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Research Article

ASSESSMENT OF SYNERGISTIC CAPACITIES OF SULBACTUM WITH A CARBAPENEM

SAYANTI MUKHERJEE,[1] SHASWATI CHAKI,[1] T.K. PAL,[2] MUSFIQUA MOOKERJEE[3], SUKHEN DAS[4] AND SUJATA G.DASTIDAR[1*]

[1]Department of Microbiology, Herbicure Healthcare Bio-Herbal Research Foundation,7&8 Metro Garden City, D. H. Road, Pailan, Kolkata-700104, India,[2]Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, India,[3]Department of Microbiology, NSHM C`ollege of Pharmaceutical Technology, NSHM Knowledge Campus, 124, B.L.Saha Road, Kolkata 700053, India,[4]Department of Physics, Jadavpur University, Raja S.C.Mullick Road, Kolkata 700032, India.Email: jumicrobiol@yahoo.co.in

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ABSTRACT

Meropenem had been successfully used independently against various types of infections when it was first discovered, while sulbactum sodium being much less potent had been given to humans more frequently in combination with ampicillin. Meropenem is now less frequently applied singly to infections caused by virulent multidrug resistant Gram negative organisms. Further potentiation of action of meropenem is possible by synergism between meropenem and sulbactum. In a study of 30 different Gram positive and Gram negative bacteria, the minimum inhibitory concentration (MIC) of meropenum was found to be varying from 1-5 µg/ml with respect to 22 organisms as determined by agar dilution technique; however, the MIC of this antibiotic was 25 µg/ml against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The MIC of sulbactum against meropenem sensitive bacteria was 25µg/ml and was between 50 and 200µg/ml against the organisms which had higher MIC values in respect of meropenem. A highly significant synergism could be observed between these two antibiotics by following Student's '*t*' test ($p<0.001$). The Fractional Inhibitory Concentration (FIC) index value of this combination with the help of checkerboard assessment procedure was found to be 0.375, confirming synergism.

Keywords: Meropenem , carbapenem, bacteria, pathogenic.

INTRODUCTION

The carbapenem antibiotic meropenem had been used primarily for the treatment of complicated skin and skin-structure associated infections, complicated intra-abdominal, chest and urinary tract infections. Being more powerful than the third generation β-lactam antibiotics cephalosporins meropenem had exhibited highly potent action against extended spectrum β-lactamase producing and AmpC chromosomal β-lactamase producing bacteria. Compared with imipenem meropenem was found to be more active against most of the deadly pathogenic Gram negative bacteria (Arrieta, 1997). The carbapenems are still used as the last resort for treating multi-drug resistant Gram negative infections in any nosocomial settings, as these antibiotics have a broad spectrum of activity and are stable to hydrolysis by β-lactamases, including ESBLs and AmpC β-lactamases. However, there have been an alarming increase in reports on carbapenem resistance in *Acenatobacter baumanii* during the past several years (Gupta *et al*., 2006; Sinha *et al* 2007; Vishnu *et al*., 2011). It is known that different antimicrobial resistance mechanisms are highly prevalent in *A. boumanii* both as constitutive and as acquired resistance (Bonomo *et al*., 2006). Carbapenemase production is the commonest mechanism of carbapenem resistance by phenotypic screening method; carbapenem hydrolyzing oxacillinase is the most likely mechanism (Vishnu *et al*., 2011).

The growing threat of antimicrobial resistance in many Gram negative bacteria rely on one hand on its extraordinary capacity to develop resistance to almost any available antibiotic through mutation in chromosomal genes and to the increasing prevalence of transferable resistance determinants, particularly those encoding class B carbapenamases, as the metallo-betalactamases and ESBLs are frequently co-transferred while genes encoding aminoglycoside modifying enzymes (Riera *et al*., 2011). Therefore in the present scenario to overcome the problem of escalating multi-drug resistance among the highly infective pathogens, the action of meropenem can be successfully accentuated by combining with a suitable drug. In 2004, Ko *et al* reported that the combination of meropenem plus sulbactam had a distinctly better applicability than meropenem alone against *A. baumanii*. The present study describes the suitability of this combination against a large number of pathogenic microorganisms.

MATERIALS AND METHODS

Bacteria

A total of 30 strains of bacteria belonging to Gram positive and Gram negative genera were tested. Many of them were received from the National Collection of Type Culture (NCTC**,** London,) or the American Type Culture Collection (ATCC, USA).The others were isolated as human pathogens in India. All the isolates were identified following standard methods (Collee *et al*., 1996).

