Evaluation of Synergism between the Aminoglycoside Antibiotic Streptomycin and the Cardiovascular Agent Amlodipine

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Amlodipine, a cardiovascular drug, exhibited remarkable antibacterial action *in vitro* against 504 bacterial strains belonging to both Gram positive and Gram negative genera, as well as *in vivo* against a mouse-virulent bacterium. Based on such findings, the present study was undertaken to determine whether the efficacy of this non-antibiotic drug could be enhanced in the presence of any antibiotic. Twelve bacterial strains, sensitive to amlodipine as well as to 6 antibiotics, *viz.*, benzyl penicillin, streptomycin, chloramphenicol, tetracycline, erythromycin and ciprofloxacin were chosen. Disc diffusion test with amlodipine and streptomycin revealed marked synergism between the combination, compared with their individual effects. The synergism was found to be statistically significant ($p<0.01$). To assess the degree of synergy, the checkerboard analysis was performed. The fractional inhibitory concentration (FIC) index of this combination turned out to be 0.24, which confirmed synergism. This antibiotic–non-antibiotic pair was then administered to mice, challenged with *S. typhimurium* to determine whether this was effective *in vivo*. Statistical analysis of the mouse protection tests suggested that the combination was highly synergistic ($p<0.001$), according to Student's *t*-test. This synergistic drug combination may help us in enhancing the scope of prolonged antibiotic therapy in various types of infections, and might open a new therapeutic approach to combat drug resistance in bacterial diseases.

Key words synergism; streptomycin; amlodipine; aminoglycoside; antimicrobial activity; non-antibiotic

Currently, there are effective medicines to cure almost all major infectious diseases, but the increasing levels of drug resistance are threatening to erode the medical advances of recent decades. Eventually the drugs may be ineffective in the battle against life threatening diseases leading to increased risk in losing these valuable existing drugs. The drugs of various pharmacological classes such as antihistamines bromodiphenhydramine and diphenhydramine,[1] methdilazine,[2] promethazine,[3] trimeprazine,[4] the tranquilizer promazine,[5] antihypertensives methyl DOPA,[6] dobutamine,[7] oxyfedrine,[8] the antispasmodic dicyclomine,[9] the psychotropics chlorpromazine,[10,11] fluphenazine[12] and local anesthetics procaine[13] have exhibited powerful antibacterial action both *in vitro* and *in vivo*. The calcium channel blocker amlodipine (Am) possesses antihypertensive activity and recently this drug was found to possess significant antimicrobial action as well.[14] The bacteria could be arranged in the decreasing order of sensitivity towards amlodipine in the following manner: *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio parahemolyticus*, *Shigella* spp., *Bacillus* spp., *Salmonella* spp., whereas *Escherichia coli*, *Klebsiella* spp. and *Pseudomonas aeruginosa* were found to be resistant to the lower concentrations of the drug. The protection offered by the drug to mice challenged with a virulent bacterium was found to be statistically highly significant. These chemotherapeutics or non-antibiotic agents have further shown that their action could be augmented by making suitable combinations with other drugs.[15,16] The present study describes the synergism between Am and the aminoglycoside antibiotic streptomycin (Sm).

MATERIAL AND METHODS

Bacteria All the bacterial strains were identified as described by Collee *et al.*[17] and preserved in freeze-dried state.

Antimicrobials Amlodipine (Am) was received from Pfizer, streptomycin (Sm) from Sarabhai Chemicals, penicillin (Pc) was obtained from Wyeth-Lederle Limited, chloramphenicol (Cm) from Parke–Davis, tetracycline (Tc) from Indian Drugs and Pharmaceuticals Ltd. and erythromycin (Em) from Abbott Laboratories. All these compounds were obtained as pure dry powder and stored at 4 °C.

Media The liquid media used in the study were peptone water (PW) consisting of 1.0% bacteriological peptone (Oxoid) plus 0.5% NaCl (Analar), Nutrient broth (NB, Oxoid) and Mueller-Hinton broth (MHB, Oxoid). The solid media used for antimicrobial tests were peptone agar (PA), nutrient agar (NA) and Mueller-Hinton agar (MHA), all of which were solidified forms of PW, NB and MHB with the help of 1.5% agar (Oxoid No. 3). PW and PA were used for assay of Gram negative bacteria, while the other media were used for the remaining bacteria.

