the Old World and *Lutzomyia* sp. in the New World). *Leishmania* spp. have more than 20 species in nature that are associated with different clinical manifestations in humans. The predominant clinical manifestation is the cutaneous *leishmaniasis* (CL), which is reported in over a million people worldwide annually. Visceral *leishmaniasis* (VL) is seen relatively less common but can be deadly in untreated patients. Leading causative agents are *L. tropica* and *L. major* for CL, and *L. donovani* and *L. infantum* for VL in the Old World. CL has been endemic especially in southeastern Anatolia in Türkiye, which has been reported from western regions as well, lately (3-6).

Treatment of *Leishmaniasis* relies primarily on pentavalent antimonials, which have been commonly used for the treatment of both CL and VL cases worldwide for the last 50 years (4-6). However, due to emerging resistance against them, pentavalent antimonials mostly remain ineffective in leishmaniasis treatment in many countries, such as India today (7). As there is no effective vaccine against leishmaniasis as well as there is an emerging resistance to treatment, there is an urgent need for new drug formulations and treatment options for leishmaniasis. Among these options, both natural and synthetic compounds have been assessed for their anti-leishmanial efficacies *in vitro* and *in vivo* (7,8).

Antimicrobial peptides (AMPs) are positively charged, small peptides with 5-100 amino acid residues, produced in several living organisms as part of the innate immunity, as well as antimicrobial activity. AMPs show large-spectrum anti-microbial efficacy, through either direct elimination of the pathogens (bacteria, viruses, fungi, and parasites) or by modulating the immune response (9-11). Many groups are present within the AMPs; among them, cathelicidins and defensins are the main groups (12). Cathelicidins have well-known antimicrobial activities against not only to bacteria, but also to fungi and protozoa, over their positively charged membranes (10-12).

Many natural AMPs are known to act by disrupting the integrity of cell membranes in protozoa (13). However, some of the AMPs can also interfere with important cellular processes of parasites. AMPs are reported to particularly disrupt Ca2+ distribution on *Leishmania* and consequently disrupt their metabolism. *Plasmodium* is the parasite with which many studies have been carried out with AMPs (14). Some fungal AMPs have an inhibitory effect on histone deacetylase (HDA) in *Plasmodium* species, leading to histone hypermethylation and subsequent alteration of gene expression in the parasite (15). When the effects of AMPs on *Trypanosoma* are evaluated, it is known that they cause the distribution and change of membrane components, stiffness of the cell membrane, and thus cause cell loss (16).

In the light of our previous studies on the antimicrobial activities of antimicrobial peptides, we designed five peptides (named as TN 1 to TN5) inspired by the natural antimicrobial peptide, cathelicidin LL-37; in other words, we designed "cathelicidine-like helical peptides" (CLHP) by imitating the phylogenetically protected sequences of cathelicidins and had them synthesized for our trials. Indeed, using *in vitro* cytotoxicity tests, we observed that the minimum inhibitory concentration values of TN peptides were below HC50 and LC50 on HeLa cells, which indicated their promising roles as antimicrobial drug candidates (17).

In the present study, our aim was to compare the anti-leishmanial efficacies of TN1-5 *in vitro*, with meglumine antimoniate (MA), the current treatment agent, on *L. tropica* promatigotes isolated from a CL patient in Türkiye.

METHODS

Design and Supply of Antimicrobial Peptides

It is well known in the literature that AMPs are generally hydrophobic and positively (+) charged (18). In our study, peptides of 10 to 20 amino acids in length forming α-helix similar to the structure of LL-37 were designed with hydrophobic and positively charged amino acids. The amide group at the C-terminal end causes the peptide to approach the membrane perpendicularly and to be taken up into the cell faster. It is very important that peptides end with an amide group, as this affects the increase in membrane permeability. 3D structures of the designed novel peptides were obtained using the PEP-FOLD3 server (19). The designed peptides were synthesized and purchased from Metabion Company in Germany, according to the guidelines of the Clinical Laboratory Standards Institute (Figure 1).