Drugs

Dry powders of meropenem and sulbactam sodium were obtained from VHB Medi Sciences Ltd, India that were soluble in water and stored at 4^0 C.

Media

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). Solid media were peptone agar (PA), prepared by solidifying PW with 1.0% agar (Oxoid No 3), nutrient agar (NA, Oxoid), and Mueller Hinton agar (MHA, Oxoid), pH 7.2-7.4; PW and PA were used for Gram negative bacteria for large inhibition zones.

Inoculum

All organisms were grown at 37^0C on PA/NA/MHA for 24 h, harvested during stationary phase and suspended in 5 ml of sterile distilled water. Turbidity of each suspension was adjusted to match against 0.5 McFarland standard (McFarland, 1907) with a spectrophotometer (Chemito UV 2600 Double Beam UV-Spectrophotometer) at 625 nm that corresponded to 2.4×10^8 colony forming units (CFU)/ml.

Preparation of discs containing meropenem and sulbactam

The discs were punched from the Whatman No. 1 filter paper and were 7.25 mm in diameter. They were sterilized in hot air oven at 160^0C for an hour in batches of hundred discs in screw capped Bijou bottles (Dasgupta *et al*., 2010).

The final concentration of meropenem to be present in each disc was either 2 µg or 5 µg; hence 2 stock solutions having 200µg/ml and 500µg/ml were prepared. The following procedure was followed to prepare drug-impregnated discs: 1 ml of the stock solution containing 200µg/ml and 500 µg/ml of meropenem were added to 2 separate bottles each containing 100 discs. Each disc absorbed 0.01ml of the solution, so that the entire 1 ml volume was absorbed thereby producing discs having 2 µg and 5 µg of meropenem (Jeyaseeli *et al*., 2012; Miles *et al*., 1996; Mukherjee *et al*., 2011). The same procedure was followed for sulbactam sodium. The final concentration of this drug to be present in a disc was 200 µg for which the stock solution containing 20 mg/ml was prepared; 1 ml of such a stock solution containing 20 mg of sulbactum sodium was added to a bottle of 100 discs. Each disc absorbed 0.01 ml of the solution so that the entire 1 ml volume was absorbed, there by producing discs each having 200 µg of the drug. Two higher concentrations of sulbactum sodium had to be made since 200 µg discs failed to produce distinct zones of inhibition with respect to many organisms; these were 400µg/disc and 800 µg/disc. The discs were used in wet condition and maintained at 4ºC until needed to retain the potency (Jeyaseeli *et al*., 2012). The discs were allowed to warm up in room temperature before being applied on prepared agar plates for determination of inhibition zone (CLSI, 2009).

Test for detection of minimum inhibitory concentration (MIC) of antibiotics, meropenem and sulbactam

This was performed by agar dilution method following the guidelines of Clinical Laboratory Standards Institute (CLSI, 2009), by spot inoculating 10^5 CFU with a 2mm loop full of 1/10 dilution of 18 h NB/MHB cultures on NA/MHA plates containing 0 (control), 1, 2, 5, 10, 25, 50 µg/ml of meropenem and 0 (control),1, 2, 5, 10, 25, 50, 100, 200 µg/ml of sulbactam sodium, plates were incubated at 37ºC overnight and observed after 24 h, and upto 72 h for appearance of growth.

***In vitro* synergism**

The method described by CLSI (CLSI, 2009) was followed. The test for combined effects of meropenem with sulbactam was carried out by disc diffusion assay with 2 µg and 5 µg of meropenem and 200 µg and 400 µg sulbactam. Test organisms were grown in PW/MHB for 18 h, flooded on PA/MHA in triplicates and dried at 37^0C for 1 h. Initially individual inhibitory effects of two agents were determined by measuring the zones of inhibition. Depending on this observation, discs containing the same agents were placed on prepared plates in such a manner that their inhibitory circles would touch each other tangentially. The zones of inhibition due to individual and mutual effects on the same plate were recorded. The increase in surface area (πr^2) due to the combination of effects was evaluated statistically with the help of χ^2 test for the level of significance (Dasgupta *et al*., 2010).

Checkerboard experiment

This was performed in micro-titre trays with MHB. Meropenem was tested at concentrations of 0.2 to 6.4 µg/well and sulbactam at 2 to 64 µg/well. The checker board was arranged as follows: in the first row all the wells contained 64 µg of sulbactam and either of 0.2, 0.4, 0.8, 1.6, 3.2 or 6.4 µg of meropenem in a final volume of 1 ml. In the second row all the wells contained 32 µg of sulbactam and increasing amounts of meropenem as described above. An identical pattern was followed in all the rows. In the last row the wells had increasing amounts of meropenem only. An inoculum of 0.5 ml McFarland standard (McFarland, 1907) was applied with the help of a multipoint inoculator, incubated aerobically and growth was recorded visually after 24 h incubation at 37^0 C. The fractional inhibitory concentration (FIC) index was calculated as given below: MIC of meropenem tested in combination / MIC of meropenem tested alone + MIC of sulbactam tested in combination / MIC of sulbactam tested alone. The resulting interaction was interpreted as synergistic when the value was ≤ 0.5 (Dasgupta *et al*., 2010; Jeyaseeli *et al*., 2012).

