The post-exposure response of Enterobacteriaceae to ceftibuten

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The responses of ten isolates of Enterobacteriaceae to ceftibuten exposure were monitored by measuring several parameters. Post-antibiotic effect (PAE), control-related effective regrowth time (CERT) and post-antibiotic sub-MIC effect (PA-SME) were determined by bacterial enumeration carried out either by impedance in combination with viable counting (IMP/VC) or by impedance in combination with bioluminescence (IMP/BIOL). Kill curves were carried out by bioluminescence, viable counting and direct microscopy and post-exposure morphology was established. Ceftibuten primarily provoked filamentation. Over 24 h, kill of up to 3.6 log₁₀ was evident by viable counting and direct microscopy at and above the MIC. Minimal kill, of up to 0.26 log₁₀, was shown by bioluminescence. PAE was found to be method dependent, with statistical differences established by Student’s t-test. PAE values of up to 0.48 h and 1.47 h (by IMP/BIOL and IMP/VC respectively) were not concentration dependent above 1 × MIC. CERT values were not method dependent, with values of up to 1.71 h also showing a lack of concentration dependence above 1 × MIC. PA-SME may reflect the situation in vivo more accurately than either PAE or CERT. In PAE and CERT studies the antibiotic is eliminated almost immediately, whereas in vivo there is gradual decrease in antibiotic levels. These persisting levels are reflected more accurately by PA-SME. Compared with PAE and CERT, significantly longer values, of up to 7.27 h, were obtained by PA-SME, although this parameter was also found to be method dependent. The results of the PA-SME studies, which may be the most clinically relevant pharmacodynamic parameter, confirm the appropriateness of the current once- or twice-daily dosing schedules despite the lack of PAE.

Introduction

Pharmacodynamics is broadly described as the study of the interaction of antimicrobial agents with microorganisms. More specifically, this area relates the concentration of an antimicrobial agent over time to the antimicrobial effect occurring at the site of infection and is thus increasingly being applied to the design of antibiotic dosing regimens. This study is an investigation of the following pharmacodynamic parameters: post-antibiotic effect (PAE), post-antibiotic sub-MIC effect (PA-SME) and control-related effective regrowth time (CERT). These parameters were studied in relation to morphology and kill induced in ten strains of Enterobacteriaceae by ceftibuten. PAE measures the delayed regrowth of bacteria after antibiotic exposure.¹ The emphasis placed on PAE is questionable as, increasingly, it is recognized that this parameter is dependent on the methods used to quantify bacterial cells. CERT, which is a measure of both PAE and kill, is independent of these methods. It has, therefore, been suggested that less emphasis should be placed on PAE data and that CERT should be used in future pharmacodynamic studies.² So far, this suggestion has not found favour, relatively few CERT studies having appeared in the literature. PA-SME is also considered by some to be a more important pharmacodynamic measurement than PAE, reflecting more closely the in vivo state of slowly declining antibiotic concentrations with long periods of persistently sub-inhibitory concentrations.

If PAE is to remain pre-eminent in the realm of pharmacodynamics, careful consideration must be given to the methodology employed in enumerating bacterial cells. Methods which have been employed in the past include the ‘reference’ viable counting method,¹ spectrophotometry,² electronic particle counting,⁴ direct microscopy,⁵⁶ bioluminescence assay of bacterial adenosine triphosphate (ATP)⁷⁸ and impedance monitoring.⁹¹⁰

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Traditional methods of bacterial enumeration employ viable counting, which relies on the growth of colony-forming units on a solid agar surface. The suitability of this method has been questioned when enumerating aberrant morphological forms induced by antibiotic exposure, as viable counting underestimates numbers of aberrant morphological forms, leading to an underestimation of the PAE. \textsuperscript{8} Previously, we have used the alternative techniques of the bioluminescence assay of ATP and impedance monitoring. We have found no significant differences in PAE measured by viable counting alone and in combination with impedance (IMP/VC) and also no significant differences by bioluminescence alone, and in combination with impedance (IMP/Biol). \textsuperscript{8} We have found significant differences in PAE measured by IMP/VC and IMP/Biol. \textsuperscript{8,11} This current study aims to further investigate PAE, PA-SME and CERT in relation to the methods used as well as in relation to post-exposure morphology and kill.

