THE *IN VITRO* ACTIVITY OF THE COMBINATION OF CEFOTAXIME AND HRE 664

M. LIMBERT, D. ISERT, N. KLESEL, C. MEICHSNER, G. SEIBERT and E. SCHRINNER

Hoechst AG, Pharma Research,
6230 Frankfurt/Main 80, FRG

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Cefotaxime (CTX) and HRE 664 (a novel penem antibiotic) possess complementary *in vitro* properties. Differences can be observed in their antibacterial spectra, their β-lactamase stability and -inhibition, and their affinity to penicillin-binding proteins. These differences suggested that combinations of the cephalosporin and the penem antibiotic would be advantageous and should be studied. The fractional inhibitory concentration values of checkerboard studies confirmed that CTX and HRE 664 act synergistically against various Gram-positive and Gram-negative bacteria. Fixed combinations containing 80% CTX and 20% HRE 664 possess broader antibacterial spectra and in certain cases higher antibacterial activities than each of the components alone. The combinations had improved activity against Staphylococci including methicillin-resistant strains, β-lactamase producing strains of *Enterobacter* sp. and *Bacteroides fragilis*. The combination as well as the single antibiotics had only limited activity against *Pseudomonas aeruginosa*.

Cefotaxime (CTX) possesses advantageous chemotherapeutic properties. The high degree of β-lactamase stability and the affinity for essential penicillin-binding proteins (PBPs 1 and 3) are the important reasons of its high antibacterial activity. The antibacterial spectrum of CTX includes most Gram-positive and Gram-negative anaerobic and aerobic bacteria. Nevertheless, substantial percentages of some important bacterial species are resistant. These bacteria usually produce CTX-destroying β-lactamases. The highest numbers of CTX-resistant strains are found among *Pseudomonas aeruginosa*, *Enterobacter cloacae* and *Bacteroides fragilis*. Considerable numbers of Enterococcal strains are also resistant to CTX. Furthermore, the MIC of CTX for Staphylococci — especially methicillin-resistant strains — are far higher than for most Enterobacteriaceae[1~3].

HRE 664 (E 83 1664) is a penem antibiotic with a broad spectrum including Gram-positive and Gram-negative aerobic and anaerobic bacteria. The *in vitro* activity of HRE 664 against Gram-positive bacteria especially Staphylococci (including methicillin-resistant strains) is higher than that of CTX. CTX-sensitive Gram-negative bacteria are also sensitive to HRE 664. However HRE 664 is much more active against the CTX-resistant, β-lactamase producing strains of *Enterobacter* sp. and *B. fragilis*[4,5]. The high antibacterial potency of HRE 664 depends on its high stability towards plasmid-coded and chromosomally-coded β-lactamases[4,5]. *In vitro* studies showed that HRE 664 is not only β-lactamase stable but is also an efficient inhibitor of these enzymes[4,5]. HRE 664 binds to PBPs 2, 5 and 6 of *Escherichia coli*[4,5]. The complementary *in vitro* properties of CTX and HRE 664 gave rise to investigations on their combined activity *in vitro*.

Conventional "checkerboard" studies were performed using a number of strains. To obtain more information we also tested the activity of a fixed combination of CTX and HRE 664 against a great number of different bacteria.

Materials and Methods

Antibiotics

CTX and HRE 664 (E 83 1664) were synthesized by Hoechst AG (Frankfurt, FRG) and Hoechst UK Ltd. (Milton Keynes, UK), respectively.

Determination of the MIC

The sensitivity of anaerobic and aerobic bacteria to the antibiotics was tested using the agar-dilution test. Anaerobes were tested on Wilkins-Chalgren agar (Oxoid) and aerobes on Mueller-Hinton (MH) agar (Difco). When testing Streptococci the MH agar was supplemented with 10% horse blood. Stationary cultures of the appropriate test strains were used as the inoculum. Agar plates containing serial dilutions of the drugs were inoculated with a multipoint inoculator. 5×10^5 cfu of anaerobes and 5×10^4 cfu of aerobes were inoculated per spot.