Bacterial Inoculum All organisms were grown overnight (24 h) on PA/NA/MHA at 37 °C and harvested during the stationary growth phase. A direct suspension of organisms was prepared in 5 ml sterile distilled water. The turbidity of the suspension was adjusted to match a 0.5 McFarland standard[18] with a spectrophotometer (Chemito UV 2600 Double Beam UV–Vis Spectrophotometer) at 625 nm, which corresponded to 2.4×10^8 CFU/ml. The suspension was further diluted 1 : 100 with sterile distilled water.

Minimum Inhibitory Concentration (MIC) of Antimicrobial Agents Agar dilution method was employed to determine the MIC of antimicrobial agents with respect to different test bacteria.[19]

***In Vitro* Synergism** Determination of the combined effects of Sm and Am by disc diffusion technique was based on the method described by National Committee for Clinical

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Laboratory Standards (NCCLS).[20] Sterile filter paper discs (7.25 mm, Whatman No. 1) containing 5 μg and 200 μg of Sm and Am, respectively, were prepared according to Cruickshank.[21] The sensitive bacteria were grown in liquid media for 18 h and used to flood on appropriate solid media in triplicate. The plates were then dried at 37 °C for 45 min. Individual inhibitory effects of Sm and Am were determined first, incubation was for 18 h at 37 °C, the zones of inhibition around each disc were measured in 3 different directions and mean diameters were recorded. For determination of combined effects of Sm and Am, the drug discs were placed on flooded agar plates in such a manner that the inhibitory circles would just touch each other tangentially. In this way, the diameters of inhibition zones produced due to individual and mutual effects on the same plate were recorded. The increase in surface area (πr^2), if any, due to a combination of effects was statistically evaluated by the χ^2 test for its level of significance. The occurrence of mutual influence/interference when the drugs were used in combination was assessed as follows: (i) indifference, when both the tangential circles of inhibition remained unaffected; (ii) antagonism, when the circles of inhibition receded and assumed kidney shape; (iii) synergism, in which there was enlargement of circles.

Checkerboard Procedure Synergy testing was performed for 12 strains by the checkerboard method in microtiter trays with MHB.[22] Streptomycin was tested at concentrations of 0.15 to 5.0 μg/ml, and amlodipine was tested at concentrations of 6.25 to 200 μg/ml. The trays were prepared with a 96-channel dispenser and stored at −70 °C until use. Sm was dispensed alone in the first row, and Am was dispensed in the first column. An inoculum of 0.5 McFarland standard was applied using multipoint inoculator. The trays were incubated aerobically overnight (standard quality control strains were included in each run).

End Point Determination The MIC was read as the lowest concentration of antimicrobial agents showing no visible growth or only a faint haze. MICs were determined for each agent individually and in combination.

Data Analysis Synergy was determined by calculating the fractional inhibitory concentration (FIC) index[22] as follows: FIC index=$FIC_A+FIC_B=[A]/MIC_A+[B]/MIC_B$, where [A] is the concentration of drug A for the organism, FIC_A is the FIC of drug A, and [B], MIC_B, and FIC_B are defined in the same fashion for drug B. Results for synergy testing were defined according to guidelines established by the American Society for Microbiology[23] so that a given FIC index was interpreted as follows: <0.5, synergy; 0.5 to 0.75, partial synergy; 0.76 to 1.0, additive effect; >1.0 to 4.0, indifference; and >4.0, antagonism. Chi-square analysis was performed to determine differing rates of synergy between the drugs.

***In Vivo* Tests**[15] Swiss albino mice maintained in our animal house were used in this study, and *Salmonella typhimurium* NCTC 74 was the test bacterium as it was naturally virulent to mice. The median lethal dose (MLD/LD_{50}) of the strain (after repeated passage through mice) was determined by using graded challenges in batches of mice and recording the mortality upto 100 h. The LD_{50} was not affected by freeze-drying and reconstitution. Reproducibility of the challenge dose was ensured by standardizing its optical density at 640 nm in a colorimeter to obtain the desired CFU on NA. The drug Am was administered at 1.5 μg/g of a mouse weighing approximately 20 g and the antibiotic Sm at 3 μg/g body weight (being calculated on the basis of standard available pharmacological data).[24] These were injected i.p. as 0.1 ml sterile solution containing 30 μg of Am or 60 μg of Sm into each mouse, 3 h before the LD_{50} challenge of *S. typhimurium* 74. The animals serving as control was given 0.5 ml sterile saline in place of the drugs. In the experiment where the *in vivo* combinations of effects of Am and Sm were determined, 20 mice were divided into 4 groups of 5 mice in each. The first group received only 30 μg of Am per mouse, second group was given 60 μg of Sm, the third had received 30 μg of Am plus 60 μg of Sm, while saline only was given to animals in the fourth group. All mice were autopsied 18 h after the challenge, their livers and spleens were removed, homogenised under sterile conditions and preserved at −20 °C for total CFU determination; 0.2—0.4 ml of heart blood was collected from each mouse for the determination of viable counts.

