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EXPERIMENTAL EVALUATION OF SYNERGISTIC ACTION BETWEEN ANTIBIOTICS AND THE ANTIPSYCHOTIC ANTIMICROBIAL TRIFLUPROMAZINE

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Abstract- Significant antimicrobial action of the antipsychotic drug triflupromazine (Tp) against various genera of bacteria has been evaluated extensively in previous studies. This present investigation was designed to study whether this phenothiazine is able to augment action of an antibiotic when tested in combination. A total of twelve different bacterial strains belonging to various genera were used and tested to be sensitive against many antibiotics and the non antibiotic Tp. The Minimum inhibitory concentration (MIC) of all the test bacteria with respect to the antibiotics ranged fron 2- 50 µg/ml. In case of Tp, MIC ranged from 25-200 µg/ml. Disc diffusion assays revealed synergism between Tp and penicillin, ampicillin, carbenicillin, streptomycin (Sm), gentamicin and ciprofloxacin. Antagonistic effect was shown between Tp and cloxacillin, erythromycin and tetracyclin. Most effective and statistically significant ($p<0.001$) synergism was observed when Tp was combined with Sm. Following checkerboard method, the Fractional inhibitory concentration (FIC) index of the duo was determined to be 0.375, which confirmed significant synergism. The pair when subjected to *in vivo* experiments in mice challenged with *Salmonella enterica* serovar Typhimurium NCTC 74, showed statistically significant ($p<0.001$) mouse protection and also resulted in reduction of the infection in internal organs. This further suggests the pair to be highly synergistic. Thus, from this study it can be concluded that the antimicrobial activity of the non antibiotic Tp can be further remarkably increased in combination with a suitable antibiotic to combat against multi drug resistant bacteria and can be used effectively as an alternative therapy.

Keywords- Non-antibiotic, triflupromazine, phenothiazine, synergism, FIC index, streptomycin, antimicrobial, antipsychotic.

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Introduction

Antibiotics and antibacterial chemotherapeutics are the most imperative weapons in combating bacterial infections. However, over the past few decades the use of antibiotics is becoming increasingly restricted due to the development of drug resistance among pathogenic microorganisms and also due to the moderate to high levels of toxicity possessed by many antimicrobials. The escalating levels of drug resistance render it indispensable to explore newer drugs with lesser degrees of toxicity and possibly fewer chances of developing resistance. There is, thus, a search for newer antimicrobial agents that can overcome these drawbacks. The drugs belonging to various pharmacological classes possess moderate to powerful antibacterial activity. Such compounds with antimicrobial properties in addition to their pre designated pharmacological properties have been recognized as 'Non-antibiotics' [1]. Examples include the antihistamines like bromodiphenhydramine and diphenhydramine, methdilazine, promethazine and fluphenazine, antipsychotic agents like chlorpromazine, promazine, thioridazine, trifluoperazine and triflupromazine, antihypertensives like propanolol and methyl-DOPA and anti-inflammatory agent like diclofenac sodium [2-6]. Several cardiovascular drugs have also shown distinct antibacterial function [7-10].

Moreover, some of these non antibiotic agents have been found to interact with conventional antibiotics resulting in distinct synergism, which could successfully render antibiotic resistant bacteria susceptible [11-16]. Such studies open up possibilities of discovering new molecules to treat problematic infections such as those of the Multi drug resistant (MDR) phenotypes. The present study describes the synergistic action between triflupromazine (Tp) and the amino glycoside antibiotic streptomycin (Sm).

Materials and Methods

Bacteria

A total of 12 different bacteria were taken in this study [Table-1]. These were preserved in freeze-dried condition and were identified according to Collee, et al. [17].

Table 1- Inhibitory spectra of antibiotics against triflupromazine sensitive bacteria

	Minimum Inhibitory Concentration (MIC) in µg/ml of antibiotics										
Bacteria	**Pc**	**Ap**	**Cx**	**Cb**	**Sm**	**Gm**	**Cm**	**Tc**	**Er**	**Cf**	**Tp**
B.subtilis UC 564	2	2	>50	2	2	2	2	5	10	2	25
S. aureus NCTC 6571	2	2	2	2	2	2	2	2	5	2	25
S. aureus ATCC 25923	2	2	2	2	2	2	10	2	2	2	25
E. coli C22	50	2	>50	5	2	2	2	2	2	2	25
E. coli ATCC 25922	2	5	2	2	5	5	2	25	10	2	50
S. typhi 57	25	25	>50	25	10	10	25	10	100	2	50
S. enterica 74	5	2	10	2	5	5	2	5	>50	2	25
S. dysenteriae 7 NCTC 519/66	10	2	50	10	2	2	2	2	5	2	50
K. pneumoniae ATCC 10031	50	50	2	2	25	10	25	2	100	2	200
P. aeruginosa APC 1	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
V. cholerae 14033	2	2	2	2	2	2	2	2	2	2	50
V. cholerae 1364	2	2	2	2	2	2	2	10	2	2	25