The MICs of the test compound for anaerobic bacteria were read after 48 hours at 37°C in anaerobic jars (Oxoid) and those for aerobes after 24 hours at 37°C. The MIC was taken as the lowest concentration of the test substance, at which no visible growth could be detected in the area of the inoculation spot after the incubation time. A haze of growth or a single colony was disregarded in the reading.

"Checkerboard" Combination Studies

The combined activity of CTX and HRE 664 was measured using the checkerboard method in conjunction with the agar-dilution test. MH agar (Difco) was used as the test medium. Stationary cultures of the test strains served as inocula after dilution by 1:10. After 18 hours at 37°C the MICs were read both from the controls containing only one of the antibiotics and from the plates containing the combination.

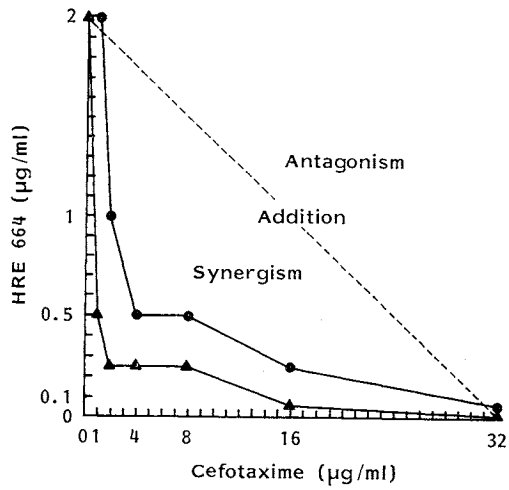
The "fractional inhibitory concentrations (FICs)"[6] were calculated from these values using the following equation:

$$FIC=\frac{A_C}{A_A}+\frac{B_C}{B_A}$$

where A_A and B_A are the MIC values obtained with the antibiotics A and B alone, and A_C and B_C are the concentrations of each compound in the lowest effective combination.

A combination of two substances is usually considered to be synergistic when the two compounds together inhibit the test strain at concentrations of 1/4 of the MIC of each compound alone or less. If this criterion is applied to the method of the "FIC", an FIC value of ≤ 0.5 shows synergism, whereas an FIC value of 1.0 expresses additive action.

Fig. 1. Isobolograms showing the effects of combinations of cefotaxime and HRE 664.
▲ *Enterobacter cloacae* M 423, ● *E. cloacae* M 447.



Results

Checkerboard Studies

Table 1 shows the MICs of CTX and HRE 664 alone and the FIC values of their combinations against various Gram-negative and Gram-positive bacteria. In 17 out of 20 strains tested, FIC values of ≤ 0.5 were obtained and indicate synergism between CTX and HRE 664.

The synergism is shown graphically in Fig. 1 for two strains of *E. cloacae*.

Table 1. Antibacterial activity of the combination cefotaxime (CTX)/HRE 664 (checkerboard technique, agar-dilution test).

Strain	MIC (μg/ml)		FIC[a]
	CTX	HRE 664	
Staphylococcus aureus SG 511	1	0.062	0.188
S. aureus 285	1	0.062	0.188
S. aureus 503	1	0.062	0.188
S. aureus 209	4	0.125	0.125
S. aureus Giorgio	2	0.125	0.156
Streptococcus durans	0.125	1	0.625
S. faecium	16.0	8	0.500
Escherichia coli 0114	0.002	0.25	0.375
Citrobacter freundii ATCC 8090	0.062	1.0	0.188
C. freundii J 81	32.0	1.0	0.313
C. freundii J 82	32.0	1.0	0.313
C. freundii 82 Cullm.	4.0	1.0	0.141
Enterobacter cloacae	0.015	0.5	0.500
E. cloacae P 99	>32.0	2.0	0.250
E. cloacae M 423	>32.0	1.0	0.188
E. cloacae M 447	>32.0	2.0	0.313
Serratia marcescens 378	0.062	2.0	0.375
Klebsiella pneumoniae 1082	1.0	0.5	0.313
Morganella morganii 938	2.0	0.015	0.750
Pseudomonas aeruginosa NCTC 10701	1.0	8.0	1.0

[a] FIC≤0.5: Synergistic activity, FIC=1.0: additive activity.

