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EXPERIMENTALLY POTENT SYNERGISM RESULTING FROM COMBINATION OF THE ANTIBIOTIC STREPTOMYCIN WITH THE NON-ANTIBIOTIC DICYCLOMINE HYDROCHLORIDE

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ABSTRACT
The anticholinergic agent dicyclomine hydrochloride (Dc) had been found to possess highly significant antimicrobial action against a large variety of Gram positive and Gram negative bacteria. Simultaneously the drug Dc also revealed statistically significant protective capacity in mice challenged with a mouse virulent strain of salmonella. In this study Dc has been combined individually with several commonly known antibiotics to determine if any of the combination could result in synergism. The antibiotics were penicillin, ampicillin, chloramphenicol, tetracycline, streptomycin, gentamicin and ciprofloxacin. A total of 12 bacteria belonging to 9 different genera and fairly sensitive to Dc were found to be sensitive to most of the antibiotics only except erythromycin to which two microorganisms were resistant. Disc diffusion assay produced synergism of Dc with streptomycin, gentamicin, penicillin, ampicillin and erythromycin. Subsequently synergism between Dc and streptomycin was confirmed by determining the fractional inhibitory concentration (FIC) index with the help of checkerboard technique in microtitre trays. The FIC index was found to be 0.375 confirming synergism. Finally the protective capacity of the combination was evaluated in a mouse model in which the challenge bacterium was *Salmonella enterica* serovar Typhimurium NCTC 74. The combination of Dc and streptomycin exhibited statistically significant ($p < 0.001$) mouse protection and also resulted in reduction of invasive bacterial cells in internal organs. Such an observation may hopefully be developed as an alternative therapy for severe bacterial infections.

KEYWORDS: Non-antibiotic, Synergism, Dicyclomine, FIC index, Streptomycin.

INTRODUCTION
Broad spectrum antibiotics had been the most essential weapons in the fight against microbial infections. However, the therapeutic use of antibiotics is becoming restricted not only due to the ever increasing problems of microbial drug resistance but also due to the high levels of toxicity present among many antibiotics. This ever growing problem of multidrug resistance among microorganisms including mycobacteria has been the focus of a large number of studies.[1-3] The frequent occurrence of penicillin resistance in pneumococci, vancomiycin resistance in enterococci, extended spectrum β-lactameses among Gram negative bacteria and quinupristin dalfopristin resistant strains of *Staphylococcus aureus* have created a panic among clinicians.[2,4,5] Although a fairly large number of compounds with potential antimicrobial function are being claimed from time to time their practical usage gets obstructed probably due to acquisition of drug resistant plasmids or mutations in the pathogens.[1] This escalation in the levels of antibiotic resistance had initiated search for newer antibacterial compounds from the known pharmacological drugs. Consequently a large number of various categories of pharmacologically diverse compounds had been repeatedly reported from different parts of the world to possess moderate to highly powerful antimicrobial activity, termed as "Non-antibiotics",[6-24] many of which were found to be acting synergistically with known antibiotics and other non-antibiotics resulting in increase in sensitivity.[11, 25-30] The

present study describes the attempt to determine the synergistic action of already known non-antibiotic anticholinergic drug dicyclomine hydrochloride (Dc)[31] with common antibiotics.

MATERIALS AND METHODS

Drugs

The non-antibiotic dicyclomine hydrochloride (Dc) was obtained in pure dry form from Cadilla Healthcare Limited, India. Its chemical formula is $C_{19}H_{35}NO_2$.HCl. The antibiotics penicillin (Pc), ampicillin (Am), chloramphenicol (Cm), tetracycline (Tc), streptomycin (Sm), gentamicin (Gm), erythromycin (Er) and ciprofloxacin (Cf) were purchased from Sigma Chemicals, USA. All these were stored at 4^0C in dry powder form and freshly prepared in sterile distilled water before use.

Bacteria

A total of 12 bacterial strains belonging to 3 Gram positive and 9 Gram negative types were included in this study. These were identified as described by Collee et al[32] and preserved in freeze-dried ampoules.