Pc-penicillin; Ap-ampicillin; Cx-cloxacillin; Cb-carbenicillin; Sm-streptomycin; Gm-gentamicin; Cm-chloramphenicol; Tc-tetracycline; Er-erythromycin; Cf-ciprofloxacin; Tp-triflupromazine

Drugs

The drug Tp was obtained as a pure dry powder from Sarabhai Chemicals, India, the antibiotics were purchased from Sigma Chemicals, USA and all the drugs were stored at 4°C. All drug solutions were freshly prepared in deionized water before use.

Media

The liquid media used in the study were peptone water (PW) containing 1% bacteriological peptone (Oxoid, UK) plus 0.5% NaCl (Analar), nutrient broth (NB, Oxoid) and Mueller Hinton broth (MHB,Oxoid).The solid media were peptone agar (PA),nutrient agar (NA) and Mueller Hinton agar(MHA), which were prepared by solidifying PW,NB and MHB, respectively with the help of 1.5% agar (Oxoid No.3).

Inoculum

All organisms were grown overnight on PA/NA/MHA at 37°C and harvested during the stationary growth phase. From these cultures the organisms were directly suspended in 5ml sterile distilled water. The turbidity of the suspension was adjusted to match with 0.5 Mc Farland standards [18] with a spectrophotometer (Chemito UV 2600 Double Beam UV-Vis spectrophotometer) at 625 nm, which corresponded to 2.4 x 10^8 CFU/ml. The suspension was further diluted 1:100 with sterile distilled water, which served as the final inoculum.

Determination of Minimum Inhibitory Concentration (MIC) of Various Agents

Agar dilution method was employed to determine the MIC of Tp and different antibiotics with respect to different test bacteria following international standard guidelines [19]. The MIC of an agent was taken to be its lowest concentration in which there was no visible growth or only a faint haze. The amounts of an antibiotic or Tp (µg/ml) were: 2, 5, 10, 25, 50, 100 and 200.

***In Vitro* Synergism between Tp and Different Antibiotics**

Effects of the combination of Tp and an antibiotic by disc diffusion technique was based on the method described by CLSI (Clinical and Laboratory Standards Institute) [20]. Sterile filter paper discs (7.25mm Whatman No.1) were prepared according to Miles and Amyes [15]. Each disc contained 5 µg of any antibiotic or 200µg of Tp. The selected bacterial strains were grown in PW/NB for 18 hrs. and flooded on PA/NA in triplicate. The plates were dried at 37°C for 45 minutes. The drug discs were placed on the flooded plates at suitable positions and the plates were incubated at 37°C for 18 h. The zones of inhibition produced by each drug were measured in 3 different directions around each disc and the mean diameter was recorded. At first the individual inhibitory effects of Tp and Sm were determined. The data obtained were used for determination of their combined effects; the drug-discs were placed on the flooded agar plates in such a manner that the inhibitory circles would touch each other tangentially. Finally, the diameter of inhibition zones produced due to individual and mutual effects of two agents were recorded on the same plate. The mutual influence/interference encountered when two drugs were used in combination was assessed as indifference, when both the zones of inhibition remained unaffected, or antagonism, when the zones of inhibition receded and assumed a kidney shape, or synergism, in which the zones have merged to form a continuous larger area of inhibition. Statistical evaluation of the increase of the surface area πr^2 a zone, due to a combination of effects, was carried out by the χ^2 test to determine the level of significance.

Checkerboard Assessment

The degree of synergism between Tp and the antibiotic Sm was confirmed by the checker board method in microtiter trays with MHB in the test solution *S.aureus* NCTC 6571. The trays were prepared with a 96-channel dispenser and stored at -20°C until use. The MIC of Sm with respect to the test organism was 2 µg/ml and that of Tp was 50 µg/ml. The concentrations tested for the Sm were 0, 0.15, 0.3, 0.6, 1.25, 2.5 and 5.0 µg and those for Tp were 0, 6.25, 12.5, 25, 50, 100 and 200 µg (using two fold dilutions). The checker board was arranged in the following manner: in the first row all the wells contained 200 µg of Tp 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 100µg of Tp 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.