**Materials and methods**

**Bacteria, antibiotics and culture media**

The bacteria used in this study were a mixture of control strains and recent clinical isolates sensitive to ceftibuten and are listed in Table I. Strains CTX 224, CTX 603 and BLR 02 are multiply resistant, exhibiting extended spectrum \(\beta\)-lactamase (ESBL) activity due to an SHV type enzyme. \textsuperscript{12} I 112 and T 767 are clinical isolates which we have studied in the past. \textsuperscript{11} Mueller–Hinton broth was used throughout the study. Ceftibuten was provided by Schering-Plough Ltd (Welwyn Garden City, U.K.).

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC (mg/L) for inoculum of</th>
<th>(5 \times 10^5) cfu/mL</th>
<th>(1 \times 10^8) cfu/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 25922</td>
<td>0.5</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>NCTC 10418</td>
<td>0.125</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>NCTC 8879</td>
<td>0.125</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 11228</td>
<td>0.016</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>NCTC 9633</td>
<td>0.125</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>I 112</td>
<td>0.125</td>
<td>(&gt;64)</td>
<td></td>
</tr>
<tr>
<td>T 767</td>
<td>0.06</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>CTX 224</td>
<td>4.0</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>CTX 603</td>
<td>8.0</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter agglomerans</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLR 02</td>
<td>8.0</td>
<td>(&gt;64)</td>
<td></td>
</tr>
</tbody>
</table>

**Materials and methods**

MICs were determined by the microdilution method according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines. \textsuperscript{13} The standard inoculum of \(5 \times 10^5\) cfu/mL was used in addition to an inoculum of \(1 \times 10^8\) cfu/mL.

**Kill curves**

Kill curves were carried out in duplicate by exposing test cultures to ceftibuten and establishing bacterial numbers by three methods. A n overnight broth was diluted 1:100 and exposed to 0.1, 1 and 10 \(\times\) MIC. A n unexposed control was obtained by diluting an overnight broth 1:1000. Counts at times 0, 1, 2, 3, 4, 5, 6 and 24 h were determined by bioluminescence and viable counting. Counts by direct microscopy were determined up to 6 h after exposure to 0, 1, 10 and 100 \(\times\) MIC.

**Determination of PAE and CERT**

Inocula of c. \(10^7\) cfu/mL were exposed to ceftibuten (0.1–100 \(\times\) MIC) for 2 h, after which antibiotic was eliminated by 1:100 dilution in test medium. Regrowth of these and a control culture was followed by impedance (eight replicates) in combination with viable counting and bioluminescence (both of which were carried out in duplicate). \textsuperscript{8} PAE was calculated as the difference in time between antibiotic-exposed and unexposed cultures to reach \(10^7\) cfu/mL (allowing for differences in their respective inocula). \textsuperscript{8} CERT was calculated as the difference in time after antibiotic exposure and elimination for test and control cultures to grow to their pre-exposure inocula plus \(1 \log_{10}\). \textsuperscript{11}

**Determination of PA-SME**

The primary method used is based on that of Odenholt-Tornqvist et al. \textsuperscript{14} and was carried out on selected test isolates as detailed in Table II. A n inoculum of approximately \(10^7\) cfu/mL was exposed to 10 \(\times\) MIC ceftibuten for 2 h, after which the cultures were diluted to achieve sub-MIC concentrations of 0.1, 0.2 and 0.3 \(\times\) MIC. After the 2 h exposure, bioassays were performed to confirm the ceftibuten concentration. Regrowth was followed by impedance (eight replicates) in combination with viable counting (carried out in duplicate). Unexposed controls were run in parallel and PA-SME was calculated in the same way as PAE.

In addition, as a comparison, test cultures were exposed to 100 \(\times\) MIC for 2 h, followed by 1:1000 dilution, leaving 0.1 \(\times\) MIC remaining in the cultures. The growth of these cultures was followed by IMP/VC and IMP/Biol and the PA-SME calculated in the same manner as PAE.
Response of Enterobacteriaceae to ceftibuten

Determination of predominant morphological forms

Inocula of c. $10^7$ cfu/mL were exposed to ceftibuten (0.1–100 × MIC) for 2 h, after which the cultures were observed by interference contrast microscopy and the predominant morphological forms present were recorded.

Results

The MIC values for the test strains against ceftibuten can be found in Table I; all fall within the sensitive range, given that ceftibuten has a breakpoint value of 8 mg/L. Although still active against the ESBL producers, ceftibuten is markedly less active against these three isolates. In common with other filament-inducing antibiotics in its class, ceftibuten showed a marked inoculum effect, with significant increases in MIC at an inoculum of approximately $10^8$ cfu/mL. The elevations in MICs which resulted ranged from 11 to two dilution steps.