RESULTS AND DISCUSSION

Of the 12 test organisms, 6 were Gram positive and 6 were Gram negative, being selected from a larger series on the basis of their sensitivity to Am and the antibiotics. The MICs of Pc, Cf, Cm, Sm and Tc ranged between 2—25 μg/ml with respect to all the test bacteria, while for Em, the MIC were from 2—100 μg/ml. However, the MIC of Am ranged from 10—50 μg/ml (Table 1). Preliminary observation on effects of combination of antibiotics and the non-antibiotic Am by disc diffusion technique revealed synergism of Am with respect to Pc, Cf, Cm and Sm. Indifference was observed between Em and Am while, in case of Tc antagonism was clearly indicated. However, best synergism was noticed between Sm and Am.

The data presented in Table 2 reveals that when Sm (5 μg) and Am (200 μg) discs were placed individually on a young culture lawn of *S. aureus* NCTC 8530, the average zones of inhibition were 14.2 and 24.8 mm respectively, which increased to 17.4 and 29.8 mm respectively when the discs were placed for combined effects. Similarly, *Sh. dysenteriae* 7 NCTC 519/66 singly produced inhibition zones of 15.0 mm due to Sm and 21.1 mm against Am, which were enlarged to

Table 1. Determination of the MIC of Various Antibiotics and Chemotherapeutic Agents against Am-Sensitive Bacteria

Bacterial strains	MICs of various antibiotics and amlodipine in μg/ml						
	Pc	Cf	Cm	Sm	Em	Tc	Am
S. aureus NCTC 8530	2	2	25	2	2	2	10
S. aureus NCTC 8532	2	2	5	5	2	5	10
S. aureus ML 123	5	2	10	5	5	5	25
S. aureus ML 324	5	2	10	10	5	5	25
B. pumilus NCTC 8241	2	2	2	2	2	2	50
B. licheniformis NCTC 10341	2	2	2	2	2	2	50
S. typhimurium NCTC 74	2	2	2	2	100	2	50
Sh. dysenteriae 7 NCTC 519/66	2	2	5	2	5	2	50
Sh. boydii 8 NCTC 254/66	2	5	25	10	100	2	25
V. cholerae 865	2	2	2	2	2	2	25
V. cholerae ATCC 14033	2	2	2	2	2	2	25
V. cholerae ATCC 14035	2	2	2	2	2	2	25

Table 2. Synergism between Sm and Am by Disc Diffusion Test

Bacterial strains tested	Diameter of the inhibition zone in mm					
	Single (A) drug effect		Combined (B) drug effect		Percentage increase on the basis of πr^2	
	Sm[a)]	Am[b)]	Sm[a)]	Am[b)]	Sm[a)]	Am[b)]
S. aureus NCTC 8530	14.2	24.8	17.4	29.8	50.15	44.38
S. aureus NCTC 8532	21.0	23.5	22.2	24.8	11.75	11.37
S. aureus ML 123	18.5	22.8	19.5	23.2	11.10	7.26
S. aureus ML 324	16.3	22.4	18.2	23.6	24.67	11.0
B. pumilus NCTC 8241	18.8	26.1	20.1	28.2	14.30	16.74
B. licheniformis NCTC 10341	18.6	21.6	20.4	23.2	20.29	8.87
S. typhimurium NCTC 74	24.8	22.2	26.2	24.2	11.60	18.83
Sh. dysenteriae 7 NCTC 519/66	15.0	21.1	22.0	25.2	115.11	42.63
Sh. boydii 8 NCTC 254/66	16.4	22.7	18.8	24.4	31.41	13.65
V. cholerae 865	20.2	20.0	21.0	22.0	8.07	21.0
V. cholerae ATCC 14033	24.6	18.8	26.4	21.0	15.17	24.77
V. cholerae ATCC 14035	18.3	20.4	19.4	21.7	12.38	13.5

Sm, streptomycin; Am, amlodipine besylate. *a*) 5 μg of the drug/disc. *b*) 200 μg of the drug/disc. Mean surface area of the inhibition zone (mm^2) was calculated as πr^2 on the basis of their mean diameter ($2r$) and % increase was calculated as (B−A)/A×100, which was highly significant ($p<0.01$). The zones of inhibition formed singly with respect to Sm and Am and those formed multiply against the same compounds were larger in size. 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.

