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The Effects of the Antimicrobial Honokiol on the Intracellular pH of *Bacillus subtilis* WB746 and *Escherichia coli* B23

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Cyclic hydrocarbons and phenolic compounds are known to insert into the cellular membranes of Gram-positive and Gram-negative bacteria, leading to expansion of membranes, leakage of ions and collapse of the proton motive force. Honokiol is a plant phenol extracted from *Magnolia officinalis* that has been used for a variety of therapeutic purposes in Chinese medicine, such as antimicrobial activity. This inhibitory action has been ascribed to membrane permeabilization based on the structure of honokiol, but the mechanism of action is yet to be determined experimentally. In this study, we sought to elucidate the molecular foundation for the observed antibacterial activity of honokiol. The minimum inhibitory concentration (MIC) was determined for the two species tested, *Bacillus subtilis* WB746 and *Escherichia coli* B23, at 25 µg/ml and 100 µg/ml, respectively. These MICs were used to assay the antibacterial action of honokiol, and correlate cell death to reduction in intracellular pH. Changes in intracellular pH were measured using the pH-sensitive fluorescent dye, carboxyfluorescein diacetate succinimidyl ester. Variations in intracellular pH were observed in both organisms which we theorized resulted in altered proton motive force and membrane energetics. Our findings indicated that the antimicrobial properties of honokiol may include disruption of cellular energy metabolism at the membrane.

The naturally forming phenolic compounds called neolignans are present in a wide variety of plants and have been demonstrated to show an extensive diversity of biological activities including a significant antimicrobial effect and cytotoxicity (5). They are synthesized in plants by converting phenylalanine to cinnamic acid followed by subsequent reduction steps to produce the desired lignan (17). Interestingly, neolignans share many structural characteristics with cyclic hydrocarbons. Cyclic hydrocarbons interact with biological membranes in a manner which can lead to altered structure and function of the membrane, resulting in impaired growth and activity of the cells (18, 19)

One such lipophilic compound, the phenolic neolignan honokiol (Hon), extracted from *Magnolia officinalis,* has been linked to a wide variety of actions including antiplatelet activity, central nervous system inhibition, anti-inflammatory effects, antimicrobial activity, antioxidative activity and free radical scavenging activity (7). In addition, Hon has shown an antimicrobial effect on many periodontal bacteria, including *Bacillus subtillis* (3). Despite the obvious valuable pharmacological attributes of Hon, little is known about the mode of action despite its use for centuries as a Chinese treatment of acute pain, diarrhoea, coughs and urinary problems (7). Based on the structural and chemical similarity of Hon to other known phenolic compounds, it is possible that the lipophilic characteristics of Hon cause disruption of membrane integrity that results in an antimicrobial response.

Membrane disruption by lipophilic compounds can occur when lipophilic phenols insert into the lipid bilayer of cellular membranes to disrupt lipid-to-lipid and lipid-to-protein interactions (19). These hydrophobic interactions may result in decreased membrane stability, repressed embedded enzyme activity and increased membrane permeability (19). The propenyl side-chains of Hon (Fig. 1A) may intensify the hydrophobicity of the molecule and hasten membrane disruption while the phenolic hydroxyl groups may aid in the diffusion of periplasmic protons into the intracellular space, weakening the proton motive force (19). Maintenance of membrane integrity is essential for maintaining bioenergetics. Carvacrol (Fig. 1B), a lipophilic phenol with similar structure to Hon, was found by Ultee *et al.* (20) to insert between lipid acyl chains causing the expansion of membranes, the leakage of ions and the collapse of the proton motive force. Ultee *et al.* (20) established that the presence of the phenolic hydroxyl group present in carvacrol, which is also present in Hon, was associated with a higher antimicrobial activity and hence important in the

chemicals mode of action. This evidence supports the model that the antimicrobial effects of Hon are caused by disruption of the cellular membrane.

