

Distinct synergistic action of piperacillin and methylglyoxal against *Pseudomonas aeruginosa*

Sayanti Mukherjee^a, Shaswati Chaki^a, Sukhen Das^b, Saswati Sen^c, Samir Kr. Dutta^c & Sujata G. Dastidar^{a*}

^aDepartment of Microbiology, Herbicare Healthcare Bio-Herbal Research Foundation, 7&8 Metro Garden City,
D.H. Road, Pailan, Kolkata 700 104, India

^bDepartment of Physics, Jadavpur University, Kolkata 700 032, India

^cDivision of Drug Development/Diagnostics and Biotechnology, Indian Institute of Chemical Biology, (IICB) CSIR,
Kolkata 700 032, India

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The dicarbonyl compound methylglyoxal is a natural constituent of Manuka honey produced from Manuka flowers in New Zealand. It is known to possess both anticancer and antibacterial activity. Such observations prompted to investigate the ability of methylglyoxal as a potent drug against multidrug resistant *Pseudomonas aeruginosa*. A total of 12 test *P. aeruginosa* strains isolated from various hospitals were tested for their resistances against many antibiotics, most of which are applied in the treatment of *P. aeruginosa* infections. Results revealed that the strains were resistant to many drugs at high levels, only piperacillin, carbenicillin, amikacin and ciprofloxacin showed resistances at comparatively lower levels. Following multiple experimentations it was observed that methylglyoxal was also antimicrobial against all the strains at comparable levels. Distinct and statistically significant synergism was observed between methylglyoxal and piperacillin by disc diffusion tests when compared with their individual effects. The fractional inhibitory concentration index of this combination evaluated by checkerboard analysis, was 0.5, which confirmed synergism between the pair. Synergism was also noted when methylglyoxal was combined with carbenicillin and amikacin.

Keywords: Antimicrobial action, Methylglyoxal, Piperacillin, *Pseudomonas aeruginosa*, Synergism

The growing problem of multi-drug resistant (MDR)¹ opportunistic human pathogen *Pseudomonas aeruginosa* have been studied and discussed extensively. The quest is on to combat this deadly pathogen which at any given chance of immune disorder in a host can be fatal. There have been a lot of investigations on the discovery and application of newer antibiotics alone and in combination, but the multiple virulence factors present in *P. aeruginosa*^{2,3} continuously try and develop ways and means to overcome their application. Continuous high rate of mutation coupled with other factors have resulted in occurrence of MDR strains. Therefore, search for different compounds, other than antibiotics which can be effective against MDR *P. aeruginosa* may open up a new pathway in its history of pathogenesis and treatment.

Methylglyoxal (Mg) which is an aldehyde form of *P. aeruginosa* glycolysis end product of pyruvic

acid can be obtained as a natural component of Manuka Honey⁴. It is a special type of honey obtained from Manuka flowers which generally grow in New Zealand and are found to be highly antimicrobial in nature. Though antibacterial activity of methylglyoxal has been reported⁵, more extensive research is needed.

Piperacillin (Pp) which is one of the recent drugs of choice against potential Gram negative pathogens is an antibiotic that can be administered alone in pseudomonal infections⁶. But with the extensive progress of MDR strains, the activity and efficacy of piperacillin is decreasing rapidly⁷. Hence the present study has been designed to estimate the potency of methylglyoxal alone and in combination with piperacillin against recent human isolates along with a standard strain of *P. aeruginosa* for evaluation of a possible synergistic combination.

Materials and Methods

Bacteria—A total of 12 strains of pathogenic *P. aeruginosa* were taken for this study. Of these ATCC 27853 was received from American Type

*Correspondent author
Telephone: 919836029744
Fax: 9133-22874042
E-mail: jumicrobiol@yahoo.co.in

Culture Collection, USA, AMRI 100, BVC 1, BVC 2, BVC 3, BVC 4 and BVC 5 are very recent isolates of severe systemic infections and C15, C17, PS7, APC1 and 732 were collected during the last two years from acute lung infections in Kolkata.

