The Comparative Survey on the Cytotoxic Effects of Amphipathic Peptide mCM11 (Modified CM11) and Metronidazole on ST1 and ST3 of Blastocystis sp.

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Abstract

Background: Blastocystis sp. is an opportunistic worldwide protozoan that is life-threatening in immunocompromised individuals; several efforts were performed against Blastocystis sp. in vivo and in vitro conditions to find an alternative for metronidazole (MTZ).

Objectives: The aim of the present study was to survey the cytotoxic effects of peptide mCM11 (modified CM11) and MTZ on ST1 and ST3 of Blastocystis sp. in vitro.

Materials and Methods: In the present study, after the culture of the parasite (in Dulbecco’s modified Eagle medium [DMEM] and Caco-2 cell lines), the anti-parasitic effect of different concentrations of either MTZ (1.25-80 μg/mL) or mCM11 (1-128 μg/mL) for 24, 48, and 72 hours was evaluated via a 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay and flow cytometry.

Results: A dose-time-dependent decrease in cell viability was detected after cell line and parasite exposure. The cytotoxic effect of the peptide on cell lines increased during 24, 48, and 72 hours, and the greatest anti-parasitic effect was observed at the highest concentration (128) after 72 hours. In addition, the ST3 showed more sensitivity to the mCM11.

Conclusion: The findings of the current research about the mCM11 peptide were promising compared to MTZ, and by further optimizing the peptide, better effects can be imagined for it, so that it can be a potential alternative to MTZ. However, more studies with a case-control design seem to be necessary in vivo conditions.

Keywords: Blastocystis sp., mCM11, Cytotoxicity, Subtypes, Cell culture

Background

Blastocystis sp. is a single-celled polymorphic parasite that is often reported as the most common eukaryotic organism in human fecal samples. However, according to numerous molecular studies, blastocysts have been subdivided into ssurDNA analyses by ST (ST1-ST28). Several subtypes have been reported in humans (ST1-9 and ST12), of which ST1 and ST3 are the most abundant.1 The exact pathogenicity of Blastocystis is still in the aura of ambiguity; however, research has discussed the possible association of this multi-faceted parasite complication with irritable bowel syndrome, gastrointestinal (GI) symptoms, and the like. Nowadays, with the increase of sensitive hosts, such as people with HIV and other immunodeficiencies, indiscriminate use of corticosteroids, and the like, the importance of little-known diseases, particularly opportunists such as Blastocystis sp., can be alarming.2 The clinical manifestations of Blastocystis sp. can vary from asymptomatic infections to annoying GI symptoms.2 The choice drug for treatment is metronidazole (MTZ), and many attempts have been made to find its alternative because of its serious and extensive side effects (e.g., neurotoxicity, neuropathy, and the like) due to long-term use; natural substances have been among the most popular objects studied in this field. In this context, the antimicrobial peptides (AMPs) with plant and/or animal origin, as well as microorganism origin, can be remarkable candidates in anti-parasitic trials. AMPs have two chief molecular approaches; the first is membrane activity, or encountering and interacting with the cell membrane, which causes perforation and, as a result, cell death. The latter is the intracellular mechanism by which the protein and/or DNA synthesis of the target cell is disrupted. Today, AMPs are potential sources of new antibiotics that include antifungal, antiviral, antibacterial, and antiparasitic properties.

CM11 is shorter than the original peptides with 11 amino acids (WKLFKILKVL-NH2) and is composed of two peptides, namely, cecropin and melittin. To reduce the toxicity of this peptide on human cells, two amino acids in the C and N terminals of this peptide sequence were changed from L to D form. This study aimed to investigate the effects of mCM11 toxicity on Blastocystis sp. ST1 and ST3 and Caco2 cells and the co-culture of Caco-2 cell and Blastocystis sp. ST1 and ST3.
**Materials and Methods**

**Peptide Design, Molecular Modeling, and In Silico Analysis**

The peptide was designed using the CM11 sequence by changing the L form to the D form of the two amino acids in the C and N terminals of the peptide; which included tryptophan (dW1) and lysine (dK1) and valine (dV1) and leucine (dL1) amino acids.

mCM11: (d-W)(d-K)WFKKILR(d-V)(d-L)-NH2.

mCM11 was synthesized and purified by high-performance liquid chromatography using a gradient of 25-75% acetonitrile in water and 0.1% trifluoroacetic acid for 30 minutes, yielding >95% purity of the peptide. In addition, ion-trap mass spectrometry was used to confirm the identity of mCM11. The powder was stored at -20 °C and dissolved in distilled water at a concentration of 4 mg/mL.