Table 2. *In vitro* activity of cefotaxime (CTX), HRE 664-mixtures against aerobic Gram-positive bacteria.

Strain	MIC (μg/ml)			
	Single compounds		Mixtures	
	CTX	HRE 664	4:1	1:1
Staphylococcus aureus SG 511	2	0.015	0.062	0.031
S. aureus Giorgio	2	0.031	0.125	0.062
S. aureus 2666	>32	1	4	1
S. aureus 3066	32	1	4	1
Streptococcus pyogenes 308 A	0.015	0.031	0.008	0.031
S. agalactiae B	0.015	0.125	0.031	0.125
S. equi 6580 C	0.031	0.031	0.015	0.031
S. faecium D	8	2	2	2
S. faecalis 10541 D	>32	8	16	16
S. durans D	0.25	1	0.25	0.5
Listeria monocytogenes 32	0.5	2	1	
Bacillus subtilis ATCC 6633	0.5	0.015	0.062	0.062
B. anthracis	8	0.015	0.25	0.062

Antibacterial Activity of Fixed Combinations

Tables 2~4 show the MICs of CTX and HRE 664 alone and of different fixed combinations (4:1 and 1:1) against a wide variety of aerobic bacteria. The MIC values show that the mixtures possess the same activity as the single compounds exhibiting the highest activity against the particular strain.

Thus Staphylococci (including methicillin-resistant strains) (Table 2) and *Enterobacter* sp. (Table

Table 3. *In vitro* activity of cefotaxime (CTX), HRE 664-mixtures against aerobic Gram-negative bacteria.

Strain	MIC (μg/ml)			
	Single compounds		Mixtures	
	CTX	HRE 664	4:1	1:1
Pseudomonas aeruginosa ATCC 9027	32	>32	>32	>32
P. aeruginosa 1771	16	32	32	32
P. aeruginosa 1771m	0.031	1	0.015	0.031
P. cepacia	0.5	0.004	0.031	0.008
Escherichia coli O 26	<0.002	0.125	<0.002	0.004
E. coli O 55	<0.002	0.125	<0.002	0.008
E. coli O 78	0.002	0.125	0.002	0.008
E. coli O 126	0.004	0.125	0.008	<0.002
E. coli TEM	0.008	0.25	0.008	0.031
E. coli 1507 E	0.015	0.25	0.015	0.031
Citrobacter freundii ATCC 8090	0.25	0.25	0.031	0.015
Salmonella paratyphi-A	0.008	0.25	0.008	0.015
S. typhimurium	0.008	0.5	0.008	0.062
S. typhi	0.008	0.25	0.008	0.008
Shigella flexneri	0.004	0.25	0.004	0.004
Klebsiella pneumoniae ATCC 10031	<0.002	0.25	0.004	0.004
K. aerogenes 1082 E	2	0.25	0.5	0.5
K. aerogenes 1522 E	0.015	0.5	0.015	0.031

Table 4. *In vitro* activity of cefotaxime (CTX), HRE 664-mixtures against aerobic Gram-negative bacteria.

Strain	MIC (μg/ml)			
	Single compounds		Mixtures	
	CTX	HRE 664	4:1	1:1
Enterobacter cloacae	0.125	0.5	0.062	0.125
E. cloacae 1321 E	0.015	0.5	0.015	0.015
Enterobacter sp. 2240	0.125	1	0.015	0.5
Hafnia alvei	0.031	0.25	0.008	0.008
Serratia marcescens A 20460	0.125	4	0.125	0.125
S. marcescens 6093	0.062	2	0.031	0.031
Proteus mirabilis 14273	0.008	1	0.004	0.008
P. vulgaris 867	0.031	0.5	0.125	0.25
Providencia rettgeri 936	0.031	0.5	0.125	0.125
Morganella morganii 939	0.125	2	0.062	0.062
Haemophilus influenzae	0.004	0.5	0.008	0.008

4) are included in the antibacterial spectra of the combinations. *P. aeruginosa* (Table 3) was the only species against which positive combination effects were not observed.

