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Involvement of oxidative stress in bactericidal activity of 2-(2-nitrovinyl) furan against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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ABSTRACT

The involvement of reactive oxygen species and oxidative stress in 2-(2-nitrovinyl) furan mediated bacterial cell death was investigated in *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Time kill assay resulted in significant decrease in the optical density and colony-forming unit (CFU) of *E. coli*, *P. aeruginosa* and *S. aureus*. The level of superoxide anion radical and nitric oxide increased significantly in concentration dependent when compared with dimethyl sulfoxide (DMSO) treated bacteria. Similar concentration dependent increase in the activity of superoxide dismutase and catalase were recorded. The non-enzymatic antioxidant glutathione decreased significantly with a concomitant increase in glutathione disulfide. The level of malondialdehyde and fragmented DNA increased significantly in the bacterial cells treated with 2-(2-nitrovinyl) furan when compared with DMSO treated cells. The CFU of *E. coli*, *P. aeruginosa* and *S. aureus* following exposure to 2-(2-nitrovinyl) furan increased significantly ($p < 0.05$) in the presence of 2,2′ bipyridyl, an Fe chelator, significantly when compared with only 2-(2-nitrovinyl) furan suggesting the involvement of hydroxyl radical in the cell death. The available data from this study showed that 2-(2-nitrovinyl) furan induced oxidative stress in *E. coli*, *P. aeruginosa* and *S. aureus* as evident from elevated levels of superoxide anion radical nitric oxides and antioxidant enzymes.

1. Introduction

Increasing death cases arising from bacterial infection calls for development of new, effective and safe antimicrobial drugs. While the development of effective antibiotics cannot be overemphasized owing to increasing resistance to the arrays of available antibiotics [1]. Pharmaceutical companies are not encouraging development of new antibiotics [2]. Antibiotics are grouped as bactericidal or bacteriostatic depending on interaction with cellular targets. Bacteriostatic antibiotics inhibit ribosome function by targeting both the 30S and 50S ribosome subunits [3–5], while bactericidal antibiotics interfere with protein translation [6,7], cell-wall synthesis, DNA replication and repair [8,9]. In addition, there are accumulating evidences suggesting the involvement of oxidative stress in bactericidal activities of all classes of bactericidal antibiotics [9–11]. Also, some antibacterial natural products have been reported to affect the activities of antioxidant enzymes, an evidence of redox imbalance mediated antibacterial activities [12,13]. Compounds such as 2-(2-nitrovinyl) furan having β-nitrostyrene moiety have attracted interest owing to ready synthesis and significant antibacterial/antifungal activity [14].

2-(2-nitrovinyl) furan (Fig. 1) is a lipid soluble derivative of nitrovinyl furan. The antimicrobial activities of this group have been documented against gram-positive and gram-negative bacteria, with gram-positive bacteria highly susceptible to this compound [14–16]. β-nitrostyrene derivatives inhibit bioenergetics processes and modify thiol groups of enzymes [17]. Furthermore, vinyl furans inhibit glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase (Glc 6-PD),

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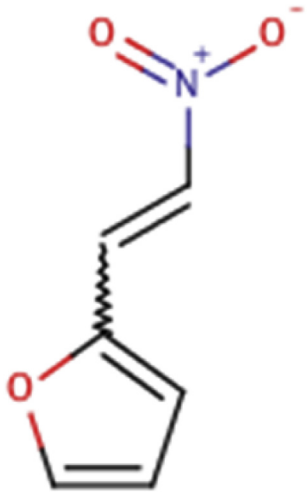

Figure 1. Structure of 2-(2-nitrovinyl) furan.

malate dehydrogenase, and glutathione reductase (GSH-red) [17]. Recently, 2-(2-nitrovinyl) furan was reported to mediate its toxicity in rats by inducing oxidative stress [18]. Despite the established antibacterial activities and inhibitory effect of vinylfuran group on bioenergetics process and redox enzymes, there is no report linking this to its bactericidal activity. Arising from these facts, this study evaluated possible involvement of oxidative stress in the antibacterial activities of 2-(2-nitrovinyl) furan against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

2. Materials and methods

2.1. Bacterial strains

E. coli (ATCC 25922), *P. aeruginosa* (ATCC 2783) and *S. aureus* (ATCC 29213) were procured from American Type Culture Collection and propagated on a lysogeny broth (LB) at 37 °C.

2.2. Materials

Diphenylamine, 5,5′-dithiobis(2-nitrobenzoic acid), guanidine hydrochloride, and N-ethyl-maleimide were procured from Research Organics (Cleveland, OH). 2-(2-nitrovinyl) furan, epinephrine, trichloroacetic acid, Hydrogen peroxide (H_2O_2), nitroblue tetrazolium, 2,2′-bipyridyl are product of Sigma–Aldrich, St. Louis, MO. All other reagents are of analytical grades.

2.3. Methods

2.3.1. Time kill bacterial susceptibility study

Susceptibility of *E. coli*, *P. aeruginosa* and *S. aureus* against 2-(2-nitrovinyl) furan was investigated using the procedure described by Samoilova et al. [12] with some modifications. Briefly, organisms were grown overnight in a liquid medium, lysogeny broth (LB), harvested by centrifugation and resuspended in 50 mL fresh medium (LB) to $OD_{600} = 0.1$ and grown aerobically at 37 °C in 250 mL flask. At mid-log phase ($OD_{600} = 0.5$), 2-(2-nitrovinyl) furan (prepared in dimethyl sulfoxide) was added to obtain concentrations (0.02, 0.04, 0.06, 0.08 and 0.10 mg/mL) or dimethyl sulfoxide (DMSO) and incubated at 37 °C for 3 h. Absorbance of the incubation medium was read at 600 nm for every 20 min interval of 3 h incubation time. For colony formation, samples of control culture (DMSO treated culture) and cultures treated with 2-(2-nitrovinyl) furan were removed at intervals (0, 20, 40, 60, 80, 100, 120, 140, 160 and 180 min) and centrifuged to collect the cells as pellet. The cells were washed and diluted with 0.9% NaCl, mixed with molten soft LB-agar (0.8%) at 42 °C and poured onto agar plates containing solid LB-agar (1.5%). Colonies were counted after 24 h at 37 °C.

2.3.2. Preparation of cell free extract

Cell free extract was prepared from the samples obtained after 3 h incubation of organisms with 2-(2-nitrovinyl) furan at the varying concentrations (0.02–0.10 mg/mL). Cells were harvested by centrifugation, washed twice and suspended in sucrose-Tris buffer (25 mmol/L sucrose solution, 10 mmol/L Tris–HCl, pH 7.4). Glass beads (2 g) was added to the bacterial suspension, homogenized and centrifuged at 3000 *g* for 10 min at 4 °C to obtain the cell free extract as the supernatant.

2.3.3. Oxidative stress assay

2.3.3.1. Superoxide radical anion and nitric oxide assay. Superoxide anion content of the cell free extract was determined using the procedure described by Becerra and Albesa [10] with little modifications. Briefly, cell free extract (0.5 mL) was incubated with 2.5 mL nitroblue tetrazolium (1 mg/mL) for 30 min at 37 °C. Then 0.5 mL of 0.1 M HCl was added. The blank was constituted same way except that the cell free extract was replaced with 0.25 M sucrose solution. The blue colour was read at 575 nm. The amount of superoxide radical anion generated was calculated using the molar extinction coefficient of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide formazan (17,000 M^{-1} cm^{-1} at pH 7.4–8.0).

The nitric oxide (NO) content of the cell free extract was determined using the colorimetric method based on the Griess reaction [19].

2.3.3.2. Superoxide dismutase. The activity of superoxide dismutase (SOD) was determined according to Misra and Fridovich [20]. Briefly, 0.2 mL of cell free extract was added to 2.5 mL of 0.05 mol/L carbonate buffer (pH 10.2) to equilibrate and the reaction was started by addition of 0.3 mL of freshly prepared 0.3 mmol/L epinephrine. The increase in absorbance at 480 nm was recorded every 30 s for 150 s. One unit of enzyme activity was defined as 50% inhibition of the rate of autoxidation of pyrogallol as determined by change in absorbance min^{-1} at 480 nm.

2.3.3.3. Catalase. Catalase (CAT) activity was determined using the method described by Aebi [21]. Briefly, 50 μL of homogenate was added to a cuvette containing 2 mL of phosphate buffer (pH 7.0) and 1 mL of 30 $mmolL^{-1}$ H_2O_2. The absorbance at 240 nm was measured for 1 min using a spectrophotometer. A molar extinction coefficient of H_2O_2 of 43.6 L mol^{-1} cm^{-1} was used to determine the CAT activity.

2.3.3.4. Glutathione reduced (GSH) and glutathione disulfide (GSSG). The level of GSH in the cell free extract was determined using the procedure described by Ellman [22]. Briefly, 1.0 mL of cell free extract was added to 0.1 mL of 25% trichloroacetic acid (TCA) and precipitate was removed by centrifuge at 5000 *g* for 10 min. Supernatant (0.1 mL) was added to 2 mL of 0.6 mM DTNB prepared in 0.2 M sodium phosphate buffer (pH 8.0). The absorbance was read at 412 nm.

GSSG level was determined using the procedure described by Hissin and Hilf [23]. Cell free extract (50 μL) was mixed with 20 μL of 0.04 M *N*-ethylmaleimide (NEM) to prevent oxidation of GSH to GSSG. It was incubated at room temperature for 30 min and 1.68 mL of 0.3 M Na_2HPO_4 solution was added to it followed by 250 μL of DTNB reagent. The absorbance of the sample was measured at 412 nm.