Strain-specific and photochemically-activated antimicrobial activity of berberine and two berberine analogs

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ABSTRACT: Berberine, a natural product alkaloid, and its analogs have been reported to have a wide range of medicinal properties, including antibacterial activity. Berberine has been shown to be a photosensitizer - photochemical excitation at the correct wavelengths generate highly reactive singlet oxygen species in situ, and this has biomedical applications in photodynamic therapy. Here, we explore antibacterial effects of photoirradiation on berberine and two semisynthetic berberine analogs, dihydroberberine and 8-methyl-7,8-dihydroberberine, across three strains of bacteria. An understanding of the photosensitizing ability of berberine may inform the design of future compounds towards the photodynamic therapy of bacterial infections.

INTRODUCTION

Widespread use of antibiotics have resulted in the emergence of strains of antibiotic resistant bacteria, which continues to present an ever-growing problem in medicine, particularly in hospital settings. Every year, tens of thousands of Americans die from infection from antibiotic resistance bacteria (1). Therefore, the development and discovery of novel antibacterial agents has been and continues to be an area of great scientific and biomedical significance.

Berberine, a naturally occurring alkaloid, is extracted from the plants in the genus *Berberis*, and has been documented to have a wide range of biological activities, including anticancer, antitumor, and antimicrobial activity (2,3,4). Berberine-containing extracts have been documented to be used in ancient cultures as a medicinal agent dating back to 3000 BC, where it was first reported to be used in ancient Chinese medicine (5). Berberine has also been shown to be a photosensitized DNA intercalating agent, generating highly reactive singlet oxygen upon photoirradiation of a berberine DNA complex (6,7). Singlet oxygen then oxidizes guanines within DNA, resulting in DNA damage and the inhibition of DNA replication, which has potential towards the photodynamic treatment of cancers (8).

Compounds with photosensitizing ability and singlet oxygen have been previously studied for antimicrobial and anticancer photodynamic therapy (9). A study on the kinetics of the photoirradiation on antimicrobial against E.coli by compounds representative of three different classes of photosensitizers found that the photochemical properties of photosensitizers and their abilities to be taken up by bacterial cells differ throughout different classes of compounds (10). Berberine has also been studied for potential applications in the photodynamic treatment of cancers. It has been previously reported that berberine, upon photoirradiation, induced anticancer effects on renal carcinoma cells (11).

Previously in this *Journal*, we reported the dose dependency and strain specific antimicrobial activity of berberine compared to five broad spectrum antibiotics representative of different structural classes (12). We found that berberine is less potent than the broad spectrum antibiotics screened (ampicillin, enrofloxacin, kanamycin, nalidixic acid, and sulfanilamide) but seemed to exhibit strain specificity.

Semisynthetic analogs of berberine have been previously demonstrated to have more potent antimicrobial activity in-vitro and in-vivo (13,14,15). Grignard additions with alkyl chains and phenyl substituents have been demonstrated to have more potent antimycobacterial effects against tuberculosis (16). 8-alkyl-12-bromo derivatives of

berberine have been previously synthesized through Grignard addition followed by radical bromination, and it was found that these compounds have more potent antimicrobial activity (17). It has also been previously reported that a borohydride reduction of berberine to dihyroberberine resulted in decreased antimicrobial activity (18).

Here, we report photoirradiation-dependent, comparative antimicrobial activity of berberine and two chemically synthesized analogs. Two separate experiments were conducted to study this: a Kirby Bauer assay based on our previous methodology and an agar infusion assay, in which the agar was infused with the compound solution and inhibition was quantified by means of cell density. We were interested in two carbon 8 analogs of the berberine: dihydroberberine and 8-methyl-7,8-dihydroberberine.

Dihydroberberine (2) was synthesized through a borohydride reduction to berberine in which the iminium ion of berberine is reduced to an enamine (Figure 1). This was conducted according to previously reported protocols (19). Dihydroberberine is an analog of berberine that also provides access to other analogs and reactions such as stork enamine reactions and 13-alkylberberine (20,21).

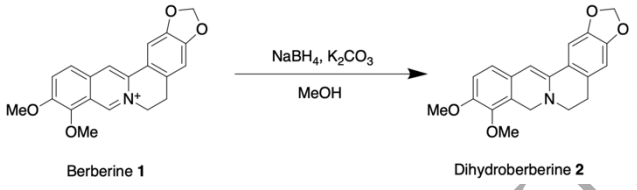

Figure 1. Synthesis of Dihydroberberine **2**. *Reagents and Conditions*: $NaBH_4$, K_2CO_3, MeOH, r.t.