RESULTS

MIC of meropenem and sulbactam

Table 1 describes a comparative assessment of the growth inhibitory spectra of 30 bacteria comprising 7 Gram positive and 23 Gram negative types. Primarily the Gram positive organisms revealed lower MIC values with respect to both the antibiotics, among the sensitive bacteria the MIC of meropenem varied from 1 to 2 µg/ml level and the MIC of sulbactam was between 10 and 25 µg/ml. However, of the Gram negative organisms, strains of *Shigella, Salmonella, E. coli* and even vibrios were more sensitive to these antibiotics than *Klebsiella, Acenatobacter* and *Pseudomonas.* The MIC of meropenem was 25µg/ml and that of sulbactam was 100 – 200 µg/ml in case of Gram negative organisms.

Table 1:Determination of Minimum Inhibitory Concentration (MIC) of meropenem and sulbactam

Bacteria	No. Tested	MIC (µg/ml)	
		Meropenem	Sulbactam
Shigella flexneri 2b NCTC 559/63, *Sh. sonni*NCTC 9774, *Escherichia.coli* C 21, *Vibrio Cholerae* 569B, ATCC 14033	5	1	10
Salmonella enterica serovar Typhimurium 2 NCTC74, *Sh. dysenteriae* 2	2	1	25
Bacillus subtiliis UC 564, *Staphylococcus aureus* NCTC 6571, NCTC 8531, NCTC 8532, *E.coli* K12 Row, C 600, *S. typhi* 59, *Enterobacter cloaca* L1, *Arizona* spp 45, *V. vulnificus* NICED1	10	2	25
Listeria monocytogenes MTCC1143, E.coli 3P/SD	2	2	50
B. pumilus NCTC 8241, *Enterococcus faecalis* 4, *Providencia* spp 11	3	5	100
Rhodococcus spp M1	1	10	100
Klebsiella pneumoniae 1, *Pseudomonas. aeruginosa* C15, 27853	3	25	100
K. pneumoniae J/1/6, *Acinetobacter baumanii* AMRI8, 536, *P. aeruginosa* APC	4	25	200

Effects of combination of meropenem and sulbactam

In the disc diffusion assay between these two antibiotics, varying degrees of synergism was observed. For the sensitive organisms, 2 µg meropenem discs and 200µg sulbactum discs were used for determining their combined action [Table 2]. When the drug discs were placed individually on the culture lawn of *S. aureus* NCTC 6571 the diameters of zone of inhibition due to meropenem was 20.0 mm and the same due to sulbactam was 14.2 mm. These increased to 21.8 mm and 15.5 mm respectively, when the discs were placed to determine the effect of combination between the two antibiotics. The increase in surface area due to the combination was 18.81 % for meropenem and 19.15 % for sulbactam. Similarly, the highly sensitive bacterium *Sh. sonnei* singly produced an inhibition zone of 19.2 mm due to meropenem and 20.1mm due to sulbactam discs,that increased to 25.0 mm and 22.6 mm respectively, in the test for effect of combination. Further studies with other bacteria with higher MIC values were carried out with 5 µg meropenem discs and 400 µg sulbactam discs [Table 3]. Tests to determine effect of combination between these two antibiotics confirmed synergism. With respect to *L. monocytogenes* the diameters of the inhibition zone due to meropenem individually was 24.9 mm and combinedly was 28.8 mm, and the % increase was calculated to be 33.78 %. The

same organism produced 19.8 mm wide zone of inhibition against sulbactam individually, that increased to 23.0 mm when tested in combination with meropenem. The resulting increase % was calculated to be 34.94 %. All the other test bacteria also exhibited substantial increase in the tests for determining the effect of combination between these two antibiotics. All the values were calculated statistically by following Student's 't' test based on the values of standard deviation and standard error obtained which showed the differences to be highly significant ($p < 0.001$) with respect to all the test bacteria [Tables 2, 3].