Earlier studies by Ho *et al.* (7) described the antimicrobial activity of Hon against a wide variety of Gram-positive and Gram-negative bacteria. Their findings indicated a minimum inhibitory concentration (MIC) of 25 μg/ml for *B. subtilis,* but no antimicrobial activity (MIC $\geq$ 100 μg/ml) for *Escherichia coli*. In light of the obvious lipophilc similarities of Hon to known compounds, we hypothesized that Hon exhibits antimicrobial activity by disrupting the cellular membrane. This was ascertained by addressing the question: does Hon inhibit the growth of *B. subtilis* WB746 and *E. coli* B23 through disruption of the cellular membrane? In doing so, we determined the MIC of Hon to *B. subtilis* WB746 and *E. coli* B23, and assessed changes in intracellular pH, which indicated variations in bioenergetics by loss of membrane integrity.

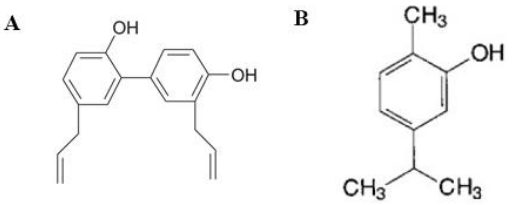

FIG. 1. Chemical structures of (A) honokiol and (B) carvacrol.

MATERIALS AND METHODS

Bacterial strain and culture conditions. Wildtype *B. subtilis* WB746 and wildtype *E. coli* B23 (obtained from the MICB421 culture collection in the Department of Microbiology and Immunology at the University of British Columbia) were cultivated aerobically at 37°C on Luria-Bertani agar [0.5% yeast extract, 1% tryptone, 0.5% NaCl and 1.5% agar, pH 7.0]. Hon was supplied by Calbiochem at 99% purity (Cat. No. 384620). The MIC determination of Hon for both cell types was carried out in trypticase soy (TS) broth [1.5% pancreatic digest of casein, 0.5% papaic digest of soybean and 0.5% NaCl] supplemented with the appropriate concentrations of Hon. For MIC determination, cells from both species were cultivated aerobically in TS broth at 37°C, shaking at 200 rpm, to 0.5 $O.D._{660}$ (Spectronic 20D+, Milton Ray). Bacteria were prepared for the intracellular pH measurements in the same manner.

Determination of MIC. *E. coli* B23 and *B. subtilis* WB746 cultures were grown to $O.D._{660}$ 0.5, and then pelleted by centrifugation (7000 x g for 10 min at 4°C using a Beckman centrifuge and Beckman JA-21 rotor). The cultures were re-suspended in fresh TS broth to a cell density of 0.1 $O.D._{660}$. Cultures were distributed into individually labeled test tubes, and Hon was added to 0, 12.5, 25, 50 and 100 μg/ml to determine MIC. Additionally, 100 nM nigericin (Nig) (Cat. No. V0627) and 100 nM valinomycin (Val) (Cat. No. N7143) (Sigma Aldrich) were added to separate test tubes as positive controls. Tubes were placed in a tube roller where cells were grown at 37°C for the remainder of the MIC tests. $O.D._{660}$ readings were taken at 0, 40, 70, 105, 140 and 180 min, and all readings were normalized with a TS broth blank.

Loading cells with fluorescent probe. The protocol for intracellular pH measurements has been described previously (3); moreover, this protocol has been adapted for use in Gram-negative bacteria (16). *E. coli* B23 and *B. subtilis* WB746 cultures were grown to $O.D._{660}$ 0.5 before being harvested by centrifugation. Harvested cells from each species were thenwashed, resuspended in an equal volume of 50 mM Tris buffer pH 9.0 and incubated at 30°C for 10 min in the presence of 2 μM carboxyfluorescein diacetate succinimidyl ester (cFDASE, Invitrogen). For *E. coli* B23, this buffer was supplemented with 1 mM EDTA. In the cell, cFDASE is hydrolysed to carboxyfluorescein succinimidyl ester (cFSE) before being conjugated to aliphatic amines (3). To eliminate non-conjugated cFSE, the cells were incubated with 10 mM glucose for 30 min at 30°C. The cells were then washed twice with 50 mM K_2HPO_4 buffer (pH 7.0), resuspended in Tris MES buffer (50 mM Tris, 50 mM MES, 140 mM choline chloride, 1 mM $MgCl_2$, 10 mM KCl, 10 mM $NaHCO_3$ and 0.5 mM $CaCl_2$, pH 7.0) and placed on ice until required. .