A racemic mix of 8-methyl-7,8-dihydroberberine 3 was synthesized through a Grignard addition with methyl magnesium bromide to berberine chloride (Figure 2). The addition of substituents to carbon 8 results in the formation of a stereocenter.

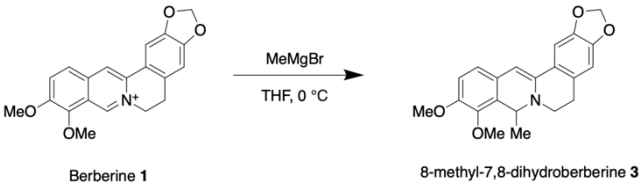

Figure 2. Synthesis of 8-methyl-7,8-dihydroberberine **3**. *Reagents and Conditions*: MeMgBr, THF, 0°C

We are interested in the effects of photoirradiation on antimicrobial activity against three bacterial strains: B. cereus, N. sicca, and S. epidermidis, a combination of gram positive and gram negative bacteria (Table 1). Strains belonging to the three species have been found to be pathogenic. Gram positive bacteria (B. cereus) have no outer lipid membrane and have a thick peptidoglycan layer, while gram negative bacteria (N. sicca and S. epidermidis) have an outer lipid membrane with a thin peptidoglycan layer (22). The selection of gram positive and gram negative bacterial strains allows us to understand the strain specific antimicrobial activity of berberine and its analogs. Based on prior literature, we hypothesize that dihydroberberine will have less potent antimicrobial activity than berberine, and 8-methyl-7,8-dihydroberberine will be more potent. Moreover, we hypothesized that strain specific antimicrobial effects might be accentuated upon photoirradiation.

Bacterial Strains	**Related Diseases**	**Characteristics**
Bacillus cereus	Food poisoning, diarrhea (23)	Can produce ATP in the absence of oxygen - Facultative anaerobic (24)
Neisseria sicca	Pneumonia, meningitis, endocarditis (25)	Oxidase-positive (aerobic)- uses oxygen as an electron acceptor in the electron transport chain (26)
Staphylococcus Epidermidis	Hospital acquired infections - nosocomial infections (27)	Forms biofilm - protects the bacteria from immune response and antibacterial agents (28)

Table 1. Strains of bacteria studied with some characteristics

The wavelength of light chosen for photoirradiation was based on the maximum wavelength of absorbance for all three compounds (Figure 3). The maximum wavelength of absorbance for the two analogs experience a blueshift in comparison to berberine.

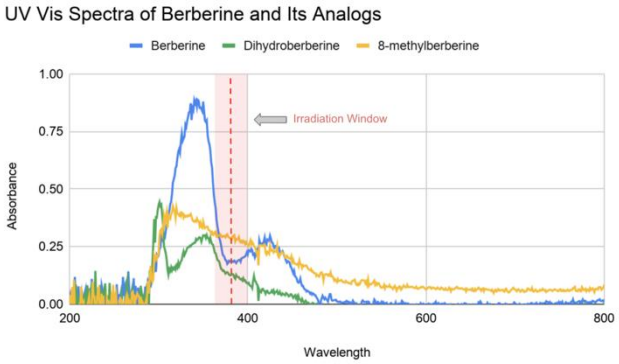

Figure 3. UV-Vis spectra of all three compounds screened in this study. The irradiation window represents all wavelengths of light that the bacteria were exposed to.

In this study, we identified the photoirradiation dependent antimicrobial activity of berberine and two semisynthetic analogs against three strains of bacteria through two separate assays.

RESULTS

Our work from the Kirby Bauer assay and the infused Mueller Hinton (MH) agar assay are aimed at understanding the photosensitizing ability of berberine analogs and

the effect of photoirradiation on their antimicrobial properties.

Radii of inhibition (ROI) from the Kirby Bauer assay (Figure 4) are shown below. Berberine and synthesized analogs had no inhibition against N. sicca at all concentrations, consistent with our previously reported results. No inhibition was observed for concentrations under 10 mM for all compounds screened. ROI against B. cereus (Figure 4a), demonstrate that berberine and 8-methyl-7,8-dihydroberberine inhibit bacterial growth at high concentrations without photoirradiation, with the 100 and 50 mM concentrations having ROI that are similar and statistically insignificant. We believe that this is a result of the maximum inhibitory effects of berberine against B.cereus being within the 10 and 50 mM concentrations. No inhibition against S. epidermidis was shown at any concentration with any of the berberine analogs in the absence of photoirradiated (Figure 4b).