One of the major gaps in the antibacterial spectrum of CTX is the limited activity against Gram-negative anaerobes, especially *B. fragilis*. β-Lactamase producers among these strains are resistant to CTX and many of the non-producers are only moderately sensitive. Table 5 shows the results of our studies with fixed combinations of CTX and HRE 664 against obligate anaerobic bacteria. The addition of only 20% of HRE 664 to CTX makes the resulting combination as active as HRE 664 alone. That means that Gram-negative anaerobes (*B. fragilis*, *Bacteroides thetaiotaomicron*) are fully sensitive to the HRE 664/CTX-combinations independent of their ability to produce β-lactamase.

Table 5. *In vitro* activity of cefotaxime (CTX), HRE 664 and CTX/HRE 664-mixtures against obligate anaerobes.

Strain	MIC (μg/ml)			
	Single compounds		Mixtures	
	CTX	HRE 664	4:1	1:1
Bacteroides fragilis 312	>16.0	0.125	0.5	0.125
B. fragilis 1313	16.0	0.5	0.5	0.5
B. fragilis 17390	16.0	1.0	2.0	2.0
B. fragilis 18125	4.0	0.125	0.5	0.25
B. fragilis 19016	4.0	2.0	1.0	1.0
B. ovatus 103	>16.0	0.031	0.125	0.031
B. vulgatus 1446	>16.0	0.5	0.5	0.25
B. thetaiotaomicron 123	>16.0	0.031	0.25	0.062
B. thetaiotaomicron 1428	>16.0	2.0	2.0	2.0
B. thetaiotaomicron 1445	>16.0	4.0	2.0	4.0
B. distasonis 1366	0.062	0.5	0.062	0.062
Sphaerophorus varius 5262	>16.0	4.0	2.0	4.0
S. varius 3085	>16.0	4.0	2.0	4.0
S. freundii 1369	>16.0	4.0	2.0	4.0
Peptostreptococcus anaerobius 932	<0.016	<0.016	0.062	<0.016
Propionibacterium acnes 6919	0.125	0.031	<0.016	<0.016
P. acnes 6922	<0.016	0.031	<0.016	<0.016
Clostridium tetani 19406	8.0	0.25	0.5	0.5

Discussion

CTX is a well tolerated broad spectrum cephalosporin with high *in vitro* and *in vivo* efficacy. Unfortunately some clinically important bacterial species (*e.g. Enterobacter* sp., *B. fragilis*) show rather high degrees of resistance against this cephalosporin.

HRE 664 is a new penem antibiotic with a very broad antibacterial spectrum. It includes — with the exception of *P. aeruginosa* — nearly all Gram-positive and Gram-negative anaerobes and aerobes tested. The MICs of HRE 664 against some bacteria are slightly higher than those of CTX, but in certain cases it is markedly lower (*e.g.* Staphylococci-including methicillin-resistant strains, *Enterobacter* sp., *B. fragilis*). The β-lactamase stability of HRE 664 and especially its β-lactamase inhibitory potency is higher than that of CTX[4,5]. HRE 664 binds preferentially to the PBPs 2, 3c, 5 and 6, whereas CTX show high affinity to PBPs 1a, 1b and 3[4,5].

The important properties of both HRE 664 and CTX are obviously complementary. This hypothesis was confirmed by the *in vitro* studies on the combined activity of both compounds. We investigated the *in vitro* activity of the combination of HRE 664 and CTX. Checkerboard studies revealed synergistic activity of both antibiotics against nearly all strains tested. Fixed mixtures of CTX and HRE 664 possess high antibacterial activity and broad antibacterial spectra, excluding *Pseudomonas*, but clearly including Staphylococci, *Enterobacter* sp. and *B. fragilis*. The mixtures combine the advantages of both single compounds CTX and HRE 664.

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