The in silico prediction hemolytic of the potency of the mCM11 was performed using the ProtParam tool (ExPASy server; http://www.expasy.org/tools/protparam.html; Gasteiger and Walker, 2005) and hemolytic peptide identification server (Hemo-PI; http://crdd.osdd.net/ragha.va/hemopi/).

**Culture and Passage of Blastocystis sp. Parasite**

*Blastocystis* sp. (ST1 and ST3) were obtained from the Parasitology Department of Tarbiat Modares University. In the cultivation process, Dulbecco’s modified Eagle medium (DMEM)/F12 medium supplemented by 150% bovine serum and ceftriaxone (100 μg/mL), and erythromycin (500 μg/mL) was utilized to remove bacteria. The initial culture was performed 72 hours after the microscopic examination of the samples. Further, the growth and morphology of the parasites were investigated with a light microscope, and cultivation was performed as well.

**Culture and Passage of the Human Colonic Epithelial Cell Line (Caco-2)**

The human colonic epithelial cell line (human colorectal adenocarcinoma; Caco-2) was also prepared from the cells stored in the nitrogen tank of the Parasitology Department of Tarbiat Modares University and cultured in DMEM/F12 (DMEM/Nutrient Mixture F-12) supplemented with 100 units/mL Pen-Strep, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum and incubated at 37 °C and 5% CO₂. The cells were trypsinized (0.25% Trypsin/EDTA) and sub-cultured in 80% of the confluency of the flask.

**3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide Assay**

The MTT solution was prepared in the amount of 2 × 10⁶ of Caco-2 cells into each well in the DMEM/F12 medium, along with 10% bovine serum, Pen-Strep, and L-glutamine, and incubated for 24 hours at 37 °C, and 5% CO₂. Thereafter, the cells were incubated with different concentrations of either MTZ (1.25-80 μg/mL) or mCM11 (1-128μg/ml) for 24, 48, and 72 hours. Then, the culture medium was emptied, MTT solution was added, and the wells were incubated for 4 hours at 37 °C. The medium was replaced with 200 μL of dimethyl sulfoxide and incubated for 15 minutes at room temperature while they were shaking. The absorbance value was measured at 570 nm and 690 nm as a test and reference, respectively. The cell viability (%) was calculated by the absorbance test/absorbance control × 100. The ratio of cytotoxicity (Half maximal inhibitory concentration IC₅₀) was calculated as the drug concentration, which inhibits the growth of 50% of samples.

**Flow Cytometry Assay**

The effects of incubation with the concentration of mCM11 on the apoptosis of Caco-2 cells after 24 hours were assessed by the flow cytometry method. Then, 5 × 10⁵ cells were cultured in a 6-well plate in a complete culture medium and incubated for 24 hours. After washing twice with phosphate-buffered saline, they were exposed to the considered concentration of mCM11 for 24 hours, and then, to show the rate of cell death, phosphatidylinerine exposure was measured by the FITC Annexin-V Apoptosis Detection Kit with propidium iodide (PI, BioLegend) according to the manufacturer’s instructions. Stained cells were immediately evaluated by a FAC Scan flow cytometer (FACSCalibur™, BD Biosciences). The resulting data were analyzed with FlowJo software (version 10.5.3).

**Cytotoxic Effects of Peptide mCM11 on Blastocystis sp. ST1 and ST3**

In this phase, 1 × 10⁵ parasites (ST1 and ST3) were added to each well of a 48-well culture plate and incubated with different concentrations of peptide (4, 8, 12, and 16 μg/mL) and MTZ (5, 10, 20, and 40 μg/mL) for 24, 48, and 72 hours. The trypsin blue staining method was used to check the viability of parasites. The sediment of the incubated samples was stained with trypsin blue 0.4% and observed under the light microscope using the Neubauer Chamber. The ratio of cytotoxicity (50% cytotoxic concentration: CC₅₀) on *Blastocystis* sp. ST1 and ST3 was calculated as well.