Media

The liquid media were peptone water (PW) containing 1.0% peptone (Oxoid) plus 0.5% Analar NaCl, nutrient broth (NB, Oxoid) and Mueller-Hinton broth (MHB, Oxoid). The solid media prepared for this study were peptone agar (PA), prepared by solidifying PW with 1.0% agar (Oxoid No.3), nutrient agar (NA, Oxoid) Mueller-Hinton agar (MHA, Oxoid), pH 7.2-7.4 . The media PW and PA were used for synergism of Gram negative organisms since such media provided larger zones of inhibition.

Inoculum

The test bacteria were grown at 37^0C in PW/NB/MHB for 18 hr and harvested during their stationary phase of growth. Each bacterium was then suspended in 5 ml of sterile distilled water. The turbidity of the organisms was then adjusted to match against 0.5 MacFarland standard[33] with the help of a spectrophotometer (Chemito UV 2600 Double Beam UV-Vis spectrophotometer) at 625 nm, corresponding to $2.4x10^8$ colony forming unit (CFU) /ml. Each culture was then further diluted 1:100. Such a suspension was the final inoculum.

Determination of the minimum inhibitory concentration (MIC) of antibiotics and Dc

The MIC was determined with the help of agar dilution technique following the guidelines of the Clinical Laboratory Standards Institute (CLSI)[34] by spot inoculating 10^5 CFU with a 2 mm loop –full of 1/10 dilution of 24 h NB / MHB cultures on NA/MHA plates containing the antibiotics or Dc. The amounts of each drug were 0 (control), 2, 5, 10, 25, 50, 100, 200 and 400 µg / ml. The plates were incubated at 37^0C for 24 h. The MIC was determined as the concentration of an agent that failed to produce any visible growth.

In vitro synergism between Dc and the antibiotics

The method described by CLSI[35] was followed. The combined effects of Dc with an antibiotic were performed by disc diffusion test. For this purpose sterile filter paper discs (Whatman No. 1, 7.25 mm diameter) containing 5 µg of an antibiotic or 200 µg of Dc were prepared following the method of Miles and Amyes.[36] Gram positive bacteria were grown in NB/MHB and Gram negative bacteria were grown in PW/MHB overnight, flooded on NA/MHA or PA/MHA in triplicates, dried at 37^0C for 1 hr. Initially individual effects of Dc or an antibiotic were determined by measuring the zones of inhibition.[25-29] The data obtained were used for determining their combined effects. The drug discs were placed on prepared plates in such a manner that the inhibitory circles would touch each other tangentially. The mutual influence encountered were first recorded, when both the zones remained unaffected, the combination was assessed as indifference, while when the two zones receded from each other the effect was considered as antagonism, whereas in case of synergism the two zones merged to form a continuous enlarged zone. Finally, the individual and combined effects of Dc with an antibiotic were performed and determined in the same plate. The increase in surface area (πr^2) due to the combination of two agents was statistically evaluated with the help of Chi-square analysis to obtain the level of significance.

Checkerboard Experiment

Synergism between two agents could be further confirmed by the checkerboard procedure[37] in microtitre trays using MHB. The checkerboard was arranged in the following manner, in the first row all the wells contained 200 µg of Dc and either of 0, 0.15, 0.3, 0.6, 1.25, 2.5 and 5.0 µg of Sm in a final volume of 1 ml of MHB. Similarly, in the second row all the wells contained 100 µg of Dc and either 0, 0.15, 0.3, 0.6, 1.25, 2.5 and 5.0 µg Sm in a final volume of 1 ml of MHB. Subsequently the following rows were given lower doses of Dc keeping the other conditions same as given above. The concentrations of Dc were 0, 6.25, 12.5, 25, 50, 100 and 200 µg (using two fold dilutions). In the last row all the wells were given increasing amounts of Dc only. Each tray was prepared with 96-channel dispenser and stored at -20^0C until use. In each well, an inoculum of 0.5 McFarland's standard[33] was applied using multipoint inoculators. This tray was kept overnight at 37^0C. For each run a standard quality control strain (*S.aureus* NCTC 6571) was included. Presence or absence of growth was noted by visual observation. Synergistic action between Dc and an antibiotic was calculated by determining the fractional inhibitory concentration (FIC) index.[37]

FIC index = (MIC of Dc in combination/MIC of Dc alone) + (MIC of antibiotic in combination/MIC of

antibiotic alone). Results of synergy were recorded following the guidelines described by American Society for Microbiology.[38] In this FIC index is interpreted as follows: synergy = < 0.5; partial synergy = 0.5-0.75; additive = 0.76 – 1.0; indifference = 1.0-4.0 and antagonism = >4.0. Finally Chi-square analysis was performed to find out different rates of synergy between the drugs.

