Experimental analyses of synergistic combinations of antibiotics with a recently recognised antibacterial agent, lacidipine

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Abstract The cardiovascular drug lacidipine (Lc) is known to possess antibacterial activity. Further potentiation of action is possible by synergism between Lc and an antibiotic or a non-antibiotic. The minimum inhibitory concentration (MIC) of antibiotics, Lc and other non-antibiotics were detected by the agar dilution technique in different bacteria. Synergism was determined by disc diffusion assay, the fractional inhibitory concentration (FIC) index through checkerboard assessment and, also, the protective capacity of the combination by administering the drugs along with 50 × LD₅₀ challenge dose of virulent Salmonella typhimurium in animal experiments. Synergism between Lc and penicillin was found to be statistically significant (P ≤ 0.01) when compared with their individual effects. The FIC index of this combination was 0.375, confirming synergism. In vivo tests suggested the statistically significant protection of infected mice with this combination. Lc exhibited synergism when combined with non-antibiotics methdilazine and triflupromazine both in vitro and in vivo. Distinct antimicrobial action of Lc and its subsequent synergism with other drugs can open up the possibility of synthesising new molecules by the structural analyses of these compounds.

Introduction

It is now known that the antibiotics and antibacterial chemotherapeutics that had been active against a very wide range of bacterial pathogens have gradually lost the battle, as mutants resistant to such agents have quickly developed throughout the world. Significant antimicrobial action has been detected in a large variety of pharmacologically distinct classes of compounds, which are grouped together as ‘non-antibiotics’ [1–5]. This has now opened up a new avenue for fighting the ever-increasing problem of drug-resistance. These non-antibiotics possess most of the characteristics of antibiotics and their antimicrobial function can often be further potentiated by suitable combinations resulting in synergism [1, 6–9]. The present study describes the augmentation of the antimicrobial function of the recently described non-antibiotic, the cardiovascular drug lacidipine (Lc) [10], by combining it with standard antibiotics and highly powerful non-antibiotics.

Materials and methods

Bacteria The strains and their sources are described in Table 1.

### References

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### Table 1 Determination of the minimum inhibitory concentration (MIC) of antibiotics, lacidipine (Lc) and other non-antibiotics

<table>
<thead>
<tr>
<th>Bacteria</th>
<th><strong>MIC of antibiotics and other non-antibiotics (µg/ml)</strong></th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pe</td>
<td>Am</td>
</tr>
<tr>
<td>S. aureus NCTC 6571</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>S. aureus NCTC 8530</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>B. pumilus NCTC 8241</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>E. coli ATCC 25922</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>S. typhimurium NCTC 74</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sh. dysenteriae 3NCTC 102</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sh. boydii 8 NCTC 254</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sh. flexneri 4s NCTC 515</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>V. cholerae ATCC 14033</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>V. cholerae ATCC 14035</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

**Drugs** Penicillin (Pe), ampicillin (Am), streptomycin (Sm), gentamicin (Gm), erythromycin (Er), tetracycline (Tc), chloramphenicol (Cm), ciprofloxacin (Cf), clindamycin (Cm), metilizline (Md), diclofenac (Dc) and triflupromazine (Tf) were obtained as pure dry powders from manufacturers in India and preserved at 4°C.

**Media** Liquid media were nutrient broth (NB; Oxoid), peptone water (PW) containing 1.0% peptone (Oxoid) and 0.5% NaCl and Mueller Hinton Broth (MHB; Oxoid). Solid media were nutrient agar (NA; Oxoid), Mueller Hinton Agar (MHA; Oxoid) and peptone agar (PA) prepared by solidifying PW with 1.0% agar (Oxoid No. 3), pH 7.2–7.4.

**Inoculum** Bacteria were grown overnight in PA/NA/MHA at 37°C, harvested during stationary phase. Suspended in 5 mL of sterile distilled water and the turbidity of each was adjusted to match against a 0.5 McFarland standard [11] with a spectrophotometer at 625 nm corresponding to 2.4 x 10^8 colony-forming units (cfu)/mL.

**Determination of the MIC of antibiotics and non-antibiotics** This was carried out according to the Clinical and Laboratory Standards Institute (CLSI) [11] guidelines by spotting 10^6 cfu with a 2-mm loop full of 1/10 dilution of 18-h broth cultures on plates containing 0 (control), 2, 5, 10, 25, 50, 100, 200 and 400 µg/mL of a drug, observed after 24 h, and up to 72 h.

**Tests for in vitro synergism** The combined effects of Lc and another drug were determined by the disc diffusion technique with sterile filter paper discs (7.25 mm, Whatman No. 1) containing 5 µg of an antibiotic and 200 µg of Lc [7–9]. Sensitive bacteria were grown in liquid media for 18 h flooded on solid media in triplicate; the plates were dried at 37°C. Drug-impregnated discs were placed on agar and incubated at 37°C, and the zones of inhibition were measured. Depending on the results, discs containing Lc and another agent were placed on prepared plates in such a way that their inhibitory circles would touch each other tangentially. The diameters of inhibition zones due to individual and mutual effects on the same plate were recorded. The increase in surface area (πr^2) due to a combination of effects was statistically evaluated by the x^2 test for its significance [8, 9].

**Checkerboard procedure** This was performed in microtitre trays with MHB [12]. Pe was tested at 0.312 to 10.0 µg/mL and Lc was used at concentrations of 1.56 to 50 µg/mL. The checkerboard was arranged as follows. In the first row, all of the wells contained 50 µg of Lc and either 0, 0.312, 0.625, 1.25, 2.5, 5.0 or 10 µg of Pe in a final volume of 1 mL of MHB. In the second row, the wells contained 25 µg of Lc and increasing amounts of Pe, as above. Similar patterns were followed for all of the rows. In the last row, the wells had varying amount of Pe only. For synergism between Lc and Tt, the concentrations of both drugs were 10, 20, 40, 80, 160, 320, 640 and 1,280 µg. An inoculum of 0.5 McFarland’s standard was applied using a multipoint inoculator, incubated aerobically at 37°C for 24 h. The presence or absence of growth was noted visually. The fractional inhibitory concentration (FIC) index [12] was calculated as follows: MIC of Pe tested in combination/MIC of Pe tested alone + MIC of Lc tested in combination/MIC of Lc tested alone. The interaction was interpreted as synergistic when the FIC index was <1.
In vivo experiments

Swiss albino male mice maintained in our animal house and
many other drugs were used. The median lethal dose
(MLD or LD₅₀) of the mouse-passaged strain was as
described earlier [10]. Drugs were given intraperitoneally,
with the doses being calculated from the available pharma-
cological data [10, 13]. To determine the synergistic effects
of Lc+Pc, 20 mice were divided into four batches with five
mice in each. The first batch received Lc (µg/mouse), the
second batch had 60 µg of Pc, while the third batch was
given Lc (30 µg) plus Pc (60 µg) and the remaining batch
received only saline. After 3 h, all of the animals were
challenged with 1.85 x 10⁵ cfu of S. typhimurium NCTC 74
[10]. The animals were autopsied 18 h after the challenge.
Their liver(s) and spleen(s) were collected individually and
homogenised for the determination of cfu count; from the
same animals, 0.2 to 0.4 mL of heart blood were collected
aseptically, allowed to clot and analysed for the size of
bacteraemia and the amount of drug in the sera. Drug
concentrations in mice were determined at 0 h with the help
of inhibition zones produced by serum-soaked filter paper
discs (7.2 mm, 3 mm thick, Millipore, absorbing about
0.03 mL volume) on a culture lawn grown on PA seeded
with 10⁵ cfu of S. typhimurium NCTC 74 [7–9]. Drug
concentrations were deduced by reference to a standard
curve calibrated with known concentrations of each drug
[8, 9].

Results

MIC of antibiotics and non-antibiotics Table 1 describes
the inhibitory spectra of ten bacteria with respect to nine
antibiotics and four non-antibiotics. Comparatively, the
three Gram-positive strains were more sensitive to
the agents than the Gram-negative strains. The MIC of Pc, Am,
Sm, Gm, Te, Cm and Cr with respect to all of the test
bacteria ranged from 2 to 25 µg/mL, while the MIC of Er
was between 2 and 100 µg/mL. The MIC of the non-
antibiotics Md, Tf, De and Lc mostly ranged between 25
and 200 µg/mL; however, both of the S. aureus strains were
more sensitive to Md, Tf and De than the other bacteria.