Leishmania Strains

The *L. tropica* isolate used in this study had been isolated from an 18-year-old female CL patient diagnosed in Manisa Celal Bayar

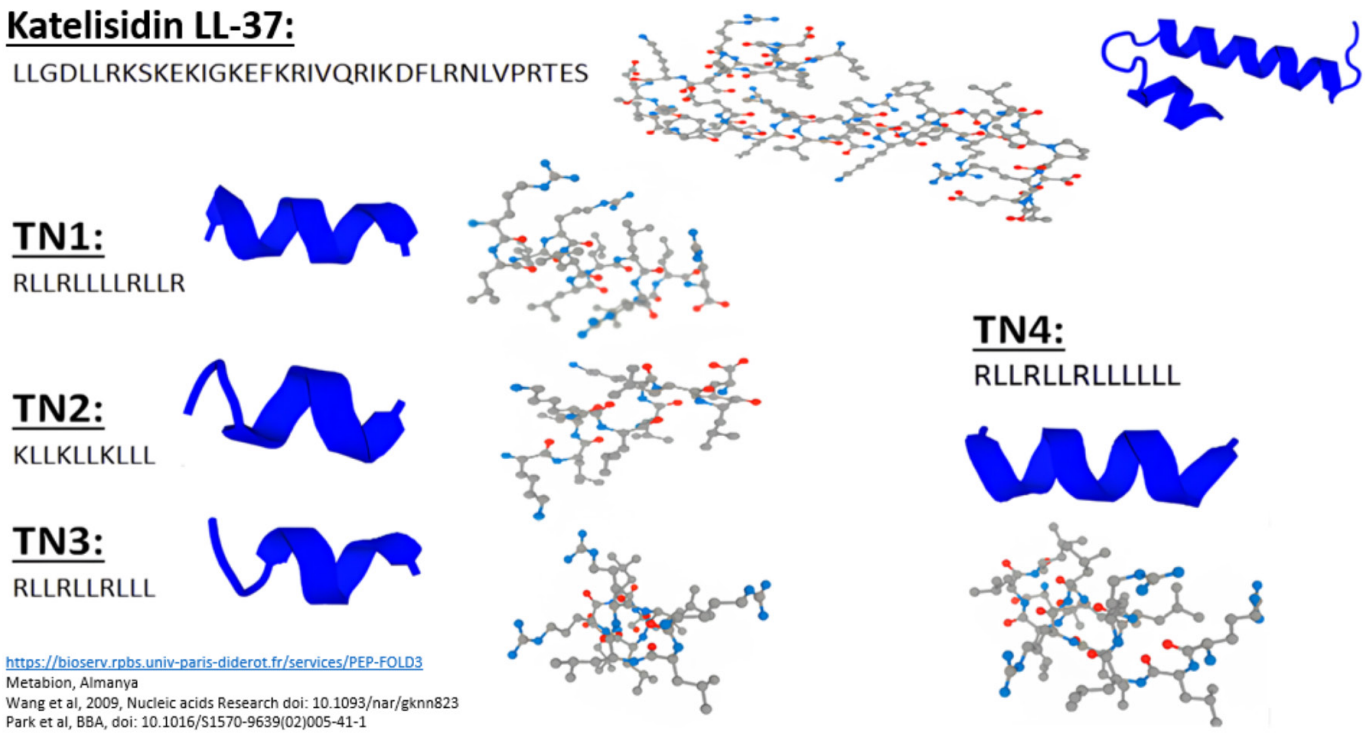

Figure 1. *In silico* figures of cathelicidins and five different cathelicidine-like helical peptides used in the study