***In vivo* experiments**

This was performed in the Swiss strain of white male mice, each weighing 18-20 gm, 3 weeks old (abiding by ethical guidelines).[39] The animals were kept under conditions of temperature (21 ± 1^0C) and relative humidity (50-60%) in a photoperiod of 14:10 h of light: darkness. The animals were provided with water and a dry pellet *ad libitum*.The test bacterium was the known natural mouse virulent orga*nism Salmonella enterica* serovar Typhimurium NCTC 74. The virulence of the organism was augmented through repeated mouse passages and then the median lethal dose (MLD or lethal dose [LD_{50}] was determined by challenging batches of mice with graded doses of the culture and recording mortality up to 100 h.[40] Reproducibility of the challenge dose was made assured by standardising its optical density in the UV-VIS Spectrophotometer (Thermo Scientific) at 640nm to obtain the desired CFU on NA/MHB. The dosages of the drugs were calculated on the basis of available pharmacological data and injected intraperitoneally. The amount of Dc to be administered to an animal was calculated as given below; human effective dose=animal effective dose (mg/kg) x 3/7. Based on this calculation the amount of Dc for a mouse weighing 20g was 30 µg.[41] The antibiotic Sm and Dc were injected intraperitoneally in the dosages based on our earlier studies. Each drug was injected intraperitoneally 3hrs before the challenge as 0.1 ml solution containing 30 µg of Dc or 60 µg of Sm. For determining the synergistic action of Dc + Sm, 20 animals were divided into four batches with five mice in each. The first batch was given 30µg of Dc, the second batch received 60µg of Sm per animal, the third batch had both the drugs while the last batch was given only saline. After 3h all the animals were challenged with the test pathogen. After 18h all the animals were killed, their livers and spleens were removed under aseptic condition homogenized individually and preserved at -20^0C, from all the organs the number of bacteria (CFU) were determined. About 0.2 – 0.4ml of blood was collected from the above animals, allowed to clot and the size of bacteraemia and amount of each drug per millitre of serum were calculated by the use of CFU counts. The amounts of each drug were analysed at 0h by measuring the zones of inhibition produced by serum – soaked filter paper disc (7.2mm, 3 mm thick, Millipore, absorbing about 0.03ml volume) over a culture lawn of PA/MHA seeded with 10^5 CFU of the challenge bacterium. Actual amounts of Dc and Sm were deduced with the help of the reference to a standard curve calibrated with known amounts of each drug.[28-29] It may be mentioned here that blood and organ homogenates obtained from normal mice in the same stock did not reveal the presence of any salmonella.

RESULTS

MIC of Dc and different antibiotics in the test bacteria

The inhibitory spectra of antibiotics and Dc are being presented in Table 1. Most of the test bacteria were sensitive to the antibiotics. However *E.coli* ATCC 25922 was resistant to Er and less sensitive to Cf, while *S.enterica* NCTC 74 was resistant to only Er. The levels of MIC of Dc in these test organisms varied from 5 to 25 µg/ml proving thereby the proficiency of Dc as a highly active non- antibiotic.

Table 1. Determination of the MIC of Various Antibiotics and the Chemotherapeutic Agent Dicylomine.

Bacterial Strains	MICs of various antibiotics and Dicyclomine in µg/ml								
	Pc	Am	Cf	Cm	Er	Tc	Sm	Gm	Dc
B. licheniformis NCTC 10341	2	2	2	2	2	2	2	2	25
B. pumilus NCTC 8241	2	2	2	2	2	2	2	2	10
S. aureus NCTC 6571	2	2	2	10	2	2	2	2	5
S. aureus NCTC 8530	2	2	2	25	2	2	2	2	5
S. aureus NCTC 8532	2	2	2	5	2	5	5	2	10
S. aureus ML123	5	5	2	10	5	5	5	5	10
E. coli ATCC 25922	5	5	50	2	150	5	5	5	25
S. enterica serovar Typhimurium NCTC 74	2	5	2	2	100	2	2	2	25
Sh. dysenteriae 7 NCTC 519/66	2	2	2	5	5	2	2	2	10
V. cholerae 865	2	2	2	2	2	2	2	2	10
V. cholerae ATCC 14033	2	2	2	2	2	2	2	2	25
V. cholerae ATCC 14035	2	2	2	2	2	2	2	2	25