Figure 1 presents mean kill-curve data for all ten test isolates. By viable counting (Figure 1a) the cultures exposed to 0 and 0.1 × MIC showed no kill although growth was slower in the culture exposed to 0.1 × MIC. The 100 × MIC culture showed kill over the 24 h test period whereas all of the other cultures demonstrated kill over 6 h followed by concentration-dependent regrowth during the 6–24 h period. Direct microscopy (Figure 1c) gave similar results, with similar kill patterns after exposure to 1, 10 and 100 × MIC over the 6 h test period. Kill curves by bioluminescence (Figure 1b) gave similar patterns for the 0 and 0.1 × MIC exposed cultures, but not for the cultures exposed to ceftibuten concentrations of 1 × MIC or above. During the first 2 h no kill was demonstrated; in fact, growth of over 1 log$_{10}$ was observed.

Over the range of test organisms, after 2 h of exposure to ceftibuten at 0.1 to 100 × MIC, mean differences in bacterial numbers by viable counting and bioluminescence are presented in Figure 2. These differences in counts were concentration dependent up to 4 × MIC, after which a plateau effect was demonstrated.

Table III presents mean PAE and CERT values as well as the predominant morphological responses exhibited by each culture in response to ceftibuten. Using Student’s t-test, the 15 mean PAE values determined by IMP/VC were compared with those determined by IMP/BIOL. The differences in PAE values were found to be highly significant (t = 8.67). By contrast, using the t-test, the mean CERT values determined by IMP/VC and IMP/BIOL were found not to be significantly different (t = 1.61).

Figure 3 illustrates how PAE, CERT and differences in counts by bioluminescence and viable counting vary with the predominant morphological form present. There is a consistent trend whereby the greatest values were measured in cultures made up of filaments and the smallest values were measured in cultures made up of bacilli/long bacilli.

Table II presents the PA-SME values obtained for the five strains tested. PA-SME values are significantly longer than PAE values and are concentration dependent. PA-SME values obtained after exposure to 100 × MIC followed by 0.1 × MIC were determined for all ten isolates by both IMP/VC and IMP/BIOL. By the former the mean value was 1.10 h (range –0.33 to 7.17) and by the latter 1.94 h (range 0.30 to 7.27). The ten PA-SME values by these two methods are significantly different (t = 4.79).

Discussion

This study found that, after exposing the test Enterobacteriaceae to ceftibuten, most of the cultures were made up of filamentous forms (Table III) as a result of bacterial numbers by viable counting and bioluminescence.
preferential binding to penicillin binding protein 3 (PBP3) and also to PBPs 1a and 1b. Braga & Piatti also found that after exposing *Escherichia coli* isolates to ceftibuten some of the filaments bore polar or spherical enlargements, although these were rarer than filaments. In the current study filaments with spherical enlargements were almost as common as filaments amongst the *Klebsiella pneumoniae* isolates tested. To date no PBP profiles have been published for *K. pneumoniae* isolates, but it seems likely that ceftibuten binds to either PBP1 or
Response of Enterobacteriaceae to ceftibuten

In addition to PBP3 in order to induce the spherical enlargements. Aberrant morphological forms, such as filaments, are clinically beneficial in that they undergo phagocytosis more easily than bacilli, but they are problematic in the laboratory. In common with other investigators, we have found that, after antibiotic exposure, different cell enumeration methods quantify aberrant morphological forms to varying degrees. This is exemplified in Figure 2, which shows the concentration-dependent nature of the differing abilities of viable counting and bioluminescence to quantify cells after ceftibuten exposure. Counts determined by bioluminescence were greater than those by viable counting, probably because although one filament may contain as many as 20 genomes, it will be represented by only 1 cfu by viable counting. Upon cell division one filament will split into separate cells, giving the impression of a rapid increase in cell numbers. In contrast, the decrease in counts by bioluminescence was not as pronounced as that by viable counting because levels of intracellular ATP were measured directly. Bioluminescence may better represent aberrant morphological forms than viable counting but it also has disadvantages. A constant ATP content of bacterial cells is assumed, but after antibiotic exposure, an enlarged bacillus may contain more ATP than a healthy bacillus. A iso, if a TFP from intact dead cells is measured, counts determined by BIOL will be falsely inflated, giving the impression of less kill than has