University Hospital (MHOM/TR/2011/CBU012). This isolate has been used anonymous (without disclosure of the patient's identity) in similar studies. Initially, the cryopreserved isolate was thawn and inoculated first in Novy-MacNeal-Nicolle medium and then in RPMI medium, including 10% of fetal bovine serum (FBS), penicillin-streptomycin 1% and 0.2% gentamycin. One mililitre of culture medium containing propagated *Leishmania* promastigotes were collected from the culture tubes using fine pipettes and added on a haemacytometer (Neubauer's Thoma slide). Here, the promastigotes seen under the microscope on the four small squares in each corner as well as the ones in the big, central square were counted, multiplied by 10.000 and divided by the number of squares to reach the promastigote number in a mililitre. The final promastigote count was adjusted to 10^8 per mililitre and used for the assessments. A total of 150 uL of CLHPs and MA were added into the first wells of the test plaques, followed by serial dilutions that revealed doses within 4 and 512 ug/mL. Then, 100 uL of cultures including $1x10^8$/mL of *L. tropica* promastigotes were added into each well. Viability of promastigotes was checked with XTT, while the promastigotes were counted under the microscope at 24^{th} and 48^{th} hours.

Assessment of Anti-*Leishmanial* Activity

Activity of AMPs against *L. tropica* promastigotes was assessed by both microscopic counting and the colorimetric cell viability XTT (2,3-bis [2-methyloxy-4- nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (Sigma Chemical Co; St. Louis, MO) assay. Promastigotes ($2.5x10^6$/100 mL per well) in the logarithmic growth phase were inoculated into a flat-bottomed 96-well plastic tissue cultured microplates, in triplicate, followed by serial dilutions of each antimicrobial peptide (100 mL). After three days of incubation at 28 °C, 25 mL of XTT (0.2 mg/mL) were added to each well, followed by an additional 3 h of incubation at 37 °C. The optimal density (OD) at 450 nm was measured using an ELISA plate reader. The anti-*leishmanial* activity was further determined by microscopic counting of the live promastigotes for each well and the growth inhibition rate of each concentration was calculated according to the control. The 50% lethal dose (IC50) was evaluated graphically by plotting concentration versus percentage growth inhibition (Figure 2). The anti-leishmanial activity was further determined by microscopic counting of the live promastigotes for each well and the growth inhibition rate of each concentration was calculated according to the control.

Statistical Analysis

Statistical analysis was performed with A Two-Way ANOVA in GraphPad Prism software to calculate the statistical probability. In statistical analyses, difference as $p<0.05$ was considered significant.

RESULTS

The results of the assessments demonstrated that one of the CLHPs, TN3, was effective against *L. tropica* at a lower dose (32 ug/mL) until the end of the 48^{th} hour. All tested CLHPs exhibited varying degrees of anti-leishmanial activities, except TN5 which expressed no efficacy against *L. tropica*, even at its highest dose, neither at 24^{th} nor at 48^{th} hours (Tables 1, 2).

The viability testing of the promastigotes revealed that TN3 managed to kill all promastigotes at the lowest dose (32 ug/mL) at 48^{th} hours. TN4 showed similar efficacy at 128 ug/mL, while TN1 and TN2 at 256 ug/mL. Again, TN5 was found to be ineffective in