Pc, penicillin; Am, ampicillin; Cf, ciprofloaxacin; Cm, chloramphenicol; Er, erythromycin; Tc, tetracycline; Sm, streptomycin; Gm, gentamicin; Dc, dicyclomine

Effects of combination of Dc with antibiotic

With the disc diffusion assay procedure Dc was found to exhibit clear cut synergism when it was combined with Sm, Gm, Pc, Am and Er. The drug Dc showed indifference with Cf and Cm while the combination of Dc and Tc produced antagonism.

Synergistic action between Dc and Sm

In the study on determination of synergism between Sm and Dc when the drug discs were placed individually on the culture lawn of *B.licheniformis* MCTC 10341 the zone of inhibition due to Sm was 17.6mm and that due to Dc was 13.8 mm [Table 2]; the diameters increased to 18.2 mm against Sm and to 14.6 against Dc when the drug discs were placed on the culture lawn for the combination. The increase in surface area due to the combination was found to be 6.93 for Sm and 11.93 for Dc [Fig 1; Table 2].

The same Sm and Dc discs when placed separately on the culture lawn of *S.enterica* NCTC 74, the inhibition zone due to Sm was 18.8 mm and that due to Dc was 16.2 mm; these zones increased to 19.2 mm and 16.9 mm respectively when the discs were placed for elucidation of their combined effect. The increase in surface area due to the combination was found to be 4.29% and 8.82% respectively. These two agents produced definite synergism in combination against all the other test organisms [Table 2]. The percentage increase on the basis of πr^2 with respect to all the Gram positive and Gram negative organisms was statistically significant.

Table 2: Synergism between Sm and Dc by disc diffusion test

Bacterial strains	Diameter of the inhibitory zone in millimetre					
	Single drug effect (A)		Combined drug effect (B)		Percentage Increase on the basis of πr^2	
	Sm	Dc	Sm	Dc	Sm	Dc
B. licheniformis NCTC 10341	17.6	13.8	18.2	14.6	6.93	11.93
B. pumilus NCTC 8241	18.0	13.6	18.7	14.2	7.92	9.01
S. aureus NCTC 8530	17.9	14.8	18.7	15.4	9.13	8.27
S. aureus NCTC 8532	18.4	13.8	19.3	14.7	10.02	13.47
S. enterica serovar Typhimurium NCTC 74	18.8	16.2	19.2	16.9	4.29	8.82
Sh. dysenteriae 7 NCTC 519/66	18.6	13.2	19.4	15.0	8.78	29.13
V. cholerae ATCC 14033	18.2	17.0	18.9	18.3	7.83	15.87
V. cholerae ATCC 14035	18.4	17.2	19.2	17.8	8.79	7.09

Sm, streptomycin (5 µg/disc); Dc, dicyclomine (200 µg/disc). Mean surface area of inhibition zone (mm^2) was calculated as πr^2 on the basis of the mean diameter (2r) and the percentage increase was calculated as (B-A)/AX100, where A=surface area due to individual effect, B=surface area due to combined effect; this was highly significant ($p<0.01$). The zones of inhibition formed, when combined with respect to Sm and Dc, 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 the test bacteria.