In each well, an inoculum of 0.5 McFarland's standard was applied using multipoint inoculators. The trays were incubated aerobically at 37°C for 24 hours. For each run the standard control strain (*S.aureus* NCTC 6571) was included. Presence or absence of growth was noted by visual observation. Synergistic action between Tp and Sm was calculated by determining the fractional inhibitiory concentration (FIC) index [21] as given below:

FIC index = (MIC of Tp in combination/ MIC of Tp alone) + (MIC of Sm in combination/MIC of Sm alone). Results of synergy were recorded following the guidelines given by American Society for Microbiology [30], in which 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 χ^2 analysis was recorded to establish synergism between Tp and Sm.

Animal Experiments

This was performed in 3-4 weeks old Swiss white male mice, each weighing 18-20 g following standard guidelines [15,22,23]. Throughout the entire period of experiment all the animals were kept in the

standard conditions of temperature at 24±1°C, relative humidity of 50-60% with a photo period of 14:10 hrs. of light: darkness. Water and dry pellets were given to the mice *ad libitum.* The mouse virulent bacterium *Salmonella enterica* serovar Typhimurium NCTC 74 was routinely given as the challenge for all the tests. The challenge dose was the same as described earlier; it was 0.95 X 10^9 cfu of the mouse passaged strain *S. enterica* 74 suspended in 0.5 ml NB [3]. Reproducibility of the challenge dose was ensured by standardizing its optical density in a colorimeter to obtain the desired CFU (colony forming units) on NA/MHA. The antibiotic Sm and the phenothiazine Tp were injected intraperitonially in the dosages based on our earlier studies. Each drug was injected intraperitoneally 3 hrs. before the challenge as 0.1 ml solution containing either 30 µg of Tp or 60 µg of Sm [12,13,24].

For determination of synergism between Tp + Sm, 20 mice were divided into 4 batches with 5 animals in each. Every animal in batch I was injected 30 µg of Tp, that in batch II received 60 µg of Sm, animals in batch III were given a combination (30 µg of Tp + 60 µg of Sm) and all the mice in batch IV received 0.1 ml sterile saline in place of the drugs. After 3 hrs., the challenge dose of *S. enterica* 74 was injected intraperitoneally into each mouse. After 18 hrs. of the administration of challenge, all the mice were autopsied; their livers and spleens were removed aseptically, homogenized individually under sterile condition and preserved at -20^0C to determine their cfu counts. The actual cfu counts from the maximum to minimum were converted to log10 [Table-3]. Heart blood was drawn from each animal, allowed to clot and serum was separated individually. The size of bacteriaemia and amount of drug/ml of serum was calculated with the help of cfu counts. Calculation of Tp or Sm in an animal was analysed by measuring inhibition zones produced by serum-soaked filter paper discs (7.2mm, 3mm thick, Millipore, absorbing about a volume of 0.03 ml) over a culture lawn of *S. enterica* 74 in PA medium. The exact amount of Tp or Sm could be deduced by referring to a standard curve calibrated with known concentrations of each drug [11,15].

Results

MIC of Antibiotics and Tp used in the Study

The inhibitory spectra of all the drugs are presented in [Table-1]. The MIC of most of the antibiotics varied between 2-10 µg/ml levels. However, the MIC of many antibiotics varied between 10 and 50 µg/ml with respect to *Salmonella typhi* 57 and *Klebsiella pneumoniae* ATCC 10031. The strain *P.aeruginosa* APC 1 was highly resistant to most of the test antibiotics. The MIC of Tp was between 25 and 50 µg/ml with respect to most of the test bacteria.

Effects of Combination of Antibiotics and Tp *In Vitro*

Initially when disc diffusion tests were performed with Tp and each of the antibiotics, synergism was revealed when Tp was combined with penicillin (Pc), ampicillin (Am), carbenicillin (Cb), streptomycin (Sm), gentomicin (Gm), and ciprofloxacin (Cf). The antibiotics which produced antagonistic effect with Tp were cloxacillin (Cx), erythromycin (Er) and tetracycline (Tc). However, chloramphenicol (Cm) when combined with Tp showed indifference. Most marked synergism was noted between Tp and Sm.

Disc diffusion tests were subsequently carried out between Tp and Sm with respect to 6 bacterial strains. The diameters of zone of inhibition, when Tp and Sm were placed individually on a culture of *Staphylococcus aureus* 6571, were 16.5 mm and 19.5mm respectively. Synergism between Tp and Sm, when the drugs were used in combination, resulted in an increase of diameters to 17.5 mm and 20.5mm respectively. The increase in surface area due to the combination was 10.52 % for Sm and 12.49 % Tp [Table-2], [Fig-1].

Table 2- Synergism between Sm and Tp by disc diffusion test

Bacteria	Diameter of Inhibition zone in mm					
	Individual effect (A)		Combined effect (B)		%increase on the basis of πr^2	
	Sm	TP	Sm	TP	Sm	TP
S. aureus 6571	19.5	16.5	20.5	17.5	10.52	12.49
B. subtilis UC564	20.5	18.0	22.0	19.5	15.17	17.36
E. coli C22	19.0	17.0	21.0	18.5	22.16	18.43
S. enterica 74	17.5	15.5	20.5	18.0	37.22	34.86
S. dysenteriae 7	15.5	17.0	20.0	20.0	66.49	38.41
V. cholerae 14033	20.0	19.0	22.5	19.5	22.56	5.33

Sm, streptomycin (5µg/disc); Tp, triflupromazine (200 µg/disc).Mean surface area of the 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 ($p<0.001$). The zones of inhibition formed combinedly between Sm and Tp, were larger in size than those formed singly against the same drugs. These were calculated statistically by determining Student's "t" test was 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.

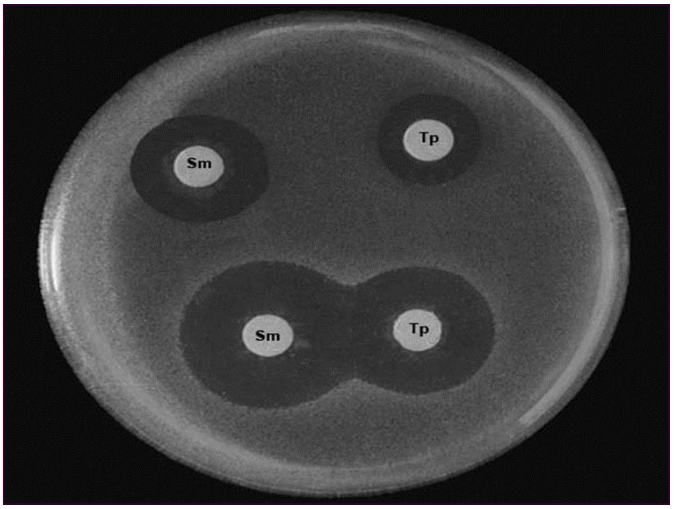

Fig. 1- Synergistic effect of Sm (5ug disc) and Tp (200 ug disc) on *S. aureus* 6571 individually (top) and combindly (below)

Individual placement of the drug discs on the culture of *Shigella dysenteriae* 7 NCTC 519/66 gave diameters of zone of inhibition as 15.5 mm and 17.0 mm for Sm and Tp respectively. This increased to 20 mm for both of the drug discs when they were placed for elucidation of combined effect. The increase in surface area due to synergism was 66.49% for Sm and 38.41% for Tp. These two drugs in combination showed statistically significant synergistic activity for the remaining test bacteria as well [Table-2].

FIC Index by Checkerboard Technique

The MIC of Tp with respect to *S. aureus* NCTC 6571 was 50 µg/ml, while that of Sm was 1.25 µg/ml. In combination the MIC values were 12.5 µg/ml and 0.15µg/ml for Tp and Sm respectively. The FIC Index was determined to be 0.375 for the Tp-Sm combination and the result was depicted on isobologram where the synergistic

antibacterial effect of the combination was shown by a concave curve [Fig-2]. The FIC Index value was <0.5, therefore proved significant synergism of the pair.

Synergism in Vivo

In animal experiments it was observed that there was a statistically significant decrease in the number of the invading pathogen in Tp or Sm treated animals with regard to the control [Table-3]. The combination of Sm and Tp could significantly reduce the number of viable bacteria in heart blood, liver and spleen samples, compared with the control mice 18 hrs. after the challenge [Table-3].Statistical analysis by student's t test showed $p<0.001$ in batches 1 and 2, and $p<0.0001$ in batch 3 versus the control, confirming their statistically significant synergism.