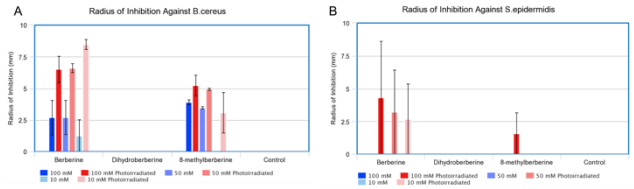

Figure 4. Radii of inhibition against *B.cereus* and *S.epidermidis*. (a) The radii of inhibition against *B.cereus* at three concentrations with both photoirradiated and non photoirradiated results represented. (b) Radii of inhibition against *S.epidermidids* at three concentrations.

Radii of inhibition upon photoirradiation indicate that photoirradiation has an effect on the antimicrobial activity of berberine analogs (Figure 5). An increase in the ROI upon photoirradiation can be observed with berberine and 8-methyl-7,8-dihydroberberine at all concentrations higher than 1 mM against both B.cereus and S. epidermidis (Figure 4). Dihydroberberine had no inhibitory effects against bacterial growth. The previous maximum inhibitory effects of berberine against B. cereus remains consistent, with the 100 mM and 50 mM resulting in statistically insignificant differences in ROI. At the 10 mM concentration, there is a significant difference between the inhibitory effects of berberine upon photoirradiation. The inhibitory effects of 8-methyl-7,8-dihydroberberine against B.cereus are also improved upon photoirradiation, with a statistically significant difference at the 50 mM concentration and antimicrobial activity at the 10 mM concentration, whereas there was no inhibition at the 10 mM concentration with no photoirradiation.

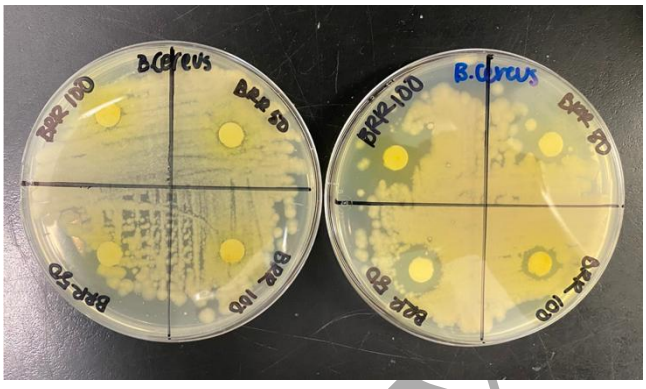

Figure 5. Bacteria growth after incubation without photoirradiation (left) and with photoirradiation (right). The compound solution here is berberine at 100 mM and 50 mM concentrations. The increased inhibition of bacterial growth can be seen in their respective radii of inhibition.

The effects of photoirradiation on the antimicrobial effects of berberine and its analogs is consistent against S.epidermidis. There was no inhibition against S.epidermidis without photoirradiation at all concentrations, but upon photoirradiation, berberine demonstrated antimicrobial effects at 100, 50, and 10 mM concentrations and 8-methyl-7,8-dihydroberberine was cytotoxic at 100 mM.

Bacterial growth from the infused Muellur Hinton agar assay was quantified by cell density. In this study, dihyroberberine demonstrated inhibitory effects against N. sicca upon photoirradiation (Figure 6). N. sicca colonies covered 30.7% of the petri dish after being subjected to photoirradiation for thirty minutes, compared to the mat of bacteria (full coverage) that grew without photoirradiation. Results for other strains of bacteria and compounds were inconclusive.

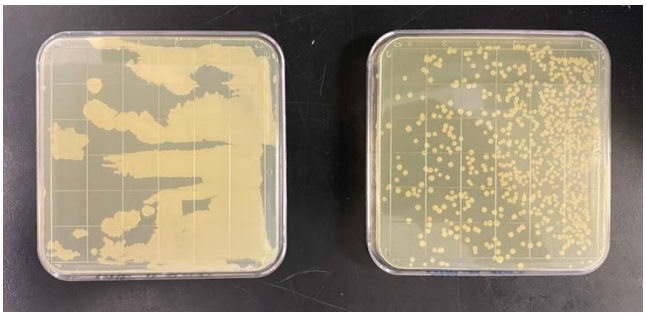

Figure 6. Petri dishes from the infused Mueller Hinton agar assay with dihydroberberine against *N.sicca*. The not photoirradiated petri dish (left), has bacterial growth in a mat, while the photoirradiated petri dish (right) has distinct bacteria colonies.