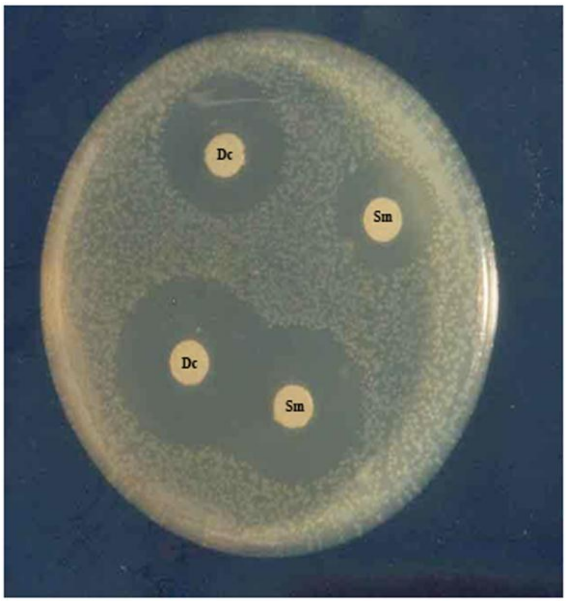

Fig. 1 Effect of streptomycin (Sm; 5µg) and dicyclomine (Dc; 200 µg) discs on *S.aureus* NCTC 8530 individually (top) and combined (below)

Determination of FIC index by checkerboard technique

The MIC of Sm with respect to *S.aureus* 8530 in MHB was 5.0 µg / ml and that of Dc was 50 µg/ml. In combination with Dc the MIC value of Sm became 1.25 µg/ml and the MIC of Dc in combination became 6.25 µg/ml. The data given above when analysed for determining the synergistic action between Sm and Dc clearly indicated that the combination was highly statistically significant as the FIC index value was 0.375. These data were depicted as an isobologram, that yielded a concave curve [Fig 2] finally proving the definite synergism between these two test drugs.

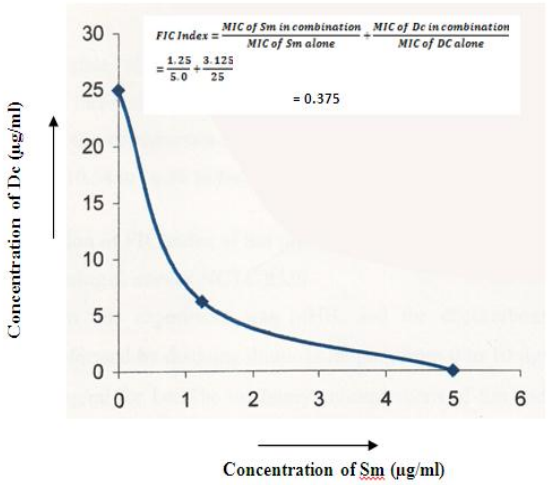

Fig 2. Isobologram plotted by determining the fractional inhibitory concentration (FIC) index of *S. aureus* 8530 using the checkerboard technique

Animal experimental evaluation of synergism between Sm and Dc

The 50 x LD_{50} dose of highly virulent *S.enterrica* NCTC 74 was 1.85 x 10^9 as described earlier.[28-29] In the control series the mice that received the challenge only the CFU counts remained between 1.8 x 10^7 and 5.4 x 10^9 in blood and the organ homogenates. Independently the animals that received either Dc or Sm the number of viable bacteria varied from 1.2 x 10^3 to7.3 x 10^4. However the animals that were given both the agents for protection there was a distinct reduction in the number of viable organisms in all the organs of the test mice [Table 3]. Statistical analysis of the data by Student's "*t*" test showed $p < 0.001$ in groups 1 and 2, while it was $p < 0.0001$ in group 3 versus the control, which finally confirmed significant synergism.

Table 3: Log difference of the reduction in CFU/ml of *S. enterica* NCTC 74 in blood, liver and spleen homogenates of mice treated with Dc, Sm and their combination with respect to the control

Group (n=5)	Drug/mouse	Log transformed values of CFU counts		
		Heart blood	Liver	Spleen
1	Dc (30µg)	1.7 x 10^3 – 4.5 x 10^4	2.3 x 10^3 – 7.3 x 10^4	3.5 x 10^3 – 4.0 x 10^4
2	Sm (60µg)	2.1 x 10^3 – 3.1 x 10^4	1.2 x 10^3 – 6.5 x 10^4	1.3 x 10^3 – 2.5 x 10^4
3	Dc (30µg) + Sm (60µg)	5.6 x 10^2 – 2.4 x 10^3	9.1 x 10^1 – 3.5 x 10^3	7.2 x 10^2 – 1.0 x 10^4
4	Control (0.1ml saline)	6.8 x 10^7 – 1.3 x 10^9	2.7 x 10^7 – 8.0 x 10^8	1.8 x 10^7 – 5.4 x 10^9

Dc, dicyclomine; Sm, streptomycin; CFU counts were significant, p<0.001, in Groups 1 and 2, and p<0.0001 in Groups 3 vs control(Group4).