Agents—A 40% aqueous solution of methylglyoxal was purchased from M.P. Biochemicals, USA. Antibiotics were obtained both in dry powder form from various Pharmaceutical Industries in India and the antibiotic discs were purchased from Pathotek Biological Labs, India; discs containing 200 μM of methylglyoxal were prepared in the laboratory.

Media—Liquid media used were nutrient broth (NB, Oxoid), peptone water (PW) containing 1% peptone (Oxoid) plus 0.5% NaCl (Analar) and Mueller Hinton Broth (MHB, Oxoid). Solid media were nutrient agar (NA, Oxoid) and Mueller Hinton Agar (MHA, Oxoid).

Minimum inhibitory concentration (MIC) of *P. aeruginosa* against antibiotics and methylglyoxal—The experiment was carried out according to the Clinical Laboratory Standard Institute (CLSI, 2010)⁸ guidelines by spot inoculating 10^5 cfu with the help of a 2 mm loopfull of 1/10 dilution of 18 h broth culture on NA and MHA plates containing 0, 50, 100, 200, 500, 1000, 1500, 2000, 3000 $\mu\text{g}/\text{ml}$ of antibiotics and 0, 100, 200, 500, 1000, 2000, 3000, 4000, 5000 μM level of methylglyoxal, the dilutions being made in sterile distilled water. All the test strains were inoculated in the same manner on NA and MHA plates, and observed after 24 h and upto 72 h. The maximum level of a drug which showed growth was taken as the resistance level against a particular strain while the next dose was taken as the MIC of piperacillin and methylglyoxal respectively.

Method of *in vitro* synergism—The combined effects of an antibiotic and methylglyoxal were determined by disc diffusion method^{9,10} with sterile filter paper discs (7.25 mm, Whatman No.1) each containing 100 μg of an antibiotic or 200 μM of methylglyoxal.

Each bacterial strain was grown in MHB for 4 h till it attained the logarithmic growth phase, then flooded on solid media in triplicate, and the plates were dried at 37°C for 2 h. Drug impregnated discs were placed on agar surface, incubated at 37°C overnight and zones of inhibition were measured. Depending on the result, discs containing methylglyoxal and an antibiotic were placed on prepared plates in such a way that their inhibitory

circles would touch each other tangentially. The diameters of inhibition zones individually and those due to the combination 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 Chi-square test for its significance.

The 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 $(B-A)/A \times 100$ where A = surface area due to the individual effect and B = surface area due to combined effect. The zones of inhibition formed in combination with respect to piperacillin (Pp) and methylglyoxal (Mg) were larger 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.

Checkerboard procedure—Synergism between methylglyoxal and an antibiotic was confirmed by performing in microtitre trays in MHB¹¹. Methylglyoxal was tested at concentrations of 0, 5, 10, 20, 40, 80, 160 and 320 μM and piperacillin was tested at concentrations of 0, 5, 10, 20, 40, 80, 160 and 320 μg . Each tray was prepared with 96 channel dispenser and stored at -20°C until use. The checkerboard was arranged as follows: in the first row, all of the wells contained 320 μM of methylglyoxal plus either of 0, 5, 10, 20, 40, 80, 160 and 320 μg of piperacillin in a final volume of 1 ml of MHB. In the second row all the wells contained 160 μM of methylglyoxal and increasing amounts of piperacillin as given above. A similar procedure was followed for all rows. In the last row, the wells had varying amounts of piperacillin only. An inoculum of 0.5 McFarland's standard¹² was applied with the help of a multipoint inoculator, incubated overnight at 37°C. The presence or absence of growth was noted visually. The MIC was recorded as the lowest amount of an agent showing no visible growth or a faint haziness. MICs were determined both individually and combinedly. Synergism was recorded by calculating the Fractional Inhibitory Concentration (FIC) index as follows: MIC of methylglyoxal in combination/MIC of methylglyoxal tested alone + MIC of piperacillin tested in combination/MIC of piperacillin tested alone.

Characterization of P. aeruginosa strains—All the organisms were identified with the help of various biochemical tests as described by Collee *et al*¹³.