Effects of combinations of antibiotics, non-antibiotics and Lc
in vivo In the disc diffusion assay tests between Lc and
antibiotics, varying degrees of synergism was revealed
between the Lc + Pc, Lc + Am, Lc + Sm, Lc + Gm, Lc +
Er, Lc + Tf, Lc + Md and Lc + Tf combinations.
Indifference was observed with Lc + Cm and Lc + De
combinations, while distinct antagonism was detected when
combination tests were performed with Lc and Te.

An elaborate study on the synergism between Lc
and Pc with respect to ten bacteria is presented in
Tables 2 and 3. When the drug discs were placed
individually on the culture lawn of S. aureus NCTC
6571, the diameter of the zone of inhibition due to Pc was
28.6 mm and the same due to Lc was 21.2 mm. These
increased to 31.4 mm and 23.5 mm, respectively, when the
discs were placed for determining the effect of combina-
tion between Pc and Lc. The increase in surface area due
to combination was 20.53% for Pc and 20.28% for Lc.
Similarly, S. typhimurium NCTC 74 singly produced an
inhibition zone of 20.0 mm due to Pc and 14.2 mm against
Lc discs, which enlarged to 21.8 mm and 15.5 mm,
respectively, in the combined test. Further studies with
combinations in all of the other test bacteria confirmed
synergism between Pc and Lc (Tables 2 and 3). This
synergistic effect obtained between Pc and Lc in terms of
percentage increase in the size of their inhibition zones
was assessed for their level of significance for both drugs.
The activity of Pc was between 5.90 and 115.11% higher
compared to their individual effects; similarly, that of Lc
varied from 8.55 to 44.38% in the tests for combination
assay vis-a-vis their individual inhibitory effect. The
percentage increase in surface area was found to be
statistically significant with respect to all of the ten test
organisms.

Checkerboard experiment for the determination of the FIC
index The MIC of Lc with respect to S. aureus NCTC
6571 in MHB was 25.0 µg/mL, while that of Pc was
2.5 µg/mL. In combination, the MIC values became 12.5
and 1.25 µg/mL for Lc and Pc, respectively. The data
presented above for the determination of the synergistic
antibacterial effect indicated that the combination pro-
duced a significant synergism of the pair Lc + Pc, as
the FIC index was calculated to be 0.375. In the case of
the Lc + Tf combination, it was found that the MIC
of Lc with respect to S. typhimurium NCTC 74 was
320 µg/mL, while that of Tf was 160 µg/mL. In
test combination, both of the values became 20 µg/mL and
the FIC index was calculated to be 0.18. Thus, synergism
was evident in the data presented here for the combination
of Lc and Tf.

Synergism in vivo The 50 x LD₅₀ dose of the virulent S.
typhimurium NCTC 74 [10] was 1.85 x 10⁵. The animal
experiments with blood and organ homogenates from
normal mice belonging to the same stock yielded no
salmonellae. The combination of Lc and Pc significantly
reduced the cfu/mL count of S. typhimurium in the organ
homogenates of mice, as determined 18 h after the
challenge; a similar significant reduction was also observed
in the in vivo tests with the Lc and Tf combination.
Table 2 Synergy between PC and LC by disc diffusion tests

<table>
<thead>
<tr>
<th>Bacterial strains tested</th>
<th>Diameter of the inhibition zone (mm)</th>
<th>Combined (B) drug effect</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single (A) drug effect</td>
<td>Combined (B) drug effect</td>
<td>% increase</td>
</tr>
<tr>
<td></td>
<td>Pc</td>
<td>Lc</td>
<td>Pc</td>
</tr>
<tr>
<td>S. aureus NCTC 6571</td>
<td>28.6</td>
<td>21.2</td>
<td>31.4</td>
</tr>
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<td>S. aureus NCTC 8530</td>
<td>31.2</td>
<td>20.5</td>
<td>34.3</td>
</tr>
<tr>
<td>B. pumilus NCTC 8241</td>
<td>18.6</td>
<td>21.6</td>
<td>20.4</td>
</tr>
<tr>
<td>E. coli ATCC 25922</td>
<td>17.2</td>
<td>16.0</td>
<td>17.7</td>
</tr>
<tr>
<td>S. typhimurium NCTC 74</td>
<td>20.0</td>
<td>14.2</td>
<td>21.8</td>
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<tr>
<td>Sh. dysenteriae 3 NCTC 102</td>
<td>20.2</td>
<td>20.0</td>
<td>21.0</td>
</tr>
<tr>
<td>Sh. boydii 8 NCTC 254</td>
<td>14.2</td>
<td>24.8</td>
<td>17.4</td>
</tr>
<tr>
<td>Sh. flexneri 4a NCTC 515</td>
<td>15.0</td>
<td>21.1</td>
<td>22.0</td>
</tr>
<tr>
<td>V. cholerae ATCC 14033</td>
<td>19.8</td>
<td>19.1</td>
<td>21.0</td>
</tr>
<tr>
<td>V. cholerae NCTC 14035</td>
<td>20.2</td>
<td>19.3</td>
<td>21.5</td>
</tr>
</tbody>
</table>

PC = penicillin (5 μg/disc); LC = lacticipine (200 μg/disc).