Table2: Synergism between meropenem and sulbactam in highly sensitive bacteria

Bacteria	Diameters of inhibition zones in mm					
	Individual drug effect (A)		Combined drug effect (B)		% increase on basis of πr^2	
	Mp	Sb	Mp	Sb	Mp	Sb
S. aureus NCTC 6571	20.0	14.2	21.8	15.5	18.81	19.15
S. aureus NCTC 8531	18.0	16.9	18.5	17.6	5.63	8.46
S. aureus NCTC 8532	31.6	20.9	34.8	22.9	21.28	20.05
E.coli K12 Row	31.6	20.9	34.8	22.9	21.28	20.05
Sh. sonnei NCTC 9774	19.2	20.1	25.0	22.6	69.54	26.42
V. vulnificus NICED 1	26.5	21.2	27.6	23.0	8.47	17.70

Mp, meropenem (2 µg /disc); Sb, sulbactam (200 µg/disc)

The 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)/Ax100, where A = surface area due to individual effect and B= surface area due to combined effect.

The zones of inhibition formed singly with respect to Mp and Sb and those formed combinedly 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.

Table3:Effect of combination of meropenem and sulbactam in drug resistant bacteria isolated from human infections

Bacteria	Diameters of inhibition zones in mm					
	Individual drug effect (A)		Combined drug effect (B)		% increase on basis of πr^2	
	Mp	Sb	Mp	Sb	Mp	Sb
L. monocytogenes MTCC 1143	24.9	19.8	28.8	23.0	33.78	34.94
K. pneumoniae 1	23.1	21.8	25.8	23.4	24.74	15.22
P. aeruginosa ATCC 27853	20.9	18.2	24.8	22.5	40.8	52.83
P. aeruginosa APC	24.8	14.2	25.4	14.5	4.9	4.27
A. boumanii AMRI 8	22.5	20.3	25.3	23.4	26.44	32.87

Mp, meropenem (5 µg /disc); Sb, sulbactam (400 µg/disc)

The 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)/Ax100, where A = surface area due to individual effect and B= surface area due to combined effect.

The zones of inhibition formed singly with respect to Mp and Sb and those formed combinedly 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.

Checkerboard test for the determination of FIC index

The MIC of meropenem with respect *E.coli* K_{12}Row in MHB was 3.2 µg, while that of sulbactam was 32 µg. In combination the MIC values decreased substantially, being 0.4 µg and 8 µg respectively. These data on the combined effect of meropenem + sulbactam revealed a significant synergistic action between the two as the FIC index was calculated to be 0.375.

DISCUSSION

Ever since its discovery meropenem was found to be highly active against Gram negative organisms, and had been applied regularly for a variety of systemic infections including septicaemia throughout the world. However, even this wonder drug started showing development of drug resistance. In view of its efficacy meropenem was combined with a less potent antibacterial agent sulbactam to determine if a synergistic combination could be achieved. Ko *et al* in 2004 reported that such a combination had produced encouraging result against *Acenetobacter baumanii,* a bacterium that can be responsible for many types of acute infective conditions.

In this study the preliminary data on the independent effect of meropenem and sulbactam on various organisms it was observed that the MICs of both the antibiotics were much higher in recent isolates of *K. pneumoniae*, *P. aeruginosa* and *A. boumanii*. The quantitative estimation using the percentage increase in the surface area of inhibition zones formed in combined tests compared to those formed by individual zones distinctly showed augmentation of action of both drugs. This *in vitro* action was statistically significant. Finally the checkerboard test provided a more definite enhancement of antibacterial action of this combination. In fact in this test for synergistic action by the FIC index, it was evident that the actual amount of each antibiotic in the test pair was much lower than that required for the individual tests, implying that a suitable combination is likely to allow a reduction in the doses of both the antibiotics. In this way the problem of break-point concentrations of these drugs may be overcome.

In an elaborate study on the mechanism of drug resistance conferred by meropenem in pathogenic isolates of *P. aeruginosa* Shashikala *et al* (2006) had emphasized on the over expression of multi-drug efflux pumps. Esterly *et al* (2011) observed that patients infected with carbapenem resistant *A. baumanii* blood stream infections were more critically ill and had greater incidences of morbidity since the inactive therapy became the predictor of death. The results suggested difficulties in treating such patients due to challenges of optimizing antimicrobial therapy in the setting of highly resistant pathogens. Combination of a carbapenem like meropenem with another antibacterial drug sulbactam may, in all probability, turn out to be highly active against the virulent threats caused by a large number of extremely virulent Gram negative pathogens as is evident from the present study. This synergistic combination of meropenem and sulbactam would hopefully open up a prospective path in the selection of antimicrobial therapeutic regimens for the continuing fight against multi-drug resistant microorganisms.

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