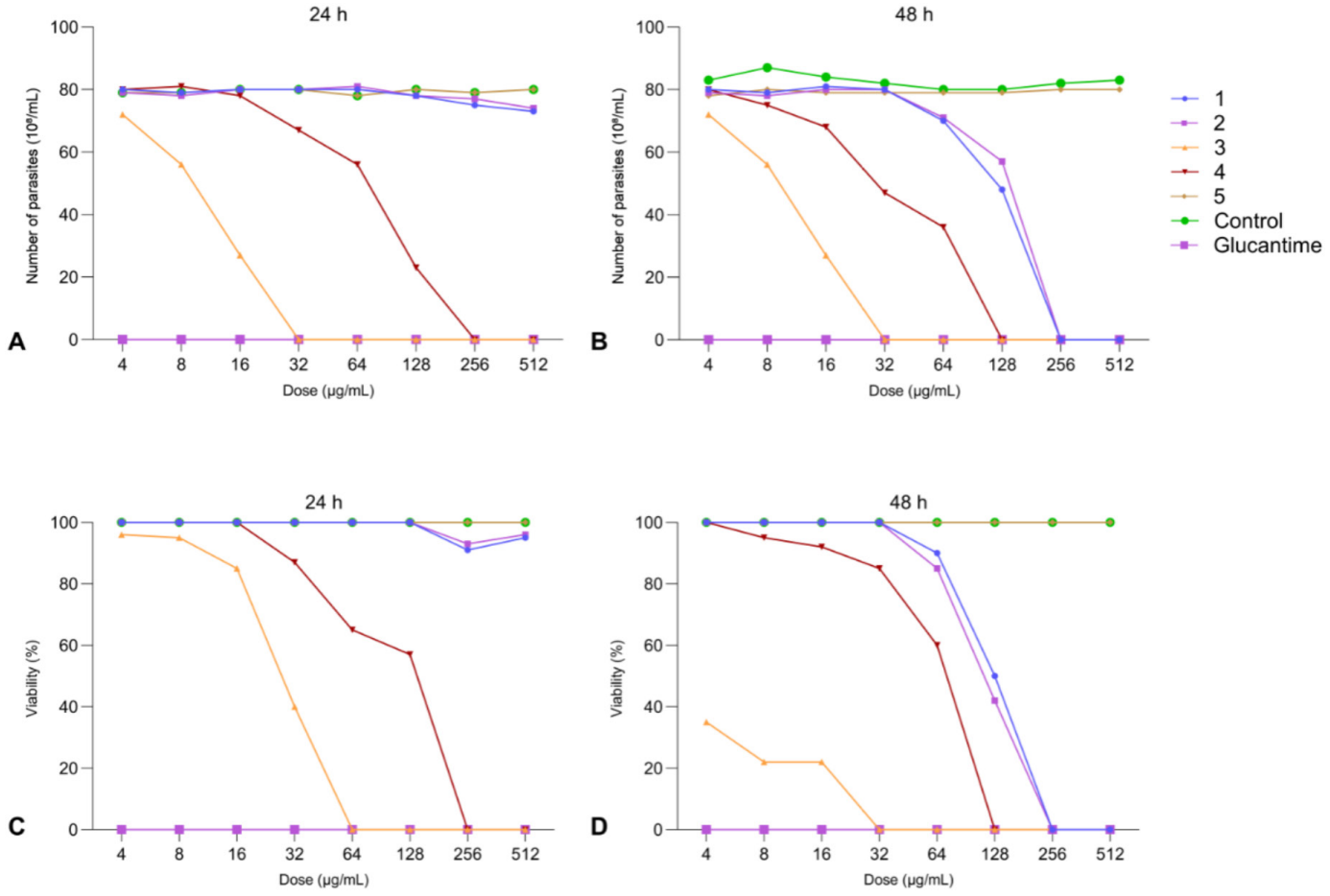

Figure 2. Growth inhibition versus peptide concentration. A and B; Effect of peptide doses on cell number. C and D; Effect of peptide doses on cell viability (%)

Table 1. Live promastigotes count at 24th hour (10^8/mL)

Agents		DOSE (µg/mL)							
		512	256	128	64	32	16	8	4
1.	TN1 RLLRLLLLRLLR	73	75	78	80	80	80	79	80
2.	TN2 KLLKLLKLLL	74	77	78	81	80	80	78	79
3.	TN3 RLLRLLRLLL	0.00	0.00	0.00	0.00	0.00	27	56	72
4	TN4 RLLRLLRLLLLLL	0.00	0.00	23	56	67	78	81	80
5	TN5 RLLLRLLLRLLLR	80.00	79.00	80.00	78.00	80.00	80.00	79	79
6	Control (no drugs)	80.00	79.00	80.00	78.00	80.00	80.00	79	79
7.	Control (Glucantime®)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

killing the parasites even at its highest dose, neither at 24th nor at 48th hours (Tables 3, 4).