**Cytotoxic Effects of Peptide mCM11 on Parasite-Caco-2 Co-culture**

In this phase, 1×10⁵ cells were seeded into each well of 48-cell culture plates to be grown as a monolayer after 24. Then, 62.5 × 10⁵ parasites (ST1 and ST3) were added to each well and treated by peptide (4, 8, 12, and 16 μg/mL) and MTZ (5, 10, 20, and 40 μg/mL) in triplicate experiments. After 24, 48, and 72 hours of incubation, all culture media of each well was poured into a 1.5 mL
microtube, and the viability of parasites was determined by the trypan blue exclusion method. The selectivity index (SI) was calculated by IC$_{50}$ (cytotoxicity on the cell) to the ratio of CC$_{50}$ (cytotoxicity on the parasite) for both subtypes.

**Statistical Analysis**

The data were obtained in triplicate and presented as means ± standard deviations (SD). The significance of differences between groups was statistically assessed by the analysis of variance (ANOVA) with post-hoc Tukey's test using GraphPad Prism 6 software. The $P$ value of ≤ 0.05 was considered the significant level.

**Results**

**Drug Toxicity on Caco-2 Cells**

To determine the toxicity of the drug on human Caco-2 cells, the cells were cultured in the presence of MTZ or mCM11 peptide. Caco-2 cells showed a decrease in viability, and this decrease was detected by the MTT assay after 24, 48, and 72 hours of exposure to MTZ and mCM11 peptide. As shown in Figure 1, the average viability percentage of Caco-2 cells exposed to the highest concentration of MTZ (80 μg/mL) after 24, 48, and 72 hours were 78%, 74.2%, and 63.5%, respectively. At the highest peptide concentration (128 μg/mL), this percentage was 19.3%, 12.6%, and 10%, respectively, for different incubation times (24, 48, and 72 hours, Figure 1).

The IC$_{50}$ of MTZ and peptide was calculated after 24, 48, and 72 hours (Table 1).

**Apoptotic Effects of the Drug on Caco-2 Cells**

The flow cytometry results of Caco-2 cells exposed to mCM11 for 24 hours were performed using Annexin-V and PI staining. The flow cytometry results of Caco-2 cells exposed to a concentration of 8 μg/mL peptide are shown after 24 hours. Cells exposed to a concentration of 8 μg/mL of peptide after 24 hours suffered 10.8% apoptosis, 12.1% delayed apoptosis, and 11.6% necrosis (Table 2).

**Drug Toxicity on Blastocystis sp. ST1 and ST3**

The viability rate of each ST was calculated at different times. For Blastocystis sp. parasite ST1, the average percentage of viability when exposed to the highest peptide concentration (16 μg/mL) after 24, 48, and 72 hours, respectively, was 40.4%, 30.1%, and 3.4%. At the lowest peptide concentration (4 μg/mL), the percentage of viable parasites was 94.5%, 75.4%, and 62.6% after 24, 48, and 72 hours, respectively. In the case of ST 3, the viability percentages were estimated as 4.7%, 0%, and 0% after 24, 48, and 72 hours at the highest peptide concentration (16 μg/mL), respectively. At the lowest peptide concentration (4 μg/mL), the viability of parasites was 29%, 26.6%, and 10% after 24, 48, and 72 hours, respectively (Figure 2). Furthermore, the viability percentages of Blastocystis sp. ST1 and ST3 were 37% and 12.1% at the highest concentration of MTZ (40 μg/mL) after 72 hours. At the lowest concentration of MTZ (5 μg/mL), 69.5% of ST1 and 38% of ST3 were viable after 72 hours (Figure 2). Table 3 presents the CC$_{50}$ value and IC$_{50}$ and SI of mCM11 (after 24, 48, and 72 hours of incubation) on Blastocystis sp.

**Drug Toxicity on Blastocystis sp. in Co-culture**

The average viability percentage of ST1 of Blastocystis sp. in co-culture with Caco-2 at the highest peptide concentration (16 μg/mL) was 6.7%, 1.1%, and 0% after 24, 48, and 72 hours, respectively. The highest viability

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**Table 1.** IC$_{50}$ Value of mCM11 and Positive Control MTZ (After 24, 48, and 72 Hours of Incubation) on Caco-2 Cell Line

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>mCM11</th>
<th>MTZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>8.72</td>
<td>32.75</td>
</tr>
<tr>
<td>48 h</td>
<td>9.29</td>
<td>11.25</td>
</tr>
<tr>
<td>72 h</td>
<td>7.25</td>
<td>244.27</td>
</tr>
</tbody>
</table>

Note: IC$_{50}$: The 50% inhibitory concentration; MTZ: Metronidazole.