Measurement of intracellular pH. The pH calibration curves for *E. coli* B23 and *B. subtilis* WB746 were created using Tris MES buffers with pH values ranging from 4 to 9. The intracellular and extracellular pH were equilibrated through the addition of 10 μM Nig and 10 μM Val, so that the measured pH values would represent those encoded by the various buffers. Flourescence intensity units (FIU) were measured with a Turner® Quantech™ digital filter fluorometer (Model No. 109323) using the following specifications: 490 nm (pH-sensitive) (NB490) and 360 nm (pH-insensitive) (NB360) excitation wavelengths, and 515 nm emission wavelength (SC515).

Intracellular pH measurements were conducted separately for both bacterial species using a formerly developed twelve-minute time course assay (5). The bacterial cell suspensions were transferred to measurement cuvettes and FIUs were measured at one-minute intervals. Glucose was added at the three-minute time point to give a 10 mM final concentration. The MIC of Hon, as determined earlier, was added at the 7-minute mark (25 μg/ml for *B. subtilis* WB746 and 100 μg/ml for *E. coli* B23). The 490/360 nm ratios were converted to intracellular pH values using the polynomial equation of the line for the calibration curve outlined above.

Error and uncertainty. Natural variation in living material results in a degree of uncertainty in our measurements. As such, 5% error is assumed for MIC determinations and fluorescence intensity values. Due to combination of uncertainty, 7% error is assumed for the calibration curve measurements and pH values resulting from the calibration curve.

RESULTS

Minimum inhibitory concentration (MIC) determination. To quantify the antimicrobial activity of Hon, the MICs for *B. subtilis* WB746 and *E. coli* B23 were determined. As expected, the *B. subtilis* control (0 µg/mL Hon) demonstrated exponential growth (Fig. 2A). However, *B. subtilis* WB746 cultures separately treated with 12.5 and 25 µg/mL Hon exhibited notable reduction in growth when compared to the control (Fig. 2A). The MIC of Hon for *B. subtilis* WB746 was confirmed to be 25 µg/mL by observing the respective cultures at 22 hours (data not shown). As expected, 100 nM Nig inhibited growth in a manner similar to Hon at the MIC; however, 100 nM Val failed to notably inhibit growth of *B. subtilis* WB746 (Fig. 2A).

The antimicrobial activity of Hon was less effective against *E. coli* B23 (Fig. 2B). The MIC of Hon was determined to be 100 µg/mL, at which the cell growth of *E. coli* B23 was significantly inhibited (Fig. 2B). In addition to exhibiting a MIC of Hon 4X greater than *B. subtilis* WB746, *E. coli* B23 was unresponsive to Val and Nig separately at 100 nM (Fig. 2B). Entry of Val and Nig into *E. coli* B23 may have been limited by their molecular size, which is significantly greater than the outer membrane porins (5).

To ensure sufficient antimicrobial activity of our control samples, the effects of Val and Nig at 10X and 100X the concentration tested in the MIC tests (1 µM and 10µM, respectively) were measured on *B. subtilis* WB746 and *E. coli* B23 separately (data not shown). Based on this data, Val and Nig were used at 10 µM during the construction of the calibration curve for the 12-minute intracellular pH assay.

The $O.D._{660}$ values obtained from the MIC determinations were corrected for background due to Hon, Val and Nig. This background value, however, may have lessened with time as the molecules were incorporated resulting in calculated $O.D._{660}$ values below the actual $O.D._{660}$.

Intracellular pH measurement. To determine the effect of Hon on the intracellular pH of *B. subtilis* WB746 and *E. coli* B23, the intracellular pH of Hon-treated (at the respective MIC) and Hon-untreated cells were contrasted for each of the cultures. The calibration curves for *B. subtilis*

