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Inhibition of *Borrelia burgdorferi* by 3,3-Diindolylmethane: A Research Protocol

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Abstract

Introduction: The spread of *Borrelia burgdorferi* is greatly exacerbated by the climate crisis. Rising global temperatures have increased the geographic range of *B. burgdorferi*-carrying ticks. 3,3-diindolylmethane (DIM) is a widely used estrogen supplement with a highly concentrated positive charge on one side, potentially inhibiting bacterial growth by attracting the negatively charged bacterial membranes, causing membrane tearing. DIM could allow for more comfortable treatment of Lyme disease, as it displays no significant side effects when consumed up to 200 mg/L. Conversely, current antimicrobial protein-based drugs may induce side effects at their minimum inhibitory concentration.

Methods: To determine whether DIM displays antimicrobial properties and could therefore be an inexpensive and ergonomic treatment, its effect on membrane-enclosed vesicles will be compared with polymyxin B, which is known to inhibit membrane structural integrity. This will be analyzed by filling vesicles with red aniline dye, then establishing three groups with different treatments. The test, positive control, and negative control groups will be treated with DIM, polymyxin B, and no treatment respectively. The resulting absorbencies of the test, positive control, and negative control groups will be compared.

Results: The test group could show significant absorbance differences when compared to the positive and negative control groups, or no significant absorbance differences when compared to these groups. Positive and negative control groups should be compared with each other to ensure the groups display substantial and negligible dye release respectively.

Discussion: If the test group shows a significant absorbance difference from the negative or both positive and negative control groups, it can be concluded that DIM displays significant antimicrobial properties. Because the results illustrate DIM is as effective or more effective than polymyxin B at inhibiting *B. burgdorferi* cell membranes, DIM will be an effective treatment for Lyme disease. These results are only reliable if the standard deviations of each group do not overlap, and the positive control group has a significantly higher absorbance than the negative control group.

Conclusion: If the results suggest DIM displays strong enough antimicrobial properties to treat Lyme disease, its structure should be modified to improve integration into the bloodstream and gastrointestinal tract.

Keywords: vector-borne diseases; Lyme disease; antimicrobial drug; antibiotic; range shifts

Introduction

Prior to the mid 1970s, no reports of tick disease were recorded in Canada due to temperatures being lower than the optimal temperature range of above 4°C for most ticks [1]. However, due to rising global temperatures, the range of black legged ticks (*Ixodes scapularis*) has shifted into Canada [1]. This has caused a major health concern in Canada because black legged ticks are carriers of *Borrelia burgdorferi*, the bacteria responsible for Lyme disease [2]. Due to the range shift of ticks, the interval of contact between human hosts and ticks has increased because of longer optimal temperatures for tick activity and outdoor activity; thus causing a trend in rising Lyme disease cases across all Canadian provinces [3].

B. burgdorferi, the bacterium causing Lyme disease, has a spiral body enclosed by two phospholipid bilayer membranes [4]. The surface of the phospholipid bilayer is primarily embedded with the negatively charged lipoprotein C (OspC), which plays an essential role in the transmission and oversees the synthesis of bacteria when the host's blood nutrients enter the tick's midgut area [5, 6]. This is essential to the bacteria's overall function. The combination of outward-facing phospholipid heads on the bilayer and the OspC lipoproteins cause the membrane to gain a strong negative charge [5].

Current treatments for Lyme disease rely on oral antibiotics such as doxycycline, amoxicillin, and cefuroxime, which inhibit bacterial biosynthetic pathways [6]. The antimicrobial protein Polymyxin B is an alternative

treatment used for patients suffering from multidrug-resistant (MDR) bacterial strains [7]. Polymyxin B is able to inhibit MDR bacteria because it contains a positively charged stripe of domains concentrated on one side of the protein [8]. Thus, its positively charged stripe will attract the negatively charged *B. burgdorferi* membrane through electrostatic interactions, tearing the membrane apart and killing the bacterium as a result [8]. However, polymyxin B must be extracted from bacteria, so the extraction process is relatively costly and time-consuming [8]. Furthermore, polymyxin B has been shown to induce side effects such as fatigue, dizziness, or muscle pain in Lyme disease patients when consumed at its minimum inhibitory concentration [8].

3,3-diindolylmethane is a widely available estrogen supplement extracted from cruciferous vegetables such as broccoli, cabbage, and cauliflower. It can be absorbed by most organ systems, although it is insoluble in the bloodstream and difficult to absorb in the gastrointestinal tract [9]. Currently, DIM is not being used to treat bacterial infections, but it exhibits several properties suggesting that it could be used as an antimicrobial alternative to polymyxin B. Firstly, the extraction process of DIM is simpler and cheaper than its counterpart in polymyxin B. Moreover, unlike polymyxin B, DIM induces no known side effects, other than slight increases in estrogen excretion, when consumed up to a concentration of 200 mg/L ($8.1x10^{-4}$ mol/L) [10].

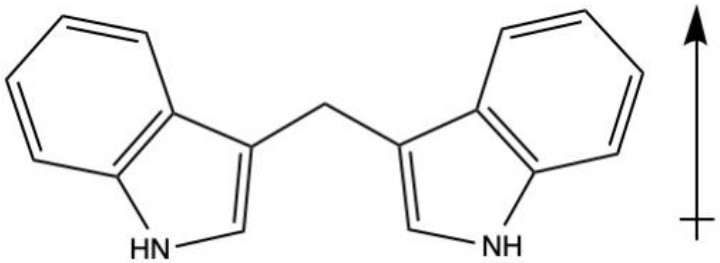

Figure 1. Structure and charge distribution of 3,3-diindolylmethane (created with ChemDraw 21.0.0).

The DIM molecule may exhibit antibacterial properties because it contains two amine groups on one side. When bonded as shown in Figure 1, the nitrogen atoms in these amine groups gain an overall positive charge [11]. Other than these nitrogen atoms from the amines, the DIM molecule is composed of uncharged carbon and hydrogen atoms [11]. Therefore, the molecule as a whole contains a highly concentrated positive charge on the entirety of its side with the bonded amine groups, which is structurally similar to the positively charged stripe of domains on an antimicrobial protein [11]. When placed into contact with *B. burgdorferi*, the positively charged stripe on DIM will potentially attract the negatively charged phospholipid heads and OspC lipoproteins on the membrane, in a similar mechanism to existing antimicrobial protein treatments [12]. A sufficiently strong positive charge from the DIM will create a large enough attraction between the bacterial membrane and the charged nitrogen atoms, disrupting the membrane's structural integrity, much like polymyxin B [12].

We propose an investigation to examine the effectiveness of DIM in displaying antimicrobial properties through inhibiting *Borrelia burgdorferi* cell membranes.

Methods

To create lipid vesicles mimicking bacterial membranes, a solution of phospholipids, OspC lipoproteins and red aniline dye dissolved in octan-1-ol solvent will be prepared [13]. This solution will be extruded through a microfluidic chip into a vessel filled with dimethylformamide (DMF) solvent [13]. DMF solvent was chosen to create these vesicles because it is nonpolar and would therefore not disturb the polarity-based interactions which would potentially tear the membrane [14]. The microfluidic chip will contain microchannels that open and close to extrude one nanoliter of solution per minute, forming the solution into uniformly shaped vesicles filled with octan-1-ol and red aniline dye, surrounded by a phospholipid membrane with embedded lipoproteins. If the membranes of these vesicles are torn, the red aniline dye will be released. Therefore, the extent of membrane rippage will be measured with the amount of red aniline dye released.

DIM's antimicrobial properties will be compared to polymyxin B, an antimicrobial protein which inhibits *B. burgdorferi* growth by tearing bacterial membranes [8]. Because DIM is insoluble in water, both solutions will use DMF as a solvent, as it dissolves DIM to a greater extent but shows no overall charge [15].

Three groups, each with five replicates, will be established: a positive control group containing dye vesicles with Polymyxin B, a negative control group containing dye vesicles only, and a test group containing dye vesicles with DIM.

1 mL of the polymyxin B solution will be pipetted into the positive control test tubes, and 1 mL of the DIM solution will be pipetted into the test group test tubes. The concentration of polymyxin B in the positive control group will be $3.32x10^{-9}$ mol/L, the minimum inhibitory concentration of polymyxin B against *B. burgdorferi*

infection [8]. Furthermore, the concentration of DIM in the test group will be $8.1x10^{-4}$ mol/L, which is the highest concentration of DIM that can be administered in vitro without the presence of any significant side effects [10].

All test tubes will then be sealed and allowed to rest for 12 hours. Then, the ripped cell membranes and intact vesicles in each test tube will be removed through centrifugation, leaving only the released red aniline dye. 2.5 mL of the contents from each test tube will be pipetted into cuvettes. The cuvettes will be inserted into a spectrophotometer, which will measure absorbance. The mean absorbances from the negative control group, the positive control group, and the DIM group will be calculated. To determine whether a statistically significant difference exists between each of the groups, a two tailed t-test will be conducted between the absorbance of the positive control group and the absorbance of the DIM group, and another two tailed t-test will be conducted between the absorbance of the negative control group and the absorbance of the DIM group. Additionally, a two tailed t-test will be conducted to compare the absorbances of the positive control and negative control groups, to detect any errors in the experimental assay that could remove the expected significant difference between the positive and negative controls. The standard deviation of the replicates in each group will also be calculated, to assess any variance between the replicates that will affect the experimental results.

To ensure the collection of reliable data, this procedure should be repeated until the standard deviations of each group consistently show no overlap with one another, and the two tailed t-test consistently shows a statistically significant difference between the positive control and negative control groups where the positive control group displays a higher absorbance than the negative control.

The treatment of the lipid vesicles is the independent variable in this investigation, with no treatment in the negative control group, polymyxin B being mixed with the lipid vesicles in the positive control group, and DIM being mixed with the lipid vesicles in the test group. The extent to which the vesicle membranes are torn, as measured by the absorbances from the negative control, positive control, and DIM groups, is the dependent variable in this investigation. Comparing the disruption of lipid vesicle membranes between the two compounds will determine the presence or absence of DIM's antimicrobial properties.

Results

Results will be obtained through first comparing the negative control group to the DIM group, then comparing the positive control group to the DIM group. The presence or absence of a statistically significant difference between the negative control and DIM groups will determine whether DIM increases or decreases membrane rupture when compared to an environment without any treatment. The subsequent presence or absence of a statistically significant difference between the positive control and the DIM group will either confirm that DIM has an insignificant effect on *B. burgdorferi* membrane rupturing, or illustrate that DIM is more effective than Polymyxin B when rupturing membranes.

To test the reliability of the results, the standard deviations of each group will be compared with each other. If the standard deviations between different groups do not overlap, the data can be considered reliable. Furthermore, comparison between the positive and negative control groups, which should show significantly different absorbances due to different levels of membrane rippage, will also serve as a test of reliability.

Discussion

If a significant difference is only found between the DIM group and the negative control group, it can be concluded that DIM displays strong enough antimicrobial properties against *B. burgdorferi*. A significant difference between the DIM and negative control groups shows that the vesicle membranes were torn to a greater extent when interacting with DIM than in an environment without any other factors, so DIM exhibited some lysing interaction with the membranes. Additionally, the similarity between the DIM and positive control groups shows that DIM's effectiveness in tearing the vesicle membranes is comparable to the effectiveness of polymyxin B, a known bacterial membrane inhibitor.

If there is a significant difference between the DIM group and both control groups, it can also be concluded that DIM displays strong enough antimicrobial properties as a Lyme disease treatment. The difference between the DIM group and the negative control shows that DIM tore the bacterial membrane to a greater extent than no interacting factors, exhibiting lysing interactions with the membrane. Furthermore, the difference between the DIM and positive control groups shows that DIM tore the membranes to a greater extent than a known membrane inhibitor. Therefore, DIM's effectiveness in tearing the vesicle membranes must be greater than the effectiveness of Polymyxin B, a known membrane inhibitor, in this case.

If the only significant difference lies between the DIM group and the positive control, it can be concluded that DIM does not display strong enough antimicrobial properties against *B. burgdorferi*. DIM tore the bacterial membrane to a similar extent as the negative control group, which is known to exhibit no significant interactions with the vesicle membrane. This shows that DIM, like the negative control, cannot attract and tear the membrane significantly. Furthermore, a significant difference between the DIM and positive control groups would imply that polymyxin B is more effective at tearing vesicle membranes than DIM, so DIM exhibits less antimicrobial properties than current treatments.

The results can be considered reliable when two conditions are met: first, the corresponding two tailed t-test

must illustrate a significant difference between the positive and negative control groups, where the positive control group displays a higher absorbance than the negative control group. This ensures that the lipid vesicle assay is correctly measuring the effect of treatments on the membrane integrity and is not compromised by experimental errors. Additionally, the standard deviations between each individual group must not overlap with each other. This ensures that the results are not affected by biological variance between the replicates in each group.

Conclusions

If the experimental results prove that DIM displays strong enough antimicrobial properties to act as a Lyme disease treatment, the chemical structure of DIM should be altered to increase solubility and absorption across the body so that it can integrate into the bloodstream and gastrointestinal system. The minimum inhibitory concentration of this more soluble DIM derivative should also be determined. Furthermore, the DIM derivative molecules should be tested with human cell cultures and organoids after alterations, to ensure that body cells and systems will not be damaged when treated with the DIM derivative's minimum inhibitory concentration.

List of Abbreviations Used

DIM: 3,3-diindolylmethane
DMF: dimethylformamide
MDR: multidrug-resistant
OspC: lipoprotein C

Conflicts of Interest

The authors declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

Our study is a research protocol proposal. No human participants were required, and no review from the research ethics board was required. All materials and substances used in this study are not prohibited.

Authors' Contributions

YJX: made contributions to the study's design and planning, and contributed by writing the manuscript.
CL: made contributions to the study's design and planning, and contributed by writing the manuscript.

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