**Table 2.** Flow Cytometry Analysis of the Cytotoxicity Effects of mCM11 Peptide Treatment on caco-2 Cells After 24 Hours

<table>
<thead>
<tr>
<th>Apoptosis (%)</th>
<th>Late Apoptosis (%)</th>
<th>Necrosis (%)</th>
<th>Live (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24 h</td>
<td>0.087</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>mCM11 (8 μg/mL)</td>
<td>24 h</td>
<td>10.8</td>
<td>12.1</td>
<td>11.6</td>
</tr>
</tbody>
</table>

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Figure 1. The Pattern of the Effects of mCM11 and MTZ on the Percentage of the Viability of Caco-2 Cells After 24, 48, and 72 Hours. Note: MTZ: Metronidazole
percentage (33.6%) was observed after 24 hours of incubation at the lowest peptide concentration (4 μg/mL). The viability of *Blastocystis* sp. ST3 was decreased by an increase in concentration and incubation time (Figure 3). The viability of ST1 and ST3 treated in co-culture conditions by MTZ was similar to other results in a time- and dose-dependent manner. The viable ST1 parasites decreased from 16% to 11.5% at the lowest concentration and from 7% to 0% at the highest concentration by increasing the incubation time (24, 48, and 72 hours). Moreover, the viability of ST3 was decreased by incubation time (24 to 72 hours) at all concentrations (Figure 3).

**Selectivity Index**
The SI of mCM11 was calculated for *Blastocystis* sp. ST1 and ST3 (Table 3).

**Discussion**
*Blastocystis* is a prevalent worldwide GI infection caused by blastocyst sp. Currently, with the increase in susceptible hosts, *Blastocystis* sp. can be considered an opportunistic infection, which can be life-threatening in immunocompromised individuals. As with other parasitic infections, efforts have been made to demonstrate the anti-parasitic effects of natural nature substances against *Blastocystis* sp. In the present study, the cytotoxic effect of the mCM11 peptide on cell lines increased during 24, 48, and 72 hours, and the greatest anti-parasitic effect was observed at the highest concentration (128) after 72 hours. In addition, the ST3 showed more sensitivity to the mCM11. Several previous studies have suggested the effects of different substances on different subtypes of *Blastocystis*; interestingly, in the present study, the effect of the peptide on ST3 was more significant.

Anti-parasitic AMPs exert their antiparasitic properties through interacting with the cell membrane, increasing its permeability, and creating holes, or by using intracellular targets and autophagy. So far, the effect of several AMPs on parasites, such as *Toxoplasma*,
Leishmania, Plasmodium, Babesia, and the like, has undergone investigation. In 2016, the effect of cathelicidin, which is an antimicrobial peptide secreted from human intestinal epithelial cells, a part of which is interleukin (IL-37), was investigated on the Blastocystis sp. parasite, and IL-37 caused a change in the membrane and inhibited Blastocystis sp. Similar to our study, Shargh et al evaluated the effect of the CM11 peptide on Blastocystis sp. (ST3) and found the therapeutic effect of the peptide in the conditions of parasite culture alone, as well as parasite culture together with the Caco2 cell line. Different species of Blastocystis, similar to most intestinal protozoa, have zoonotic potential that can be transmitted from animals to humans; therefore, the treatment of animal species that live close to human habitations should be taken into consideration.