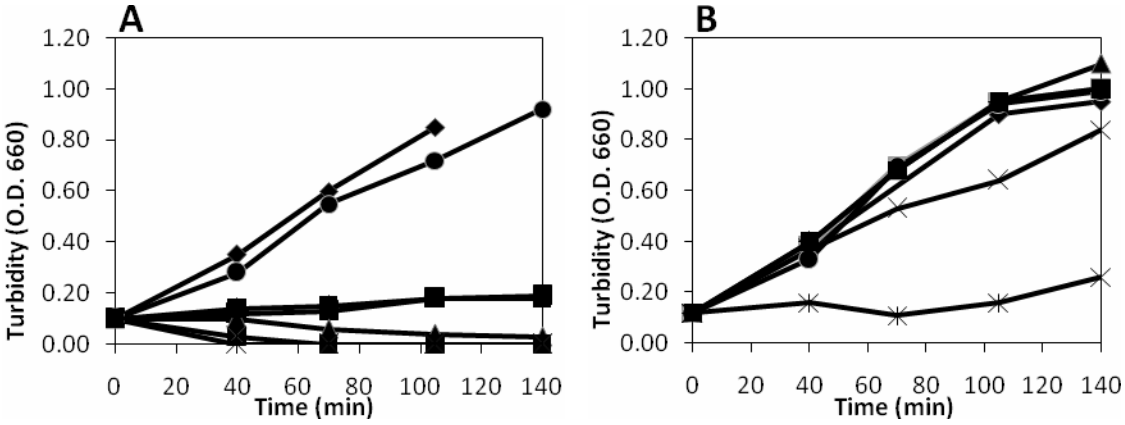

FIG. 2. MIC determination of honokiol for *B. subtilis* WB746 (A) and *E. coli* B23 (B). Cells suspensions were monitored at 37°C with shaking. Honokiol at 0 µg/mL (♦), 12.5 µg/mL (■), 25 µg/mL (▲), 50 µg/mL (×), 100 µg/mL (*), valinomycin at 100 nM (●), and nigericin at 100 nM (ı) were separately added at 0 min. The turbidity ($O.D._{660}$) of the cultures was observed at regular time-intervals.

