Synergistic interaction of cinnamic acid with amikacin against *Escherichia coli* under *in vitro* conditions

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Abstract: - One of the ways of reducing the amount of antibiotics is to add a phytochemical which would produce a synergistic interaction with the former. Effect of combination of cinnamic acid and amikacin against *E.coli* is reported in this paper. Checkerboard assay indicated that the amount of antibiotic could be reduced by a factor of 5 by adding 1 mM of the phytochemical. Time kill curve studies indicated a twofold decrease in colony forming units when the two compounds were used in combination, when compared to them being used individually. The post antibiotic effect (i.e., persistant suppression of bacterial growth following a brief exposure to an antibiotic) of amikacin was enhanced when cinnamic acid was used. This study indicates that cinnamic acid acts synergistically with amikacin, indicating that the therapeutic dose of the later could be considerably decreased leading to reduction in its toxicity and prevention of drug resistance.

Key-Words: - Cinnamic acid, amikacin, *Escherichia coli*, interaction, Checkerboard, Time kill curve, Post antibiotic effect.

1 Introduction

The use of botanical medicines is widespread and continues to grow. As a result, the potential for drug-herb interactions is a major concern for many health care practitioners. The risk of interactions is not limited to herbal supplements. Certain foods can interact with medications. Synergistic interaction of natural compounds with anticancer agents and antibiotics has been extensively reviewed [1-3]. Hydroxycinnamic acids are widely available in foods and beverages, so there is always a chance of their interaction with synthetic drugs and such an interaction can either be positive or negative.

The toxicities of aminoglycosides include those to the kidneys and ears (vestibular and auditory) and, rarely, neuromuscular blockade and hypersensitivity reactions [4,5]. These adverse reactions can be reduced when aminoglycosides are given in combination with some phytochemicals. Cinnamic acid is a compound which can be found in nature. It occurs throughout the plant kingdom, especially in flavour compositions and products containing cinnamon oil [6]. It was observed that trans-cinnamic acid enhanced the activity of isoniazid, rifampin, ofloxacin, amikacin and clofazimine against *M.avium* and *M. tuberculosis* [7,8]. The focus of the present study is to study the interaction of trans-cinnamic acid with amikacin against the Gram negative bacteria, *Escherichia coli*.

2 Materials and method

2.1 Bacterial strain

*Escherichia coli* (NCIM 2931) is specifically grown in Ca supplemented Muller Hinton Broth (Himedia, India) at a temperature of 37°C with aeration, for 16-18 h. The Minimum Inhibitory Concentration (MIC) of the antibiotics and the phytochemical was determined as described below with 10⁵ CFU/ml of colonies.

2.2 Antimicrobial compounds

Amikacin and trans-cinnamic acid was purchased from Himedia (India). The antibiotic was dissolved in sterile distilled water and the phytochemical in dimethyl sulphoxide (DMSO).

2.3 Assessment of MIC

Susceptibility of the bacterial strain was determined by broth microdilution method in accordance with the procedure recommended by the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) [9]. Briefly, a bacterial inoculum of 20 µl corresponding to 5 × 10⁵ CFU/ml, was added to 100 µl of serially twofold diluted antibiotic in Muller Hinton Broth (MHB) in the wells of a microtiter plate. The mixture was incubated at 37°C for 20 h. The MIC₉₀ of a compound is defined as the concentration of the compound that inhibits the bacterial growth by 90 %. The growth is determined by measuring the optical density of the broth at 600 nm using a microtiter plate reader (Spectramax Plus 384, Molecular Devices, USA). The MICs were determined...
in triplicate. In all the experiments DMSO and ethanol were maintained at concentrations less than 5%, since at this level they were found to be not inhibitory against this organism.

2.4 Checkerboard test

Checkerboard test was performed in duplicate for all the combinations, as described previously [9]. Two fold dilutions of the phenylpropanoid was added to columns of a 96 well plate at concentrations ranging from 10 to 0.001 mM. Two fold serially diluted concentrations (with initial concentrations starting at its MIC) of the antibiotic was then added to each row. MHB with an inoculum of approximately $5 \times 10^5$ CFU/ml was used for this assay. The MIC$_{90}$ for the combination was determined at 600 nm.

The antagonistic, additive, or synergistic effect of the phytochemical in combination with the antibiotic was determined by plotting them as isobologram [10]. The graph is represented with the concentration of phenylpropanoid in the X-axis and the ratio of the MIC of the antibiotic in combination to the MIC of the antibiotic when used alone in the Y-axis. A straight line that connects the ratio 1.0 (in the ordinate) and MIC of the phenylpropanoid in the abscissa indicates the line of additivity. MIC of combination, if located considerably below this line (below the 95% confidence band), indicates synergy and if it is above the line it indicates antagonism.

2.5 Time Kill Curve

Tubes containing 10 ml of MHB broth with amikacin and cinnamic acid (at 2x, 1x, 0.5x times their MIC) alone and in combinations and one tube without antibiotics were inoculated with 100 µl of actively growing E. coli culture adjusted to yield a final inoculum of $10^7$ cells per ml. They were incubated at 37°C. At 0, 4, and 24 h, the tubes were gently vortexed and the bacterial growth in each tube was estimated. Aliquots of 100 µl were removed from each tube and were serially diluted 10-fold in MHB medium. Aliquots of 100 µl were diluted and directly subcultured in duplicate by plating them on MH agar plates. The plates were examined and the colonies were counted after 24 h of incubation [11].