From this study, we found that the antimicrobial activity of berberine and related analogs are affected by photoirradiation, and the inhibitory activity remains strain specific when photoirradiated.

DISCUSSION

In this study, we report the photoirradiation-dependent antimicrobial activity of berberine and 8-methyl-7,8-dihydroberberine 3, a semisynthetic analog of berberine.

We found that photoirradiation results in superior antimicrobial activity at all concentrations of berberine and 8-methyl-7,8-dihydroberberine 3, and that the antimicrobial activity remains strain specific.

Differences between this study and our previously reported study, including the concentrations of the compound solutions necessary to induce inhibitory activity, can be attributed to the modification in the composition of our Mueller Hinton agar. It has been previously reported that differences in agar composition can result in significant differences in bacterial growth rates and colony density (29).

The results of the Kirby Bauer assay and the infused agar assay suggest that the mode of administration of the compound affects its antimicrobial activity. Dihydroberberine demonstrated no inhibition in the Kirby Bauer assay, which is a disc diffusion assay, while it exhibited inhibition of bacterial growth in the infused agar assay. We believe that this may be attributed to dihydroberberine having a poor ability to diffuse, while other compounds studied were able to diffuse better, resulting in inhibition in the Kirby Bauer assay.

Our synthesized analog, 8-methyl-7,8-dihydroberberine, had antimicrobial activity that was comparable to that of berberine. Further studies on the structure-activity relationship of the length of the alkyl chain on carbon 8 of berberine and dihydroberberine and the antimicrobial activity and strain specificity, along with analogs with substituents on different carbon positions, would be instrumental to understanding the structure relationship activity of berberine analogs and antimicrobial activity. Such structures are synthetically accessible with alternate Grignard reagents. Additionally, future optimizations to the method of delivery of berberine analogs would be instrumental to gaining insight on the effects of photoirradiation to the antimicrobial activity of berberine and the effects of diffusion versus infusion. This could guide the design of future analogs of berberine and the possible identification of novel antibacterial agents.

MATERIALS AND METHODS

Chemical Synthesis

Synthesis of 7,8-dihydroberberine To a vacuum dried 50 mL round bottom flask charged with a teflon stir bar was added berberine chloride 1 (1.000 g, 2.690 mmol, 1.0 eq.), sodium borohydride (0.112g, 2.959, 1.1 eq.), and potassium carbonate (1.100 g, 7.959 mmol, 3.0 eq.) in MeOH (30 mL). The reaction mixture was stirred at room temperature for one hour and monitored by thin layer chromatography (10% MeOH in DCM). Upon disappearance of the starting material by TLC, the reaction mixture was filtered with a Buchner funnel. The resulting material was recrystallized in ethanol to give dihydroberberine as yellow crystals (0.799 g, 2.36 mmol, 75.4% yield)

Synthesis of 8-methyl-7,8-dihydroberberine To a vacuum dried 25 mL round bottom flask charged with a teflon stir bar and cooled in an ice bath was added 0.2 g (0.538 mmol, 1.0 eq) of berberine chloride in tetrahydrofuran (THF). A 3M solution of MeMgBr (1.793 mL, 5.379 mmol, 10 eq.) in THF was added dropwise through a syringe. The reaction mixture stirred for 15 minutes in the ice bath and monitored by thin layer chromatography (25% hexane in ethyl acetate). Upon disappearance of the starting material by TLC, the reaction mixture was quenched with water, and extracted with brine and ethyl acetate. The organic layer was collected and dried over anhydrous magnesium sulfate, and concentrated in vacuo to give 8-methyl-7,8-dihydroberberine (0.126 g, 0.359mmol, 66.7% yield).

Characterization All compounds were characterized by 1H nuclear magnetic resonance (NMR) spectroscopy (Nanalysis, NMReady, 60 MHz), Fourier-transform Infrared spectroscopy (Thermo Nicolet iS5, iD5 ATR assembly) and UV-visible spectroscopy (BioRad Smartspec 3000).