DISCUSSION

There had been a steady rise in the incidence of bacterial infections not only among hospitalised patients but also among patients in the general population. However, for any patient the first criteria of treatment start with isolation and identification of the causative organism followed by determination of its antibiogramme pattern. Therapy begins with the application of the antibiotic that shows maximum sensitivity. In case of an acute infection by a known virulent bacterium treatment often begins with a simultaneous administration of two antibiotics that had revealed distinctive action against the invading pathogen. In this way combination therapy is now a common feature among clinicians. Combination therapy can be highly successful when the selected antibiotics are synergistic. In this study a search was made to find out the antibiotics that may be synergistic to the non-antibiotic Dc whose antimicrobial potentiality has already been demonstrated.[31] The drug Dc although proved to be synergistic with Sm, Gm, Pc, Am and Er in *in vitro* studies, but most promising action was noted when Dc was allowed to interact with Sm. The synergism between these two drugs was even more convincing in the *in vivo* system. It may be pointed out here that the amount of Dc required for protection in mouse mortality test was less than that of Sm [Table 3]. Both these drugs are in routine use for various infections among all populations throughout the world and their entire toxicity profiles and safety margins are well documented.[42]

Several studies by different researchers have repeatedly revealed that phenothiazines possessing tricyclic benzene rings are often highly active antibacterial agents.[7-23] However, several different pharmacological compounds possessing two benzene rings also showed antimicrobial action both *in vitro* and *in vivo*.[25-29] The action of these non-antibiotics may well be compared with that of the chemotherapeutics sulphonamides, nalidixic acid and nitrofurantoin. Many of these non-antibiotics produced synergistic action when they were combined with antibiotics[11, 19, 25-30] and their positive interaction could be proved both *in vitro* and *in vivo*. It may be pointed out further that the degree of synergism for Dc plus Sm among the test organism was comparable.

The mechanism by which the non-antibiotic organisms has not been ascertained as yet. However, several studies have repeatedly shown that phenothiazines possessing tricyclic benzene rings are capable of inhibiting the action of efflux pump in multi-drug resistant bacteria.[43-48] The test drug Dc being structurally similar to phenothiazines may possess a similar mechanism of action against bacteria. Furthermore the combined action of Dc plus an antibiotic may have a definite role on the intrinsic efflux pump activity resulting in the reduction of MICs of both the two drugs, probably below the

break-point concentration, resulting in synergism between the two. In the study for determination of synergism between Dc and Sm by FIC index it was observed that the actual amount of each drug in the test pair was always lower than that required for individual test. Thus this study shows that in a suitable combination of a non antibiotic with an antibiotic producing synergism actual amount of each agent may be much reduced to produce visible action. The *in vivo* experiments were performed in Swiss albino mice with intraperitonial infection by the known mouse virulent pathogen *S. enterica*. Although viable cells of *Salmonella* are quickly phagocytosed by neutrophils as soon as they come to inside the peritoneum they may not be killed immediately after.[49] The low pH of phagolysozome can activate the two- component regulon Pmr A and Pmr B resulting in further activation of nine genes responsible for synthesis and insertion of LipidA into the newly formed lipopolysaccharide larger in size than that of the bacterium; at this stage salmonellae become totally resistant to any adverse condition.[49] Due to such sequential events salmonella can invade beyond the epithelium of the colon into the mucosa which is a common situation in case of colon resection in a hospital. In such cases therapy becomes genuinely difficult. However, that the combination therapy wth Dc plus Sm became highly effective in the infected mice may be due the fact that antibiotics (in this case Sm) accumulate in the phagolysosome of macrophages and active against invading bacteria while a non antibiotic (like Dc) accumulate within the lysosomes of macrophages.[50-51] As the experimental animals receiving Dc and Sm, can accumulate such drugs in the lysosome, these can efficiently inhibit the two component regulon response of phagocytosed cells of salmonella. Although it may not be possible to clearly prove the exact mechanism of killing of salmonellae by the test drugs, the results fully prove beyond doubt the efficacy of simultaneous introduction of Sm and Dc as a highly potent antimicrobial combination.

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