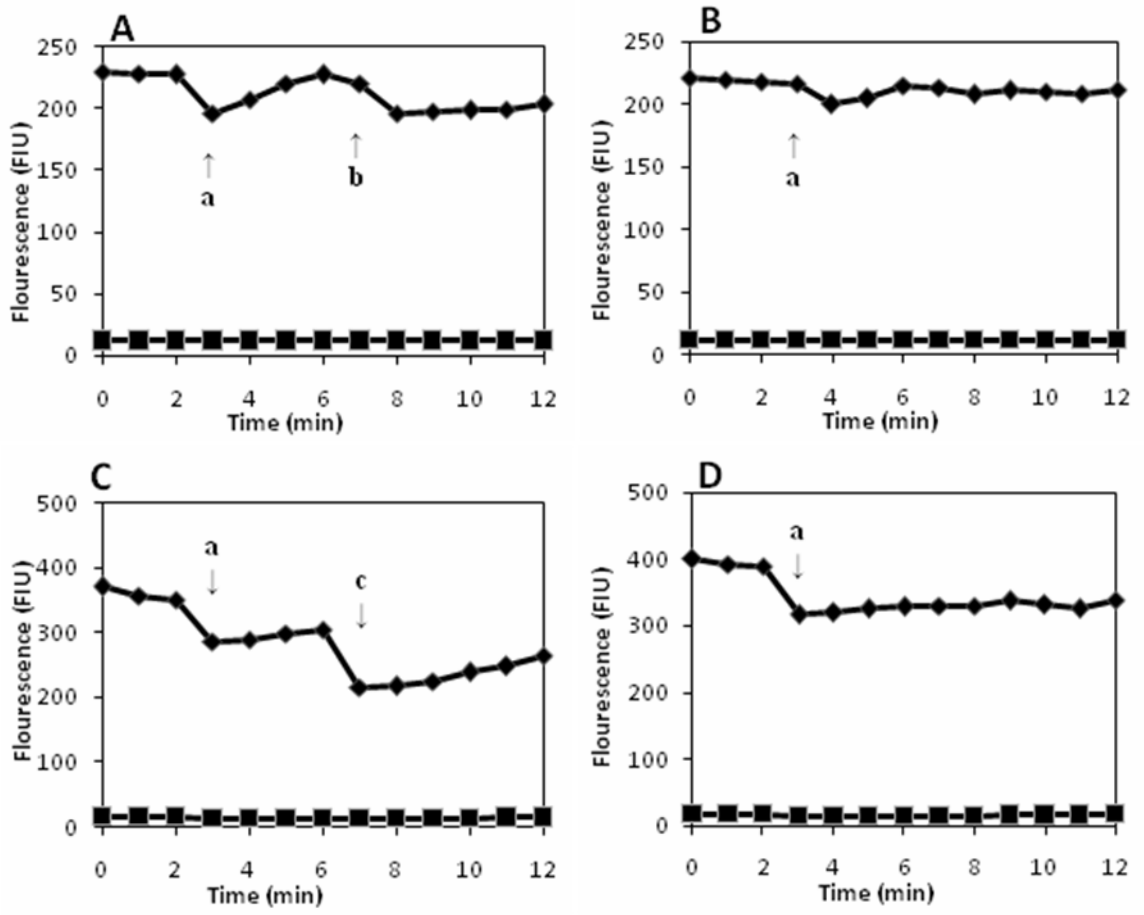

FIG. 3. Flourescence intensity of (A) honokiol-treated *B. subtilis* WB746 and (B) -untreated *B. subtilis* WB746, (C) honokiol-treated *E. coli* B23 and (D)-untreated *E. coli* B23 at excitation wavelengths of 490 nm (♦) and 360 nm (■). The emission wavelength was 515 nm. The following additions were made at times indicated by the arrows: a, glucose (10 mM); b, honokiol to honokiol-treated *B. subtilis* WB746; c, honokiol to honokiol-treated *E. coli* B23.

WB746 (A) and *E. coli* B23 (B) were produced with the following polynomials equations, respectively: $y = 1.237x^2 - 9.912x + 20.67$ and $y = 0.831x^2 - 5.469x + 10.31$ (data not shown). The emissions at 490 and 360 nm excitation wavelengths for Hon-treated and Hon-untreated *B. subtilis* WB746 (Fig. 3A, Fig. 3B) and *E. coli* B23 (Fig. 3C, Fig. 3D) were presented in FIU. Previous studies have demonstrated that fluorescence emissions of cFSE at 490 and 360 nm excitation wavelengths are pH-sensitive and pH-insensitive, respectively (5). Based on the polynomial equations derived from the calibrations curves, the ratios of fluorescence at 490 to 360 excitation wavelengths were processed and presented as pH_{in} for Figure 4.

The effects of Hon on *B. subtilis* WB746 and *E. coli* B23 were monitored over 12-min time-course assays (Fig. 3). In *B. subtilis* WB746, the addition of glucose at 3 min resulted in a reduction of the fluorescence emission at the 490 nm excitation wavelength of approximately 10% in both the Hon-treated and Hon-untreated cultures, prior to the addition of Hon (Fig. 3A and B). The fluorescence recovered to levels comparable to pre-glucose addition within 3 min (Fig. 3A and B). Another reduction of in fluorescence emission at the 490 nm excitation wavelength of about 10% was observed after the addition of Hon in the Hon-

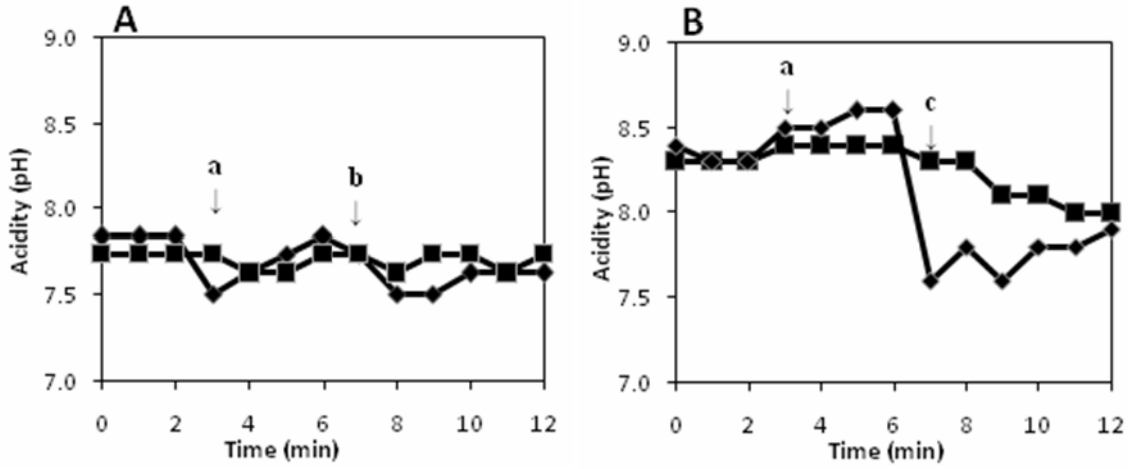

FIG. 4. The intracellular pH of honokiol-treated (♦) and –untreated (■) (A) *B. subtilis* WB746 and (B) *E. coli* B23 cell suspensions, as calculated by the appropriate calibration curve. The following additions were made to appropriate cultures at times indicated by the arrows: a, glucose (10 mM); b, honokiol to honokiol-treated *B. subtilis* WB746 (25 µg/mL); c, honokiol to honokiol-treated *E. coli* B23 (100 µg/mL).

treated culture (Fig. 3A). This reduction in fluorescence did not recover and was not observed in the Hon-untreated culture (Fig. 3A and B).

In contrast, the addition of glucose to *E. coli* B23 at 3 min resulted in a reduction in fluorescence at both excitation wavelengths of approximately 20% in the Hon-treated and Hon-untreated cultures (Fig. 3C and D). The addition of Hon at 7 min to the Hon-treated culture resulted in a reduction in the fluorescence emission at the 490 nm excitation wavelength of approximately 30% (Fig. 3C).

The intracellular pH of *B. subtilis* WB746 prior to the addition of glucose and Hon was approximately 7.8 (Fig. 4A), which falls within the expected range of 7.5 – 8.0 (5). A pH reduction of 0.33 and 0.11 was observed in the Hon-treated and –untreated cultures, respectively, with the addition of glucose at 3 min. The addition of Hon at 7 min resulted in a pH reduction of 0.1, followed by another reduction of 0.23 at 8 min. Note that the combined reduction of 0.33 pH units is substantial but not significant relative to the Hon-untreated culture due to the broad uncertainty range.

The intracellular pH of *E. coli* B23 was comparable and constant at 8.4 between the Hon-treated and –untreated cultures prior to the addition of Hon (Fig. 4B). Previous findings indicate the pH of *E. coli* B23 to typically be 7.5 - 8.0. The observed drop in intracellular pH of 1 with the addition of Hon in the Hon-treated culture of *E. coli* B23 implies the collapse of the pH gradient normally sustained across the cytoplasmic membrane (Fig. 4B). A comparable drop was not observed in the Hon-untreated culture (Fig. 4B).

DISCUSSION

The MICs of Hon for *B. subtilis* WB746 *and E. coli* B23 were determined to be 25 and 100 ug/ml, respectively. This corresponds with values reported in other studies which describe similar MICs (7).

There are several possible reason for the difference in susceptibility between the two species. As Gram-

negative bacteria, *E. coli* B23 possesses a lipopolysaccharide (LPS) layer on its outer membrane which may impede the action of insertion of Hon, as suggested by Sikkema et al (20). Furthermore, an increased tolerance to phenols has been observed in Gram-negative bacteria, as they have the ability to convert *cis* fatty acids to *trans*, resulting in increased membrane fluidity (20). This would suggest a larger dose-dependent effect in *E.coli* B23 versus the Gram-positive *B.subtilis* WB746. Porin channels of *E.coli* B23 often exclude molecules greater than 3.8 nm, the size of a porin at its largest point (6, 21) so Hon (33 nm) may need to pass through the membrane using alternate mechanisms (14).