Considering the need for new drugs and the disadvantages of MTZ treatment, researchers have sought to investigate the effects of AMPs against Blastocystis species. The present study comparatively evaluated the effects of peptides mCM11 and MTZ on two subtypes of the Blastocystis parasite ST1 and ST3. Consequently, it was found that the viability of ST1 and ST3 in confronting mCM11 is dependent on dose and time. In this way, the lethal effect of the peptide will increase with the increase in the peptide concentration and the passage of time; this is similar to what happened with MTZ. However, this increase is not up to 48 hours because the effect of MTZ disappears after 48 hours. From the comparative examination of the results on these two STs, it is clear that Blastocystis sp. ST1 is more resistant to mCM11 peptide than Blastocystis sp. ST3; it is possible that this issue is related to the wall structure of the parasite in these two STs. This resistance is due to the wall structure and many pores in ST1, increasing the pathogenicity of this subtype. Differences in the surface coverage of three Blastocystis sp. isolates were observed by an electron microscope, and these differences can be related to the pathogenic differences of Blastocystis sp. subtypes. This sensitivity of Blastocystis sp. ST3 to confronting MTZ can be detected as well. In the investigation of the effect of CM11 peptide on ST3, which was conducted by Shargh et al, the peptide at the highest concentration (24 μg/mL) had 71.3% toxicity after 24 hours, while the WR11 peptide on ST3 at the highest concentration (16 μg/mL) had 95.3% toxicity after 24 hours, indicating an increase in peptide toxicity.
The anti-parasitic effect of the CM11 peptide (Cecropin A (2−8)-Melittin (6−9)) has been tested in another previous study; in this regard, Abbasi et al examined the effect of the mentioned peptide on *Toxoplasma gondii* tachyzoites in the *in vitro* and *in vivo* conditions. The results of this study revealed that CM11 has a beneficial effect on the tachyzoites of *T. gondii in vitro*. The result of the animal model suggests that CM11, either alone or in combination with other chemotherapeutic agents, could be a potential therapeutic for toxoplasmosis. Therefore, the studied peptide has shown good potential in terms of its effect against protozoa. After examining and comparing the average viability percentages of Blastocystis sp. ST1 in the presence of the mCM11 peptide in co-culture, we noticed an improvement in the therapeutic effect of the peptide in co-culture, leading to a reduction in some of the resistance of ST1 to the peptide. Based on the results of the average viability percentage of both STs when exposed to MTZ, in both cases of the parasite culture and co-culture, it is true that the response to MTZ is variable in different STs of the parasite.

To investigate the toxicity of this peptide on Caco2 cells, the flow cytometry method was performed when the cells were exposed to a concentration of 8 μg/mL obtained from the IC50 after 24 hours. The percentages of early apoptosis, delayed apoptosis, and necrosis were calculated as 10.8%, 12.1%, and 11.6%, respectively. Moreover, in the study conducted by Sharh et al, the percentages of early apoptosis, delayed apoptosis, and necrosis in the exposure of Caco2 cells to a concentration of 24 μg/mL peptide after 24 hours were reported as 6.16%, 47.8%, and 4.3%, respectively. These results showed the induction of low apoptosis and necrosis, and since mCM11 peptide has high toxicity on Blastocystis sp.; therefore, mCM11 peptide has high toxicity on Blastocystis sp. and can be a suitable option for *in vivo* studies. Optimizing peptides for anti-parasitic studies can enhance their delivery and effectiveness so that, for example, combining these materials with nanoparticles can make the mechanism of action of the peptide more targeted.

The present study has some limitations, including the impossibility of investigating the parasite mortality rate with death gene (apoptosis) expressions, as well as the mechanism of the effect of the peptide on the parasite with the electron microscope method. In addition, it was impossible to investigate and generalize the findings to the *in vivo* environment.

**Conclusion**

Our findings demonstrated that mCM11 has a more toxic effect on Blastocystis sp. ST1 and ST3 than MTZ. Considering the results of the present study, it is expected that mCM11 will be a suitable alternative to MTZ against Blastocystis sp. in the future. The results of the anti-parasitic effect of mCM11 are promising and can be considered a potential candidate to substitute MTZ. However, it seems that more studies are needed in *in vivo* environments. As a suggestion for future studies, it is recommended that studies with *in vivo* designs are also conducted to check or confirm the effect of the peptide. Additionally, the use of *in silico* tools can be helpful for peptide optimization. It seems to be useful to use different GI cell lines to test the effect of the peptide and confirm or reject the results; finally, the use of methods with higher sensitivity/specificity at the cellular-molecular levels (measuring the expression levels of genes with real-time polymerase chain reaction) will lead to more decisive results.

**Authors’ Contribution**

**Conceptualization:** Majid Pirestani.

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**Formal analysis:** Majid Pirestani.

**Funding acquisition:** Majid Pirestani.

**Investigation:** Abdolhossein Dalimi.

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**Project administration:** Majid Pirestani.

**Resources:** Abdolhossein Dalimi.

**Software:** Zahra Ghazi.

**Supervision:** Majid Pirestani.

**Validation:** Majid Pirestani.

**Visualization:** Abdolhossein Dalimi.

**Writing—original draft:** Zahra Ghazi, Majid Pirestani, Abdolhossein Dalimi.

**Writing—review & editing:** Abdolhossein Dalimi.

**Competing Interests**

The authors declare that they have no conflict of interest.

**Ethical Approval**

This study was approved by Research Ethics Committee of Tarbiat Modares University (IR.MODARES.REC.1400.014).

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