Discussion

The present study clearly shows that Tp is not only potentially antimicrobial alone; its action can be much enhanced when this phenothiazine is combined with a highly active antibiotic Sm both *in vitro* and *in vivo.* The first requirement to treat a bacterial infection is to determine the MIC of a large number of antibiotics against the invader. In this era of escalating frequency of prevalence of multidrug resistant human pathogens, a clinician often has no alternative other than application of a combination of antibacterial drugs, the choice of which is based on MIC values of several antibiotics in respect of the invading organism, since monotherapy may lead to sub-optimal treatment or even treatment failure. Combination therapy has become a very common practice for various systemic infections, particularly those caused by the deadly pathogens like *P. aeruginosa, K. pneumoniae and A. baumanii.*

Earlier studies have shown the powerful antimicrobial action of Tp both *in vitro* and *in vivo* [3] and the present study has proved further augmentation of the action of Tp by combination with Sm. It may be noted that although the *in vitro* action of Tp with respect to test organisms was higher than that of Sm, the amount of Tp required to protect the animals was much less than for Sm [Table-3]. Since these drugs have been used against various types of animals for the last few decades, their safety margins and toxicity profiles are noteworthy [7,8,15].

Enhancement and promotion of antibacterial activity of the antibiotic Sm could be achieved with the help of the non-antibiotic Tp both against Gram positive and Gram negative bacteria. Quantitative estimation of significant synergism between Tp+Sm could be evaluated statistically by determining percent increase of surface area of inhibition zones produced by the discs of the two agents [Table-2]. The checkerboard titration became the final confirmation of the in vitro synergism [Fig-2].

Subsequent *in vivo* studies additionally confirmed the synergistic activity between Tp and Sm [Table-3]. Earlier studies had established that prominent synergism could be observed both *in vitro* and *in vivo* when Sm was combined with either diclofenac sodium or amlodipine [12,13].

The antipsychotic phenothiazines possessing tricyclic benzene rings are often moderate to highly powerful antimicrobics [25]. However, several other pharmaceutical compounds containing two benzene rings can also be potent antimicrobics [6-10]. Many of these agents have been found to reveal synergism when combined with suitable antibiotics [11-16]. Our observation on antimicrobial action of Tp and its subsequent synergism with several common antibiotics indicate that much like the sulfonamides, nalidixic acid and nitrofurantoin this non-antibiotic also exhibits antibacterial property independently and also when combined suitably with a known antibiotic. The mechanism of action of the non-antibiotic diclofenac sodium revealed that it is able to interfere actively with synthesis of bacterial DNA. However, the mechanism by which the tricyclic phenothiazines produce their antibacterial action has not been ascertained satisfactorily. According to Amaral and his colleagues [25,26] the phenothiazines are able to inhibit action of efflux pump of bacteria responsible for MDR phenotypes. Being a phenothiazine Tp may possess a similar mechanism of action. It may be possible that the combination of Tp with Sm can affect the intrinsic efflux pump of the test bacteria resulting in reduction of the MIC levels of the two drugs in combination, probably below their break-point concentration, resulting in distinct synergism between the two. While determining the FIC index between Tp and Sm and between other non-antibiotics plus antibiotics [11-15] it was observed that the actual amount of each drug in the test pair was always lower than that required in individual tests. It may therefore be implied that a suitable combination between a non-antibiotic and an antibiotic allow reduction in amounts in both the drugs.

It has been suggested by Gunn [27] that although viable cells of *Salmonella* are liable to be quickly phagocytosed by neutrophils, soon after they are introduced intraperitoneally in mice, phagocytosis of salmonella cells by the neutrophils may not always result in cell lysis. This is due to the induction of the two component regulon Pmr A and Pmr B resulting in further activation of nine genes. These genes are responsible for the synthesis and insertion of Lipid A into the nascent lipopolysaccharide layer of salmonella [27].Thus in the present study when 50 MLD dose of viable cells of *S. enterica* 74 were inside the peritoneum of a mouse they revealed resistance to killing by neutrophils on one hand, and simultaneously encountered the environment where Tp and Sm were both available in a suitable combination.

The phenothiazines are known to accumulate within the lysozomes of macrophages [28] while the antibiotics retain activity against pathogenic bacteria by accumulating within the phagolysozomes of macrophages [29,30]. Since the drugs are present in the peritoneum when the organism is introduced, the lysozomal presence of accumulated non-antibiotic and antibiotic are in a sufficiently active state to inhibit the two-component regulon response of recently phagocytosed salmonella. Thus regardless of which mechanism is responsible for protection of mice from a virulent infection by salmonella, the combination of Tp and Sm protect the animals in a highly statistically significant manner.

Conclusion

It may be concluded from this study that like other non-antibiotics Tp may be explored as a lead compound for the creation of new antimicrobial agents with significant activity against virulent pathogenic bacteria and finally action of such a compound can be further potentiated by combining with a suitable antibiotic or even another non-antibiotic to help us combat against the multi drug-resistant bacteria.

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