The results of this study indicated a decrease in intracellular pH in both *B. subtilis* WB746 and *E.coli* B23 following treatment with Hon at the appropriate MIC. Intracellular pH measurement

was achieved by treatment with cFDASE, which has been previously used in both *B. subtilis* and *E. coli* (3, 16). It is of note that EDTA was used to facilitate entry of the cFDASE into *E. coli* B23 cells. EDTA is useful for permeablizing membranes of Gram-negative bacteria, but must be used sparingly to maintain cell viability. Note that EDTA treatment may have increased the effect of Hon. The intracellular pH of *B. subtilis* WB746 and *E. coli* B23 is typically 7.5-8.0 (1); most of our determined pH values fall within that range. Notably, *E. coli* B23 was measured to have an initial intracellular pH higher than expected, which may be explained by differences between strains. Nevertheless, a significant drop in intracellular pH was observed upon treatment with Hon. Since Hon results in a drop in intracellular pH, as observed in *B. subtilis* WB746 and *E.coli* B23, it will result in a drop in the PMF and energy synthesis. This dysregulation after Hon treatment would require disruption of the membrane (5).

There are several possible explanations for how Hon can disrupt the membrane. Similar molecules, such as carvacrol, have shown a mechanism wherein the molecule associates with a given cation and can then cross the membrane (20). Once across the membrane, hydroxyl groups become deprotonated and leave the cytoplasm as an anion, where it can again be protonated and repeat the cycle (20). This may result in a decrease in the intracellular pH and dysregulation of energy metabolism. The similarity of Hon, with its two phenolic hydroxl groups, and its ease of deprotonation, suggest that it may readily bring protons into the cell through this mechanism.

Alternatively, Hon may alter intracellular pH by self-insertion into the membrane, causing a loss of the PMF. Not only would this result in death via loss of energy, but could also result in death by inducing complete collapse of membrane integrity and the transfer of required metabolites both into and out of the cell (19). It has been shown that lipophilic phenols can partition themselves into the lipid bilayer of cellular membranes (19, 20). This partitioning results in membrane expansion and disruption of cellular function (18, 20). Therefore, it is possible that the effect of Hon is through direct insertion into the membrane.The potential of multiple modes of action of Hon cannot be overlooked. This study has addressed action via disruption of the PMF and death by loss of membrane integrity; however, several other mechanisms may exist. In eukaryotes, nor-isoguaiacin and dihydroguaiaretic, known lignans, have been shown to cause inhibition of NADH-oxidase resulting in the disruption of the mitochondrial electron transport system (15). Given the large similarity between lignans, it can be argued that Hon has the potential to disrupt electron transport chains in aerobes by similar mechanisms. There is also the potential for lipophilic molecules to cause a change in activation of membrane bound and embedded proteins, and that this interaction may result in a change in enzyme signaling (19). Hon is also a heavily researched small molecule in fields of cancer immunotherapy and has been shown to induce apoptosis, cell cycle arrest, anti-inflammatory activity as well as an anti-depressant effect (8, 9, 13, 21). This multitude of actions in eukaryotic cells demonstrates that Hon may also exhibit a large array of intracellular effects on bacterial cells. This is substantiated by the observed killing of *B. subtilis* WB746 and *E. coli* B23 by Hon (MIC), and the observed drop in intracellular pH in both organisms.

This study has established a link between reduced cell viability and a decrease in intracellular pH upon exposure of *B. subtilis* WB746 and *E.coli* B23 to the neo-lignan Hon. A MIC of Hon at 25 ug/ml and 100 ug/ml of was determined for *B.subtilis* WB746 and *E.coli* B23, respectively, and the intracellular pH of both organisms was shown to drop upon the addition of the molecule. Overall, these observations demonstrate the potential for Hon to act by disrupting membrane homeostasis and increasing cell mortality. Further work is needed to elucidate the exact mechanism of Hon, of which there may be several.

FUTURE EXPERIMENTS

In order to cause the observed reductions in intracellular pH, we suggest that Hon inserts into cellular membranes to cause disruption of PMF. Therefore, we would expect to see expansion of *E. coli* B23 and *B. subtilis* WB746 membranes as Hon destabilizes their structure under this model. In previous works, the self-quenching fatty acid octadecyl rhodamine β chloride (R_{18}) has been used to measure liposomal-membrane expansion (20). This method is based on the relief of fluorescence self-quenching of this probe resulting from dilution, which is caused by membrane expansion. That is, liposomes from *B. subtilis* WB746 and *E. coli* B23 could be extracted through sonication, labeled with R_{18}, treated with Hon and visualized using a fluorometer to show the expected increase in R_{18} fluorescence as Hon disrupts the liposomal membranes.

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