Dihydroberberine ^{1}H NMR (60 MHz, $CDCl_3$): δ 5.82-7.02 (m, 5H), 5.84 (s, 2H), 4.18 (d, J = 13.4 Hz, 2H), 3.80 (s, 6H), 2.59-3.65 (m, 4H); FTIR (ATR, cm-1): 2930.24, 2833.66, 2249.37, 1607.91, 1494.35, 1484.76, 1457.74, 1427.17, 1388.49, 1333.92, 1277.43, 1246.80, 1221.93, 1163.74, 1131.16, 1083.90, 1039.00, 990.70, 938.65, 908.41, 860.13, 799.16, 772.73, 730.86, 647.90; UV (iPrOH) λmax: 301, 354

8-methyl-7,8-dihydroberberine ^{1}H NMR (60 MHz, $CDCl_3$): δ 5.75-7.14 (5H, m), 4.69 (1H, m), 3.74 (3H, s), 3.68 (3H, s), 2.67 (2H, m), 2.02 (2H, m), 1.38 (3H, d, J = 6.9 Hz); FTIR (ATR, cm-1): 2931.25, 2359.69, 1681.92, 1597.43, 1482.63, 1416.18, 1343.49, 1266.07, 1229.80, 1167.99, 1096.48, 1037.44, 932.42, 847.36, 810.53, 738.87, 612.23; UV (H_2O) λmax: 316

Bacteria Cultures Live bacteria cultures of Bacillus Cereus, Escherichia coli, Neisseria sicca, and Staphylococcus epidermidis were acquired from Carolina Biological. Overnight cultures were grown in falcon tubes with 10 mL LB media (1% tryptone, 1% NaCl, 0.5% yeast extract, 97.5% water) at 37°C for 12-14 hours.

Compound Solutions Solutions of berberine, dihydroberberine, and 8-methyl-7,8-dihydroberberine were made at 6 different concentrations (100 mM, 50 mM, 10 mM, 1 mM, 0.1 mM, 0.01 mM). Compounds were dissolved in solutions of 10% DMSO in deionized water. A solution of 10% DMSO in deionized water served as our control. Solutions were sonicated to help with dissolution. The appropriate dilutions were performed, and the appropriate amounts of DMSO were added to maintain a 10% DMSO solution.

Kirby Bauer Assay Bacteria from the overnight cultures were inoculated on Petri dishes plated with modified Mueller Hinton Agar. Filter paper discs saturated with the compound solution were used to administer the compound solutions. The bacteria were incubated overnight at 37 ℃, and radii of inhibition measurements were taken in mm with an electric caliper. All plating was done in a sterile laminar flow hood. Triplicates with technical replicates were completed, and the results were averaged.

Infused Agar Assay To modified Mueller Hinton agar was added 1% of the 100 mM compound solutions, resulting in a 0.1 mM concentration of the compounds in the agar. Petri dishes were plated with the infused Mueller Hinton agar, and bacteria were inoculated. The petri dishes were incubated overnight at 37 ℃. Results were quantified by bacteria density, which was measured by colonies per cm2. The average size of a colony was found. All plating was done in a sterile laminar flow hood.

Photoirradiation Petri dishes were photoirradiated at 380 nm for thirty minutes while incubated at 37 ℃. After initial photoirradiation, all petri dishes were incubated overnight at the same temperature. A nail curing lamp set up inside of the incubator was used as the light source (Figure 7). The wavelength of emitted light was determined by a spectrometer (Ocean Optics).

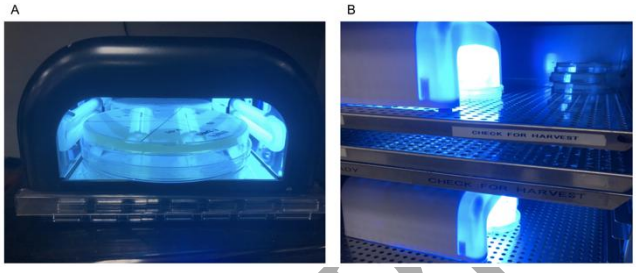

Figure 7. Experiment setup for the photoirradiation of the petri dishes. (a) Petri dishes placed inside the nail curing lamps. Each petri dish was photoirradiated for 30 minutes. (b) Nail curing lamps placed inside the incubator, at 37℃

Statistical Analysis Radius of inhibition measurements were acquired in millimeters using an electronic caliper. The ROI from all three experiments were averaged, and standard error was calculated using the follow equation: $\frac{\sigma}{n}$,where σ is equal to the standard deviation and n is equal to the number of values.

Unpaired t-testing was completed using the following equation: t=x̄1-x̄2s2(1n1-1n2), where x̄ represents the population means, s2 represents the sample variances, and n the populations. The sample variance (s2) was calculated using the following equation: s2=i=1n1(xi-x̄1)2-i=1n2(xi-x̄2)2n1+n2-2, where the sum of the variances squared were subtracted and divided by the population. The t-distribution critical values table was then used to derive the statistic's corresponding critical value at the 95% confidence level, whereupon if the t-testing result was less than the critical value, the p-value was determined to be less than 0.05. Results with a p value less than 0.05 were considered statistically significant.

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