Table 3. FIC Index of Sm and Am Combinations against 12 Sensitive Bacteria

Name of the organisms	FIC index of Sm–Am
S. aureus NCTC 8530	0.50
S. aureus NCTC 8532	0.50
S. aureus ML 123	0.63
S. aureus ML 324	0.56
B. pumilus NCTC 8241	0.38
B. licheniformis NCTC 10341	0.50
S. typhimurium NCTC 74	0.50
Sh. dysenteriae 7 NCTC 519/66	0.28
Sh. boydii 8 NCTC 254/66	0.38
V. cholerae 865	0.75
V. cholerae ATCC 14033	0.56
V. cholerae ATCC 14035	0.63

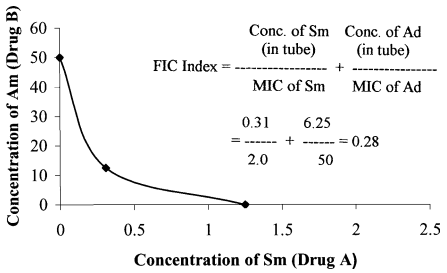

Fig. 1. Synergistic Effect of Sm (5 μg) and Am (200 μg) on *Sh. dysenteriae* 7 NCTC 519/66 Individually (Top) and Combinedly (Below)

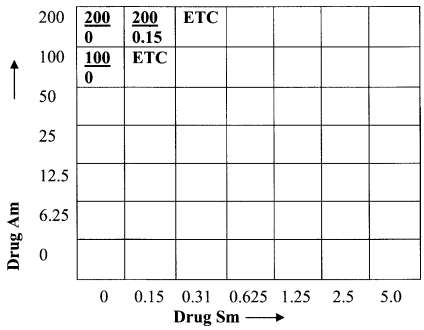

Fig. 2. Determination of FIC Index of Sm+Am Combination by Checkerboard Technique with Respect to *Shigella dysenteriae* 7 NCTC 519/66

22.0 mm, and 25.2 mm respectively, in combined test. The drugs in combination also showed synergistic activity for the remaining bacteria (Table 2). The synergism observed between Sm and Am in terms of % increase in the size of their inhibition zones was assessed for their level of significance for both the agents. The activity of Sm was 11.10 to 50.15% higher in respect to Gram positive ones in comparison with their individual effects. Similarly, the activity of Am was 7.26 to 44.38% in case of Gram positive bacteria and 13.5 to 42.63% higher in Gram negative ones when compared with their individual effects. The % increase in surface area with respect to all the test bacteria was found to be statistically significant.

The inhibitory concentrations of Sm singly to all the strains tested ranged from 2 to 10 μg/ml and those of Am were 10 to 50 μg/ml, while in combination these became 0.31 to 1.25 μg/ml to Sm and 6.25 to 25 μg/ml for Am. The FIC index values ranged from 0.24 to 0.75 for the test bacterial strains. Synergism was observed with respect to *S. aureus* 8530, 8531, *B. pumilus* 8241, *B. licheniformis* 10341, *S. typhimurium* 74, *Sh. dysenteriae* 7 NCTC 519/66 and *Sh. boydii* 8 NCTC 254/66 with FIC values ranging from 0.24 to 0.5, while partial synergy were observed with respect to other bacterial strains (0.5 to 0.75). However, best synergism could be observed for *Sh. dysenteriae* 7 with FIC index of 0.28 (Table 3; Figs. 1, 2).

The median lethal dose (or LD_{50}) was determined for the strain *S. typhimurium* 74 based on the mortality ratio data and was found to be 0.9×10^9 CFU/ml. The *in vivo* studies of blood and organ homogenates studies of 24 h normal mice belonging to the same stock yielded no *S. typhimurium* or other salmonellae. The combination of Sm and Am significantly reduced the CFU/ml counts of *S. typhimurium* in the organ homogenates of the mice 18 h after challenge. The data were analyzed statistically by employing Student's *t*-test and

Table 4. CFU/ml of *S. typhimurium* NCTC 74 in Organ Homogenates and Blood Samples of Mice

Time of sampling	Group	Mouse No.	Drug/mouse	CFU/ml counts in		
				Heart blood	Liver	Spleen
18 h	1	1	Am 30 μg	2.0×10^{6}	2.5×10^{6}	1.5×10^{6}
		2		3.5×10^{6}	2.8×10^{6}	2.0×10^{6}
		3		1.8×10^{5}	9.0×10^{5}	8.5×10^{5}
		4		7.6×10^{5}	8.7×10^{5}	7.0×10^{5}
		5		8.5×10^{5}	9.5×10^{5}	7.5×10^{5}
18 h	2	1	Sm 60 μg	8.0×10^{5}	7.6×10^{5}	2.2×10^{5}
		2		3.0×10^{6}	8.1×10^{5}	8.0×10^{5}
		3		9.5×10^{5}	2.4×10^{6}	4.5×10^{5}
		4		7.5×10^{5}	6.5×10^{5}	6.5×10^{5}
		5		6.8×10^{5}	7.5×10^{5}	5.8×10^{5}
18 h	3	1	Am 30 μg	8.8×10^{3}	2.5×10^{4}	8.5×10^{3}
		2		3.5×10^{4}	3.8×10^{3}	9.5×10^{3}
		3	+	5.0×10^{4}	6.7×10^{3}	4.5×10^{4}
		4	Sm 60 μg	2.6×10^{3}	7.8×10^{3}	4.0×10^{4}
		5		7.9×10^{3}	6.5×10^{3}	5.5×10^{4}
18 h	4	1	Saline	6.5×10^{7}	4.5×10^{7}	1.2×10^{8}
		2	(Control)	7.0×10^{7}	2.5×10^{7}	6.8×10^{7}
		3		7.5×10^{7}	1.1×10^{8}	4.5×10^{7}
		4		1.2×10^{8}	9.5×10^{6}	7.2×10^{7}
		5		4.8×10^{7}	5.0×10^{7}	2.1×10^{8}

The mice autopsied 18 h after the challenge, their blood samples collected and livers and spleens homogenised and checked for viable bacteria. The data were analyzed statistically employing Student's *t*-test and found to be significant; $p<0.01$ in Am/Sm and $p<0.001$ in Am plus Sm *versus* control.

was found to be significant; $p<0.01$ in Sm/Am and $p<0.001$ in Sm+Am versus control (Table 4).

The susceptibility profile or MICs are the primarily tools or information utilized by clinicians in selecting appropriate antimicrobial therapy for a particular infection. It is a standard to practice to use two agents to treat an infection caused by pathogens and the clinician's selection of antimicrobial agents is guided by the susceptibility results for the pathogens. Unfortunately, with the increased incidence of multidrug-resistant organisms, the clinician is often faced with only one agent (or one class of agents) that the pathogen is susceptible to (on the basis of the MICs) and monotherapy may be chosen, which might result in sub-optimal treatment of the infection and even treatment failure. Selecting only antibiotics to which the organism is interpreted as being susceptible and not those to which it is considered to be resistant by the MIC implies that the benefit of combination therapy (preferably synergism) is only obtained when the organism is susceptible to both agents.

We have been able to show enhancement and promotion of antimicrobial activities of the non-antibiotic Am in combination with the antibiotic Sm to both Gram positive and Gram negative bacteria. Quantitative estimation using percent increase in surface area of the individual inhibition zones compared with those produced by the combination showed a significant enhancement of activity. This *in vitro* activity was significant statistically. The checkerboard titration yielded significant synergy with respect to all the test organisms employed in this study. The checkerboard method is a relatively early test to perform; however, it is merely a gauge of inhibitory activity. The checkerboard method has been questioned as an appropriate technique by some investigators.[25)]

In vivo studies additionally indicated the synergistic activity of Sm with Am. It may be pointed out that the amount of Am required to inhibit the bacteria *in vitro* is considerably greater than that required for Sm, however, the amount of Am required to protect an animal is much less than Sm. Since both these drugs have been used satisfactorily for a long time in clinical medicine for other purposes, their rather low toxicity and safety margins observed during human application are encouraging.

Evidence of antibacterial activity possessed by Am[14)] and the synergistic response of such activity in combination shows that this non-antibiotic, like many other chemotherapeutics, *e.g.*, sulphonamides, nalidixic acid and nitrofurantoin independently and in suitable combinations, manifest a broader spectrum of activity by inhibiting both Gram positive and Gram negative organisms. Of the various classes of the pharmacological agents the phenothiazines, which contain tricyclic benzene rings possess moderate to powerful antimicrobial action.[26)] In amlodipine, one benzene ring is attached to another that may be considered as incomplete phenothiazine ring. Moreover, the presence of a halogen (chlorine) moiety may be playing a key role in conferring antimicrobial activity to this compound. Our results suggest that rates of synergy for Sm and Am for the test organisms in this study are comparable and it may be concluded therefore that such a synergistic combination of drugs, having the potentiality to act against a larger variety of bacteria, is expected to assist us in general battle against drug resistance and further serve as a potentially powerful tool to aid in selection of an appropriate antibiotic therapy.

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