Results and Discussion

Resistance pattern of P. aeruginosa with respect to antibiotics and methylglyoxal—In a comparative study of growth inhibitory action of a range of antibiotics against *P. aeruginosa* strains it was observed that the antibiotic penicillin was highly inactive against all the strains. For piperacillin and carbenicillin the resistance level was between 100 to >1000 µg/ml. For imipenem, ceftazidime, ceftriaxone and cefotaxime the resistance level was between 250 and >1000 µg/ml while ceftazidime was found to be a better drug as far as sensitivity towards most of the MDR strains was concerned. Amikacin also surprisingly showed high levels of sensitivity towards almost all the strains. The pseudomonads, on the other hand, were largely resistant to tetracycline, chloramphenicol and azythromycin at rather high levels (between 1500 and >3000 µg/ml). The level of resistance to methylglyoxal was much like the antibiotics, varying between 2000 and 5000 µM.

Effect of combination of an antibiotic and methylglyoxal in vitro—Synergism between methylglyoxal and the antibiotics piperacillin, carbenicillin and amikacin is presented in Table 1. When synergism tests were performed between two compounds the percentage increase in surface area was found to be statistically significant with respect to methylglyoxal plus piperacillin in all the 5 experimental strains. The actual action between piperacillin (100 µg) and methylglyoxal (200 µM) discs with respect to *P. aeruginosa* 732 is being

presented in Fig. 1. There was definite synergism when the antibiotics carbenicillin and amikacin were combined with methylglyoxal (Table 1).

Checkerboard experiment for the determination of the FIC index—The MIC of methylglyoxal with respect to *P. aeruginosa* 27853 in MHB was 160 µM while that of piperacillin was 40 µg. In combination the MIC values became 80 µM and 20 µg for methylglyoxal and piperacillin respectively. The data presented here for determining synergistic antibacterial effect indicated that the combination produced a significant synergism of this pair, as the FIC index was calculated to be 0.5. Such significant values of FIC

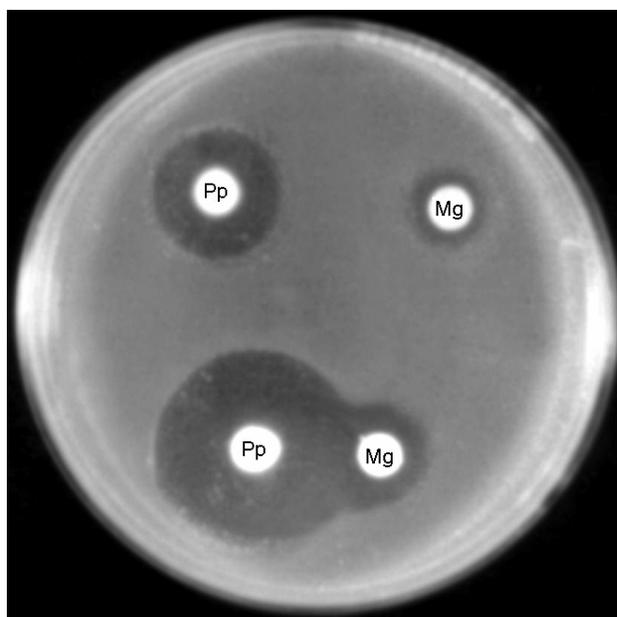


Fig.1—Effect of Pp (100 µg) and Mg (200 µM) discs on *Pseudomonas aeruginosa* 732 illustrating distinct synergism between the two

Table 1—Effects of combination of methylglyoxal with the antibiotics piperacillin, carbenicillin and amikacin

<i>P. aeruginosa</i> strains tested	Individual effect		Combined effect		Diameter of the inhibition zone (mm)		Individual effect		Combined effect	
	(A)		(B)		(A)	(B)	(A)	(B)		
	Mg	Pp	Mg + Pp		Cb	Mg + Cb	Ak	Mg + Ak		
ATCC 27853	15.5	17.0	17.5 (16.6)*	19.0 (25.22)	14.2	16.4 (9.33)	18.4 (29.6)	15.2	15.9 (6.0)	17.4 (14.5)
C15	19.5	19.0	21.3 (17.4)	21.0 (22.26)	18.0	20.2 (3.6)	19.5 (8.33)	18.4	20.5 (5.12)	19.8 (7.6)
C17	13.5	13.0	15.6 (15.5)	19.75 (130.8)	12.5	14.5 (7.4)	18.2 (45.6)	12.5	14.0 (3.7)	13.5 (8.0)
732	12.9	21.58	15.0 (35.21)	24.9 (33.13)	20.4	14.6 (13.2)	23.2 (13.7)	21.2	14.2 (10.10)	22.5 (6.1)
APC1	14.6	18.5	15.0 (5.74)	22.3 (45.6)	17.5	14.8 (1.3)	21.2 (22.3)	17.7	15.0 (2.73)	19.6 (10.7)