The mean surface area of the inhibition zone (mm²) was calculated as πr² on the basis of the mean diameter (2r), and percentage increase was calculated as [(B - A)/A]x100, where A = surface area due to the individual effect and B = surface area due to the combined effect. The zones of inhibition formed in combination with respect to PC and LC were larger in size than those formed singly against the same compounds. These were calculated statistically by determining Student's t-test based on the values of standard deviation and standard error obtained, which showed the differences to be highly significant (P<0.01) with respect to all of the test bacteria.

Discussion

Antimicrobial action of the cardiovascular drug LC has already been previously reported [10] and now we are able to demonstrate clearly the enhancement and promotion of its activity when combined with a suitable antibiotic like PC or a non-antibiotic like TF. Quantitative estimation using the percentage increase in the surface area of inhibition zones produced in combined tests compared with those produced by the individual zones clearly indicated a significant augmentation of action. This in vitro estimation was found to be statistically significant. Checkerboard titration further provided synergistic action between LC + PC and LC + TF combinations. Animal experiments additionally proved synergism between these drugs. It may be pointed out that the amount of LC necessary to inhibit bacteria in vitro is considerably larger than that required by PC, but in vivo tests, it is evident that the protective dose of LC is distinctly smaller compared to PC. Since both of these drugs have been used in humans satisfactorily for many years in clinical medicine for different purposes, their rather low toxicity and large safety margins observed during human application appear to be encouraging [13].

Extensive studies on non-antibiotics [1–5] have revealed that various phenothiazines possessing tricyclic benzene rings are often highly powerful antimicrobics. However, pharmacologically distinct chemical compounds having two benzene rings can also be potential antimicrobics [5, 8, 9]. Definite antimicrobial action in LC and its subsequent synergism with antibiotics indicate that, like sulphonamides, nalidixic acid, nitrofurans and other antibacterial chemotherapeutics, this non-antibiotic may exhibit a broad spectrum of activity independently and also in suitable combinations. It is noted that synergy between LC and PC against a test organism is a unique phenomenon. Although the exact mechanism of action of most non-antibiotic drugs have not yet been fully analysed, there is a possibility of multiple factors interfering with cellular biosynthesis [13]. The same factors could reduce the MIC of two drugs in a combination to even below their break-point concentrations, thereby, making the pair synergistic. In the combination test for synergism by the FIC index, it was evident that...

Table 3 Log difference of the reduction in cfu/ml in Salmonella typhimurium NCTC 74 in the organ homogenates of mice treated with LC, PC and their combination with respect to controls

<table>
<thead>
<tr>
<th>Group (n=5) Drug/mouse</th>
<th>Log difference of cfu/ml in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart blood</td>
</tr>
<tr>
<td>1</td>
<td>LC (30 μg)</td>
</tr>
<tr>
<td>2</td>
<td>PC (60 μg)</td>
</tr>
<tr>
<td>3</td>
<td>LC (30 μg) + PC (60 μg)</td>
</tr>
</tbody>
</table>
the actual amount of each drug in the test pair was much lower than that required for the individual tests, implying that a suitable synergistic combination like that of Lc and Pe or Lc and Tf would allow a reduction in the doses of both drugs, thus, overcoming the problem of unrealistic break-point concentrations of these drugs for prolonged use. However, since the concentration of most non-antibiotics cannot be reduced to as low as existing antibiotics, a shorter duration of therapy may be achieved by administering their combinations, e.g. Lc + Md or Lc + Tf, thereby, reducing the hazards of toxic effects. The synergistic combinations as presented in this study, along with our earlier related work [1, 7–9], would, hopefully, open up a prospective path in the selection of antimicrobial therapeutic regimens to contribute to the continuing fight against multi-drug-resistant microorganisms.

References