2.5 Determination of Post antibiotic effect

Twenty millilitres of 1:20 diluted overnight culture (OD of 0.1 at 600 nm = $10^6$ cfu) in MHB was incubated for 2 h at 37°C with (at concentrations of 2x, 1x, 0.5x) and without the antibiotics. In order to remove the antibiotics, the exposed bacteria were washed twice with phosphate-buffered saline (pH 7.2) by centrifugation for 10 min at 7000 rpm. Control was handled similarly. The pellets were resuspended in 20 mL of MHB broth followed by incubation at 37°C and samples (1 mL) were obtained at 2 h of exposure to antibiotics for corrections that were made to ensure that all the cultures would start with the same bacterial count. Samples were removed at time 0 (immediately after washing and after correction) and then hourly up to 7 h and the OD values were determined (reflecting the bacterial growth). The OD values were converted into cfu (bacterial growth) by using a standard curve which was constructed relating the bacterial count to the OD. The PAE is defined, according to Craig & Gudmundsson [12] as, PAE = $T - C$, where $T$ is the time required for the viable counts of the exposed bacteria to increase by $1 \log_{10}$ above the counts observed immediately after washing and $C$ is the corresponding time for the sample unexposed to antibiotic (control).

3 Results and discussion

Fig.1. Isobologram depicting the interaction of cinnamic acid and ampicillin

The MIC of amikacin and cinnamic acid was 0.0025 and 16.75 mM respectively. The natural product had a higher MIC when compared to the antibiotic. For the interaction studies with checkerboard method combinations of different concentrations of cinnamic acid and amikacin ranging from four to 1/32 times its MIC were used. The isobologram shows that the MIC of the combination falls well below the line of additivity. All the combinations were exhibiting synergistic activity (Fig 1). The amount of amikacin could be reduced to 0.3 µM, from 2.5 µM by adding 1.05 mM of cinnamic acid. Time–kill studies have been used to investigate the activity of numerous antimicrobial agents. They are also often used as the basis for in vitro investigations in pharmacodynamic drug interactions. Fig 2A shows the effect of 1x MIC of cinnamic acid with various concentrations of amikacin on the growth of E. coli at different time points. Fig 2B and Fig 2C show similar growth curves with 0.5x MIC and 0.25x MIC of cinnamic acid respectively. 1x MIC of cinnamic acid (1x CIN) is bactericidal, since more than 3 logs decrease in the cfu/ml is noted when compared to initial inoculums (Fig 2A). Even though there is a 2 logs decrease in the cfu/ml, it cannot be defined as synergy because as per definition the CFU/ml with the combinations are not less
than the most effective compound when used alone (1x CIN). The three combinations namely (2x AMI + 0.5x CIN), (1x AMI + 0.5x CIN), (0.5x AMI + 0.5x CIN) could be termed as synergy, where more than 2 log_{10} decrease in the cfu/ml is observed when compared to the most active drug i.e., 1x AMI (Fig. 2B). The combination (0.25x CIN and 2x ami) is synergistic, whereas the other two combinations (0.25x CIN and 1x AMI; 0.25x CIN and 0.5x AMI) are not (Fig. 2C).

Post-antibiotic effect (PAE) is a well-established pharmacodynamic parameter that reflects an arrested bacterial growth following the removal of the active antibacterial agent from the growth medium [13]. The duration of the PAE is mainly influenced by the bacterial species, and the nature of the antibacterial drug and its concentration. Environmental factors such as temperature, pH, pO₂, growth medium, the kind of body fluid, etc also affect PAE. Addition of cinnamic acid seems to prolong the post antibiotic effect of amikacin. Interestingly amikacin or cinnamic acid when used alone does not depress the growth of the microorganism after they were removed.

**Table 1. Post antibiotic effect of cinnamic acid and amikacin alone and in combination.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>PAE (in hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5x Cin</td>
<td>0</td>
</tr>
<tr>
<td>0.25x Cin</td>
<td>0</td>
</tr>
<tr>
<td>2x Ami</td>
<td>0.5</td>
</tr>
<tr>
<td>1x ami</td>
<td>0</td>
</tr>
<tr>
<td>0.5x ami</td>
<td>0</td>
</tr>
<tr>
<td>2x Ami + 0.5x cin</td>
<td>2</td>
</tr>
<tr>
<td>2x Ami + 0.25x Cin</td>
<td>1</td>
</tr>
<tr>
<td>1x ami + 1x cin</td>
<td>1</td>
</tr>
<tr>
<td>0.5x ami + 0.5x cin</td>
<td>1</td>
</tr>
</tbody>
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**4 Conclusion**

Aminoglycoside antibiotics are considered to be of special value by clinicians by virtue of their excellent and rapid bactericidal activity against gram-negative bacilli, their pharmacokinetic properties, and their ability to interact synergistically with beta-lactam or other cell-wall active drugs. The use of these agents in therapy is however limited by the fact that they possess weak activity against gram-positive organisms or bacteria that code for aminoglycoside modifying enzymes. They also may have undesirable side effects such as ototoxicity and/or nephrotoxicity in vivo. Our study shows a positive interaction of cinnamic acid with amikacin which is evident from checkerboard and time-kill curve studies. The MIC of the antibiotic could be considerably reduced by the addition of the phytochemical. Cinnamic acid when used in combination improves the post antibiotic effect of amikacin. Oral dosage of cinnamic acid along with amikacin could improve the treatment strategy against *E.coli*. 

Fig.2. Time kill curves

(A) 

(B)
References:


