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Assessment of antibacterial activity of the cardiovascular drug nifedipine

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SUMMARY

The cardiovascular drug nifedipine exhibited significant *in vitro* and *in vivo* antibacterial activity against 331 strains of bacteria belonging to three Gram-positive and twelve Gram-negative genera. The minimum inhibitory concentration of the drug, as determined both by agar and broth dilution methods, was seen to range from 25 - 200 μg/ml against most test bacteria, including several pathogenic ones, in the *in vitro* studies. Nifedipine was bacteriostatic in action. *In vivo* studies with this drug showed that it could offer statistically significant protection ($P < 0.001$) to mice challenged with a virulent bacterium. Therefore, nifedipine has the potential of an antibacterial agent, which may be developed after further pharmacological studies.

Key words: Antibacterial activity; *In vitro; In vivo;* Non-antibiotic; Nifedipine

INTRODUCTION

The most authoritative weapons against microbial infections have been the antibiotics and theantibacterial chemotherapeutics. However, these health benefits are now at risk due to the emergence and swelling incidence of drug-resistant bacteria over the past decades. It is evident that there is an urgent need to explore newer agents to counter the problems of drug-resistance. Studies in this line have disclosed notable antimicrobial action in drugs belonging to different pharmacological classes, such as antihistamines like bromodiphenhydramine and diphenhydramine (Dastidar *et al.*, 1976), methdilazine (Chattopadhyay *et al.*, 1998), promethazine (Chakrabarty *et al.*, 1989), trimeprazine (Dastidar *et al.*, 1997), tranquilizers like promazine (Dash *et al.*, 1977), antihypertensives like propranolol (Manna *et al.*, 1984), methyl-DOPA (Dastidar *et al.*, 1986), dobutamine (Sarkar *et al.*, 2003), amlodipine (Kumar *et al.*, 2003), oxyfedrine (Mazumdar *et al.*, 2003), antispasmodics like dicyclomine (Karak *et al.*, 2003, 2004), antipsychotics like chlorpromazine (Amaral *et al.*, 1991), fluphenazine (Dastidar *et al.*, 1995), thioridazine (Radhakrishnan *et al.*, 1999) and the anti-inflammatory agent diclofenac (Annadurai *et al.*, 1998, 2002; Dastidar *et al.*, 2000, 2003) Such drugs, having antibacterial activity in addition to their predesignated pharmacological action, have been grouped together under the banner of "Non-antibiotics"(Kristiansen, 1992). The present paper describes the detailed *in vitro* and *in vivo* activity of such a non-antibiotic-the cardiovascular drug nifedipine.

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MATERIALS AND METHODS

Drugs

Nifedipine (Fig. 1) is a yellow crystal, having a molecular weight 346.34. Chemically, it is 3, 5-Pyridinecarboxylic acid, 1, 4-dihydro-2, 6-dimethyl-4-(2-nitrophenyl)-, dimethyl ester, ($C_{17}H_{18}N_2O_6$), melting at about 174°C. Practically insoluble in water and slightly soluble in alcohol, this compound is very soluble in chloroform or acetone. It was dissolved in Dimethylformamide (DMF). The solution was extremely light sensitive.

Nifedipine was obtained from Stadmed Pvt. Ltd., benidipine from Stancare, clonidine and dipyridamole from German Remedies, digoxin from Cadila Pharma, enalapril from Nicholas Piramol, felodipine was procured from Cipla, nimodipine from Torrent and nitrendipine from Concept. All the drugs were obtained in pure dry powder form and dissolved in distilled water, dimethyl sulfoxide (DMSO) or DMF, depending on their solubility, and kept at 4°C.

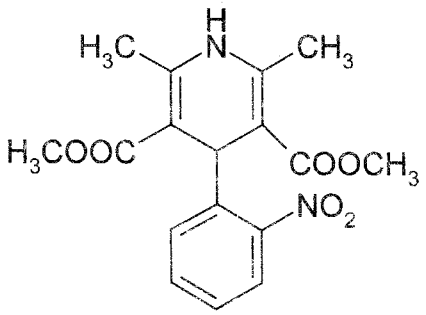

Fig. 1. Structure of nifedipine.

Bacteria

A total of 331 bacteria belonging to 15 genera were tested, comprising 111 Gram-positive and 220 Gram-negative types was tested (Table 1). These were of human origin, identified as described by Barrow and Feltham (Barrow and Feltham, 1993) and preserved in freeze-dried state.

Media

Liquid media used for this study were peptone water [PW; Oxoid brand bacteriological peptone 1% (w/v) plus Analar NaCl 0.5% (w/v)], nutrient broth (NB; Oxoid), Mueller Hinton broth (MHB; Difco). Solid media were peptone agar (PA), bromothymol blue lactose agar media (BLA), nutrient agar (NA) and Mueller Hinton agar (MHA),

Table 1. Source of bacterial strains

Name	Source
Bacillus pumilus NCTC 8241	S. P. Lapage, London, U.K.
Staphylococcus aureus NCTC 6571, 8530, 8531, 8532	S. P. Lapage, London, U.K.
Escherichia coli K12 Row, *E. coli* C 600	J. D. Abbott, U.K.
E. coli PBR 322, *E. coli* ATCC 259222	S. Palchaudhuri, U.S.A.
Salmonella typhimurium NCTC 11, NCTC 74, *S. viballerup*, *S. choleraesuis* 37, *S. choleraesuis* NCTC 101, *S. uganda* 101, *S. paratyphi* 85, *S. london* NCTC 76, *S. typhi* 57, 59	J. Taylor, London, U.K.
Shigella boydii 5 NCTC 541/60, *Sh. boydii* 8 NCTC 254/66, *Sh. boydii* 9 NCTC 304/67, *Sh. dysenteriae* 3 NCTC 102/65, *Sh. dysenteriae* 7 NCTC 519/66, *Sh. dysenteriae* 8 NCTC 599/52, *Sh. sonnei* NCTC 5/59	K. Patricia Carpenter, London, U.K.
Vibrio cholerae ATCC 14033, 14035	S. Mukerjee, Calcutta, India.
V. cholerae 80, 540, 546, 566, 569 B, 590, 738, 764, 824, 838, 906, 1003, 1021, 1023.	National Institute of Cholera & Enteric Diseases, Calcutta, India.
V. parahaemolyticus 4750, 9369, 72001, 72006. *Klebsiella pneumoniae* 14, ATCC 10031. *K. oxytoca* ATCC 130988	Y. Miyamoto, Japan A.N. Chakrabarty, Calcutta, India M.K. Lalitha, Christian Medical College, Vellore, India.

obtained by solidifying the liquid media with 1.2%(w/v) agar (Oxoid No.3). In case of BLA, bromothymol blue indicator 1.2% (w/v) and lactose 1% (w/v) were added. The pH was maintained at 7.2 - 7.4 for all the media. NA agar was used for tests with Gram-positive bacteria and PA and BLA were used for the rest of the bacteria as needed.

Determination of minimum inhibitory concentration (MIC) of different drugs

The MIC of all the drugs with respect to different test bacteria was accurately determined both by broth and agar dilution methods. For broth dilution (NCCLS, 1993), 0.1 ml of standardized suspension of a strain (10^6 cfu/ml) was added to each tube containing a drug at concentrations of 0 (control), 2, 5, 10, 25, 50, 100 and 200 μg/ml in MHB. The tubes were incubated at 37°C for 24 h, and looked for visible growth after vortexing the tubes gently. For agar dilution, the drugs were added at concentrations of 0 (control), 2, 5, 10, 25, 50, 100 and 200 μg/ml in molten NA and poured in Petridishes (Koneman, 1997). The organisms were grown in PW, and the overnight culture was spot-inoculated on the NA plates such that each inoculum contained 2×10^6 cfu. The plates were incubated at 37°C, examined after 24 h and incubated further for 72 h, if necessary. Since one NA medium containing a drug can be used for inoculation of a large number of bacteria at a time, the results of this method are being presented here, as the total number of test bacteria was 331. The lowest concentration of a drug in a tube or plate that failed to show any visible macroscopic growth was considered as its MIC. The MIC determination was performed in duplicate for each organism, and the experiment was repeated where necessary. The MIC values for a given isolate were either identical, or within ± one dilution.

Determination of cidal/static action of nifedipine on *Shigella (Sh.) boydii* 8 and *Salmonella* (S.) *typhimurium* NCTC 74

For this purpose, *Sh. boydii* 8 NCTC 254/66 and *S. typhimurium* NCTC 74 were individually grown in NB overnight at 37°C. From each such culture, 2 ml was added to 4 ml of fresh NB and incubated for 2 h so that the culture could attain the logarithmic growth phase. The number of viable cells was then determined and nifedipine was added at a concentration higher than its MIC value with respect to the test bacterium (25 μg/ml for each strain). The cfu counts were determined up to 6 h at intervals of 2 h and then after 18 h (Krogstad and Moellering, 1990).

***In vivo* tests**

Swiss strain of male white mice weighing 18 - 20 g was used for the *in vivo* studies. Animals were maintained at standard conditions at 21 ± 1°C and 50 - 60% relative humidity with a photoperiod of 14 : 10 h of light-darkness. Water and a dry pellet diet were given *ad libitum*. The virulence of the test strain *S. typhimurium* NCTC 74 was exalted by repeated mouse passage and the median lethal dose (MLD or LD_{50}) of the passaged strain corresponding to 0.95×10^9 cfu/mouse suspended in 0.5 ml NB served as the challenge dose (Reed and Muench, 1938) for all the groups of animals. Reproducibility of the challenge dose was ensured by standardization of its optical density in a Klett-Summerson colorimeter at 640 nm and determination of the cfu count in NA.

To determine the toxicity of nifedipine, 40 mice were taken, 20 of which were injected 60 μg of the drug, while the rest 20 received 30 μg of nifedipine. They were kept under observation upto 100 h.

The protective capacity of nifedipine was judged as follows: Two groups of mice, 20 animals per group, were kept in separate cages. Group I was intraperitoneally administered 30 μg nifedipine per mouse, and Group II was given 60 μg of the drug per mouse. After 3 h, each Group was challenged with 50 MLD of *S. typhimurium* NCTC 74. A control group of 40 mice was also injected similarly with the same bacterial strain, and 0.1 ml sterile

saline instead of nifedipine. The protective capacity of the drug was determined by recording the mortality of the mice in different groups up to 100 h of the treatment, and statistically by χ^2 test.

In another experiment, 4 groups of mice, 5 animals per group, were taken. Groups 1 and 3 were administered 60 μg of nifedipine, while groups 2 and 4 were given 0.1 ml sterile saline. After 3 h, all the groups were given a 50 MLD challenge of *S. typhimurium* NCTC 74. After 2 h, groups 1 and 2 were sacrificed. Their heart blood was collected aseptically; their livers and spleens were removed aseptically and homogenized in tissue homogenizes. Cfu counts of the individual organs were determined separately. The same procedure was applied on groups 3 and 4, 18 h after the challenge. Statistical analysis of the *in vivo* data was done by Student's *t*-test.

RESULTS

In vitro antimicrobial action of cardiovascular drugs

All the bacterial strains tested were found to be resistant to clonidine, digoxin, dipyridamole, enalapril and nitrendipine, while benidipine, felodipine and nimodipine produced moderate inhibitory action. Nifedipine showed strong antimicrobial action against all the bacteria (Table 2).

Bacterial inhibitory spectrum of nifedipine

Table 3 shows that out of 9 strains of *Bacillus* spp., 2 were inhibited by nifedipine at 10 μg/ml, 4 at 25 μg/ml, 2 at 50 μg/ml and the remaining 1 strain at 100 μg/ml. Among 99 strains of *Staphylococcus aureus* tested, 73 were inhibited within 50 μg/ml, 12 strains at 100 μg/ml and the rest 6 strains at 200 μg/ml. Strains of *E. coli*, *Salmonella*, klebsiellae and *Pseudomonas* were moderately sensitive to resistant to the drug. Out of 32 strains of *Shigella* spp., 23 stopped growing by 25 μg/ml, 5 at 50 μg/ml and 4 strains at 100 μg/ml. For the vibrios the MIC of nifedipine ranged from 10 - 100 μg/ml for most of the strains. Certain enterobacteria like *Proteus*, *Providencia*, *Citrobacter*, *Bordetella* and *Arizona* were less sensitive to nifedipine (MIC 50 - 200 μg/ml).

Kinetic studies on the action of nifedipine

The MIC of nifedipine against both *Sh. boydii* 8 NCTC 254/66 and *S. typhimurium* NCTC 74 was 25 μg/ml; in the logarithmic growth phase, their cfu counts were 5.5×10^8 and 6.1×10^8, respectively. At this zero (0) h, 50 μg/ml of nifedipine was added to each of the culture tubes. Nifedipine was

Table 2. Primary screening of cardiovascular drugs *in vitro* for presence of antibacterial action

Bacteria	Minimum inhibitory concentration (μg/ml) of the drugs		
	Clonidine, dipyridamole, digoxin, enalapril, nitrendipine	Felodipine, benidipine, nimodipine	Nifedipine
Bacillus pumilus 8241		200	25
Staphylococcus aureus NCTC 6571		200 - 400	10
S. aureus NCTC 8530		200 - 400	25
Escherichia coli K12Row	R	200 - 400	> 200
Salmonella typhimurium NCTC 74	E	200 - 400	50
Salmonella typhi 59	S	100 - 200	25
Shigella dysenteriae 7	I	25 - 200	10
Shigella sonnei 1	S	200	50
Shigella flexneri 4a24	T	100 - 200	25
Shigella boydii 8	A	200	10
Klebsiella pneumoniae 14	N	200 - 400	50
Vibrio cholerae 569B, 14033, 14035	T	100 - 200	25
Pseudomonas aeruginosa APC		> 800	> 200

Table 3. *In vitro* activity of nifedipine on Gram-positive and Gram-negative bacteria

Bacteria	No. tested	No. of strains inhibited by nifedipine (μg/ml)						
		5	10	25	50	100	200	> 200
Bacillus spp.	9	-	2	4	2	1	-	-
Staphylococcus aureus	99	8	14	36	23	12	6	-
Streptococcus spp.	3	-	-	1	-	2	-	-
Escherichia coli	31	-	-	2	3	5	10	11
Salmonella spp.	23	-	-	2	1	1	3	16
Shigella spp.	32	3	3	17	5	4	-	-
Klebsiella spp.	6	-	-	-	1	3	-	2
Proteus spp.	7	-	-	-	-	2	4	1
Providencia spp.	1	-	-	-	-	1	-	-
Citrobacter spp.	1	-	-	-	1	-	-	-
Arizona spp.	1	-	-	-	1	-	-	-
Pseudomonas spp.	9	-	-	-	-	-	-	9
Bordetella bronchiseptica	1	-	-	-	-	1	-	-
Enterobacter cloaca	1	-	-	-	-	-	-	1
Vibrio cholerae	93	-	18	18	10	17	23	7
Vibrio parahaemolyticus	14	-	2	2	-	10	-	-
Total	331	11	39	82	47	59	46	47

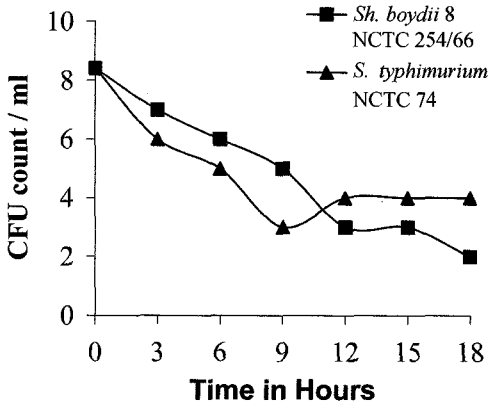

Fig. 2. Action of nifedipine on the changing kinetics of the growth of *Sh. boydii* 8 and *S. typhimurium* NCTC 74.

found to be bacteriostatic with respect to both the strains (Fig. 2).

Protective capacity of nifedipine *in vivo*

Table 4 shows that in the control group, 29 out of 40 animals died within 100 h of the challenge and no mortality was recorded in those groups of mice that received different doses of nifedipine only. There was significant protection ($P < 0.001$) in the drug-treated groups by nifedipine.

In Table 5, it is seen that nifedipine significantly reduced the number of viable bacteria in heart blood, liver and spleen of mice, both at 2 h and 18 h after challenge, compared with the control (saline

Table 4. Determination of protective capacity of nifedipine *in vivo*

Control group[a]		Test group[a]	
Drug injected per mouse	Mice died (out of 40)	Drug (μg) injected per mouse	Mice died (out of 20)
0.1 ml sterile saline	29	30	13
	-	60	4

[a]Received a challenge dose of 0.95×10^9 cfu in 0.5 ml NB of *S. typhimurium* NCTC 74. None of the animals died when 30 μg or 60 μg nifedipine was injected to 2 separate groups of mice (20 mice in each), i.e., nifedipine was found to be non-toxic to mice. $P < 0.001$, according to Chi-square test.

Table 5. Reduction in cfu/ml of *S. typhimurium* NCTC 74 in organ homogenates of mice treated with nifedipine

Time of sampling	Group	Mouse No.	Drug /mouse	Cfu/ml counts in		
				Heart blood	Liver	Spleen
2 h	1	1	Nifedipine 60 μg	2.1×10^3	1.1×10^3	4.3×10^3
		2		2.3×10^3	3.0×10^3	4.6×10^3
		3		2.5×10^3	6.5×10^4	1.2×10^3
		4		3.1×10^4	2.1×10^3	6.2×10^3
		5		5.6×10^3	1.2×10^4	2.5×10^4
2 h	2	1	Saline (Control)	5.7×10^6	2.8×10^6	8.4×10^6
		2		4.0×10^5	4.6×10^6	1.2×10^5
		3		5.8×10^5	6.0×10^6	5.4×10^6
		4		6.9×10^6	7.0×10^6	8.6×10^5
		5		7.8×10^6	8.5×10^6	8.8×10^6
18 h	3	1	Nifedipine 60 μg	3.6×10^4	5.8×10^3	7.8×10^5
		2		2.6×10^3	7.3×10^4	3.5×10^3
		3		4.5×10^4	3.8×10^4	7.2×10^3
		4		1.1×10^3	2.3×10^4	4.0×10^4
		5		7.0×10^3	7.1×10^4	3.4×10^4
18 h	4	1	Saline (Control)	4.7×10^9	5.8×10^8	5.0×10^8
		2		5.4×10^8	5.2×10^9	5.4×10^9
		3		6.8×10^8	2.7×10^9	8.2×10^9
		4		5.6×10^9	3.9×10^9	4.9×10^8
		5		7.2×10^9	8.0×10^3	1.8×10^8

Viable counts between two groups significant; $P < 0.05$ in 2 h samples and $P < 0.01$ in 18 h samples.

treated) mice. Statistical analysis showed $P < 0.05$ for 2 h samples and $P < 0.01$ for 18 h samples.

DISCUSSION

Nifedipine is a potent peripheral vasodilator. This property, coupled with its lack of significant effect to decrease S.A. node automaticity, causes some reflex tachycardia. Sympathetic reflex activity also tends to negate the negative intropic effects of nifedipine. It currently is approved for treating essential hypertension (prolonged release) and vasospastic and stable angina. For hypertensive crisis, the standard capsules are punctured and the contents are swallowed. It is also effective in treating migraine and Renauds syndrome. It is likely to produce dizziness, nervousness and headache. Gingival hyperplasia, gynaecomastia, dermatological reactions, eye pain and neuropathies have occurred rarely.

In addition to these pharmacological actions, nifedipine has significantly inhibited several Gram-positive and Gram-negative bacteria *in vitro* and *S. typhimurium in vivo*. The antibacterial activity of the drug was noteworthy with respect to different strains of *Staphylococcus, Vibrio* and *Shigella*, but it was less so against most strains of *E. coli, Klebsiella* and *Pseudomonas* (Table 3).

The mode of action of nifedipine on Gram-negative bacteria was determined to be bacteriostatic (Fig. 2).

Nifedipine was non-toxic to mice. *In vivo* studies using Swiss albino male mice and *S. typhimurium* NCTC 74 were found to be statistically significant (Table 4 and 5).

The animal experiments were carried out in order to ascertain the importance of nifedipine to human therapeutic application and finding the equivalent of mouse dose to possible human dose.

Conventional dose for human consumption orally is 10 mg thrice a day at an initial stage, to be increased gradually to 22 to 30 mg 3 or 4 times a day, if necessary (doses exceeding 180 mg a day are not recommended); sustained release, initially 30 or 60 mg once a day, titrate to maximum (20 mg a day over 7 to 14 days period). About 90% of an oral dose is absorbed, but its bioavailability is 65 to 70%; there is significant hepatic first-pass metabolism. Greater than 90% of the drug is bound to plasma protein. It is metabolized to inactive metabolites, probably by the liver. Most (80%) of the inactive metabolites are excreted in urine; 15% are excreted in the stool. The half-life is 2 to 6 h; sustain release tablets provide longer effective plasma levels [Dose range for the experiment was calculated keeping the human dose range in mind].

Previous examination among various classes of pharmacological agents has revealed that in general, the tricyclic phenothiazines possess discernable antimicrobial action (Bourlioux, 1992). The structure of nifedipine reveals two benzene rings attached to each other, almost resembling a phenothiazine. This may explain its antibacterial property. Nifedipine is already in standard usage, convincing therapeutic requirements, and it is in compliance with human toxicity levels. Thus, this drug stands a chance of being developed as an antimicrobial agent against common bacterial infections with a view to designing a new generation of promising non-antibiotic drugs to combat bacterial drug-resistance.

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