*% increase on the basis of πr^2

Mg : methylglyoxal (200 µM/disc); Pp : piperacillin (100 µg/disc); Cb : carbenicillin (100µg/disc); Ak : amikacin (100 µg/disc)

index were also obtained in the pairs of methylglyoxal + carbenicillin and methylglyoxal + amikacin.

Methylglyoxal is a unique compound formed as a metabolic intermediate during the various biochemical pathways in microorganisms. It is a dicarbonyl compound, both an aldehyde and a ketone; which is an integral part of the natural product of Manuka honey. This honey exhibits strong antibacterial action compared to normal honey commonly available in the market. This unique property of Manuka honey is attributed to the presence of methylglyoxal in it. Reports further reveal that in bacteria the synthesis of protein, DNA and RNA are all strongly inhibited by methylglyoxal⁵.

The anticancer property of methylglyoxal has also been known for a long time. From the early 1960s, there were ample evidences being produced by scientists on the anticancer property. Eglylid *et. al*¹⁴. suggested that the anticancer property of methylglyoxal is mediated through growth inhibitory effect of the compound, which in turn, is due to the inhibition of protein synthesis. These observations guided us to look for the antibacterial potentiality of methylglyoxal against MDR *P. aeruginosa* for experimentation in this study. It was observed that piperacillin, carbenicillin, ceftazidime, amikacin and ciprofloxacin show much better sensitivity towards almost all the *P. aeruginosa* strains at comparatively much lower levels than rest of the antibiotics tested. The results are in accordance with the present therapeutic treatment against *P. aeruginosa*, as all the antibiotics are drugs of choices against this pathogen. The recent isolates AMRI 100, BVC 3 and BVC 5 were resistant to all antibiotics including the drugs of choice at very high levels. The earlier isolates were less resistant to these drugs, but maintaining them as freeze dried cultures did not seem to have lost their drug resistance properties, as was clearly observed when the resistance pattern was studied. Therefore it can be concluded that *P. aeruginosa* fails to lose its resistance easily with time or subculturing. All the isolates were simultaneously distinctly highly resistant to many common antibiotics including chloramphenicol and tetracycline.

The present results further revealed that the resistance levels towards piperacillin and methylglyoxal were distinctly much more individually than when they were combined to determine their effect together proving thereby the synergism between the two drugs. Such results proved that

methylglyoxal is a potential drug with profound synergistic activity with ability to kill *P. aeruginosa* strains. The toxicity study of methylglyoxal¹⁵ as a potential drug has already been carried out quite extensively showing no alarming hazardous effect in *in vivo*. Thus if a small dosage of methylglyoxal is used as a drug with piperacillin the combined effect can induce the beginning of a new era in the search for effective handling of MDR *P. aeruginosa*. Piperacillin continues to be the drug of choice for treatment against this pathogen but for MDR strains the drug alone seems to be incompetent even in higher doses. Therefore if methylglyoxal can be supplemented with piperacillin in a low dose, the effectiveness of piperacillin as a drug will increase convincingly.

In this study piperacillin, carbenicillin, amikacin and ciprofloxacin¹⁶ were selected for synergism tests along with methylglyoxal since these antibiotics are established drugs¹⁷ for treatment of acute *P. aeruginosa* infections.

It is known that methylglyoxal is a very simple compound structurally and there should be no difficulty in synthesizing such a compound in a pharmaceutical industry. In view of the acute problem of antibiotic resistances not only in *P. aeruginosa* but also among various other pathogenic organisms application of methylglyoxal as a highly potent antimicrobial drug may now be taken up for the treatment of different infections.

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