DISCUSSION

World Health Organization describes emerging resistance to antibiotics as a global threat for humanity, today (20). It is estimated that antimicrobial resistance (AMR) is directly associated with 1.27 million deaths in the world in 2019, while it will bring an extra cost of health expenditures around 1 trillion USD by the year 2050 (1,20). Main reason of AMR is the abuse and extreme usage of antibiotics, not only in humans but also for animals raised for humans. This abuse of antibiotics may cause not only resistance emergence but also toxicity in humans (2,21,22). AMR is associated with bacteria as well as protozoal infections. For example, pentavalent antimonial compounds which have been used primarily in the treatment of *Leishmaniasis* in the world, are almost non-effective due to emerging resistance in India today, which may have already exceeded 60% (3,7,8). This is also an emerging problem in Türkiye; in addition to unpublished data on

Table 2. Live promastigotes count at 48th hour (10^8/mL)

Agents		DOSE (µg/mL)							
		512	256	128	64	32	16	8	4
1.	**TN1 RLLRLLLLRLLR**	**0.00**	**0.00**	**48**	**70**	**80**	**81**	**79**	**80**
2.	**TN2 KLLKLLKLLL**	**0.00**	**0.00**	**57**	**71**	**80**	**80**	**78**	**79**
3.	**TN3 RLLRLLRLLL**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**27**	**56**	**72**
4	**TN4 RLLRLLRLLLLLL**	**0.00**	**0.00**	**0.00**	**36**	**47**	**68**	**75**	**80**
5	**TN5 RLLLRLLLRLLLR**	**80.00**	**80.00**	**79**	**79**	**79**	**79.00**	**80.00**	**78.00**
6	**Control (no drugs)**	**83.00**	**82.00**	**80.00**	**80.00**	**82.00**	**84.00**	**87**	**83**
7.	**Control (Glucantime®)**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

Table 3. Colorimetric rates of viability of the promastigotes at 24th hour, using XTT method (%) (10^8/mL)

Agents		DOSE (µg/mL)							
		512	256	128	64	32	16	8	4
1.	**TN1 RLLRLLLLRLLR**	95	91	100	100	100	100	100	100
2.	**TN2 KLLKLLKLLL**	96	93	100	100	100	100	100	100
3.	**TN3 RLLRLLRLLL**	0	0	0	0	40	85	95	96
4	**TN4 RLLRLLRLLLLLL**	0	0	57	65	87	100	100	100
5	**TN5 RLLLRLLLRLLLR**	100	100	100	100	100	100	100	100
6	**Control (no drugs)**	100	100	100	100	100	100	100	100
7.	**Control (Glucantime®)**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

Table 4. Colorimetric rates of viability of the promastigotes at 48th hour, using XTT method (%) (10^8/mL)

Agents		DOSE (µg/mL)							
		512	256	128	64	32	16	8	4
1.	**TN1 RLLRLLLLRLLR**	0	0	50	90	100	100	100	100
2.	**TN2 KLLKLLKLLL**	0	0	42	85	100	100	100	100
3.	**TN3 RLLRLLRLLL**	0	0	0	0	0	22	22	35
4	**TN4 RLLRLLRLLLLLL**	0	0	0	60	85	92	95	100
5	**TN5 RLLLRLLLRLLLR**	100	100	100	100	100	100	100	100
6	**Control (no drugs)**	100	100	100	100	100	100	100	100
7.	**Control (Glucantime®)**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**

the increase of longer treatment requirements of *Leishmaniasis* patients, there is also an increase in publications on antimonial resistant cases (23-25).

AMPs may become either alternatives or complementary options to conventional antiprotozoal drugs due to their broad-spectrum activity, lower toxicities, and different action mechanisms (26,27). They can exhibit various activities that may directly inhibit the microbial growth or modulate the immune response through the activation of immune cells. They can be totally synthesized or modified chemically, after which they gain higher resistance to proteolytic enzymes (9). Thus, AMPs were initially used to fight the antibiotic resistance of microorganisms, since these compounds were not affected by the mechanisms of bacterial resistance to conventional anti-microbials. Despite their disadvantages such as current high production costs, lower activity in certain conditions (interaction with proteases, etc.) (27,28).

There are almost 3000 natural AMPs identified predominantly from eukaryotes (18,27,28). Among the 990 active registered AMPs today, only 83 of them were assessed as anti-parasitic agents. The leading parasites assessed in AMP studies are *Plasmodium* sp., *Leishmania* sp., *Toxoplasma gondii, Trypanosoma cruzi* and *Cryptosporidium* spp. (26,27).

Previously, various AMPs were assessed for their anti-*Leishmanial* activities, and shown to be effective against clinically-relevant *Leishmania* species, such as *L. amazonensis* (29,30), *L. donovani* (19,31), *L. major* (31), *L. mexicana* and *L. tropica* (32) and *L. infantum* (33). Cathelicidins are important AMPs and human cathelicidin, LL-37, has well-known antimicrobial effects. They interact with the negatively-charged cell membranes of bacteria, fungi and protozoa and kill them, either directly or through pore formation (34). The role of cathelicidins has been investigated in many studies in *Leishmaniasis* as well, mainly using *in vitro* assays, especially in the promastigote stage (34-36). They were found to be involved in the restriction of *Leishmaniasis* in macrophages of CL patients (19), and augmentation of Amphotericin B's macrophage-activating effects (37). In addition, human cathelicidin was shown

to induce an apoptosis-like phenotype in a dose dependent manner in both *L. major* and *L. aethiopica* promastigotes as well as in *L. aethiopica* amastigotes, while they are also involved in the innate immune responses against *Leishmaniasis* in a human primary cell model (34,37).

Here, in the present study, anti-*Leishmanial* efficacy of cathelicidin-like alpha-helical peptides we designed was investigated on *L. tropica* promastigotes *in vitro*. The results of our assessments indicated that one of the assessed AMPs, TN3, showed efficacy against *L. tropica* at a lower dose (32 µg/mL) compared to MA, *in vitro*. Other peptides, TN1, TN2 and TN4 showed efficacy against *L. tropica* as well, but in higher doses, while TN5 exhibited no efficacy in our trial against *L. tropica* even in its highest dose.

In the literature, it is seen that most of the studies conducted with parasites are with natural AMPs such as mellitin, temporin, cathelicidin (10). When the antileishmanial activities in these studies were evaluated, it was seen that melittin inhibited *L. major* at 74.01 mg/mL (34). It has been stated that the antileishmanial effect of cecropin, another antimicrobial peptide, on *L. aethiopica* was greater than 250 mg/mL (35). It was also stated that the antileishmanial effect of temporin antimicrobial peptide was 11.6 uM on *L. major* (36). It has been stated that cathelicidin, another antimicrobial peptide, can kill *L. major* and *L. donovani* by 50% even at high concentrations (37). Here, we observed that TN3 exhibited particular antileishmanial efficacy at a relatively lower dose (32 ug/mL), which is similar to natural derivatives and even more effective than cathelicidin and cecropin.

CONCLUSION

The results of this *in vitro* study indicate TN3 as a promising anti-*Leishmanial* agent. Further studies involving its *in vivo* efficacy and toxicity are warranted to unveil its potential as a treatment option for leishmaniasis in future. Regarding their efficacy in resistant microorganisms, AMPs may soon become the leading weapons of our arsenal against life-threatening microbial agents.

***Information:** This isolate has been used anonymous (without disclosure of the patient's identity) in similar studies.

*Ethics

Ethics Committee Approval: The *Lesihmania* strains used in this article are study materials that have been stored in liquid nitrogen for research purposes for many years, and are research samples that have been stored and used with the identities of the patients from whom they were isolated deleted. In this context, there is no need to obtain ethics committee approval.

Informed Consent: Not necessary.

*Authorship Contributions

Concept: N.Ü., İ.Ç., T.P., Ö.K., A.Ö., T.K., Design: A.Ö., T.K., Data Collection or Processing: N.Ü., İ.Ç., T.K., Analysis or Interpretation: N.Ü., İ.Ç., T.P., Ö.K., A.Ö., T.K., Literature Search: N.Ü., Ö.K., Writing: N.Ü., Ö.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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