### Table III. Summary of mean (± s.e.m.) PAE, CERT values and predominant morphological forms

<table>
<thead>
<tr>
<th>PAE (h)</th>
<th>CERT (h)</th>
<th>Predominant morphological formsa (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IMP/BIOl</td>
<td>IMP/Vc</td>
</tr>
<tr>
<td>E. coli (three strains)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 × MIC</td>
<td>0.40 ± 0.31</td>
<td>-0.30 ± 0.20</td>
</tr>
<tr>
<td>4 × MIC</td>
<td>0.27 ± 0.15</td>
<td>-0.44 ± 0.14</td>
</tr>
<tr>
<td>1 × MIC</td>
<td>0.31 ± 0.31</td>
<td>-0.37 ± 0.25</td>
</tr>
<tr>
<td>0.1 × MIC</td>
<td>0.04 ± 0.24</td>
<td>-0.28 ± 0.50</td>
</tr>
<tr>
<td>K. pneumoniae (six strains)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 × MIC</td>
<td>0.68 ± 0.57</td>
<td>-0.34 ± 0.22</td>
</tr>
<tr>
<td>4 × MIC</td>
<td>0.64 ± 0.61</td>
<td>-0.50 ± 0.18</td>
</tr>
<tr>
<td>1 × MIC</td>
<td>0.60 ± 0.37</td>
<td>-0.45 ± 0.20</td>
</tr>
<tr>
<td>0.1 × MIC</td>
<td>-0.13 ± 1.01</td>
<td>-0.79 ± 0.41</td>
</tr>
<tr>
<td>E. agglomerans (one strain)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 × MIC</td>
<td>1.47</td>
<td>0.48</td>
</tr>
<tr>
<td>4 × MIC</td>
<td>1.47</td>
<td>0.33</td>
</tr>
<tr>
<td>1 × MIC</td>
<td>1.44</td>
<td>0.52</td>
</tr>
<tr>
<td>0.1 × MIC</td>
<td>0.14</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Table III. Summary of mean (± s.e.m.) PAE, CERT values and predominant morphological forms

*Key: B, bacilli; LB, long bacilli; F, filaments; BF, filaments with bulges.

Figure 2. Mean differences in counts determined by bioluminescence and viable counts.

Figure 3. Predominant morphological forms versus mean differences in counts by bioluminescence and viable counts ( ), mean PAE (IMP/BIO, □) and mean CERT (IMP/BIO, □).
MIC after a plateau effect was seen. This correlates
with the morphological responses shown by the test
cultures in response to ceftibuten exposure. It was only
above 4 × MIC that all of the cultures showed a
predominance of filaments (Table III). The largest
differences in counts after ceftibuten exposure and the
longest PAE and CERT values were found in cultures
made up of filaments. PAE and CERT may represent the
time required for filamentous forms to resynthesize new
PBPs, or the time during which antibiotic which has
accumulated in the periplasmic space continues to inhibit
newly formed PBPs. They may also represent the time
during which the cells regener ate active enzyme molecules
after the bound antibiotic has dissociated from the target
site. The rate of synthesis varies for different bacteria and
this could account for corresponding variations in the
duration of both the PAE and CERT.

Impedance monitoring brings with it the associated
benefits of an automated system. The only disadvantage is
that the initial enumeration of bacterial cells must be
performed by an additional method and this value used as
a baseline. PAE and CERT values determined by IMP/VC
and IMP/BIOL are presented in Table III. Differing
abilities of viable counting and bioluminescence to
e numerate aberrant morphological forms translated into
highly significant differences between PAE values
determined by IMP/VC and IMP/BIOL (Student’s t-test,
t = 8.67). Negative PAE values determined by IMP/VC
are clearly artefactual and a function of the methodology.
In contrast there were no significant differences between
the CERT values (t = 1.61). As discussed previously, viable
counting of aberrant morphological forms probably
yields falsely low counts, which leads to an inflation of the
bactericidal activity and an underestimation of PAE when
calculated by viable counting, both alone and in combi-
nation with impedance monitoring.

Different strains of the same species do not respond in
the same way to a specific antibiotic as demonstrated by
the large standard deviation values in Table III. To illustrate
this further, the CERT values for the three isolates of
E. coli are presented in Figure 4. Differences in these
values are most marked at 100 × MIC with a range of
0.25–4.11 h. A s PAE is method dependent but CERT is
not, CERT values would yield a more meaningful pharma-
codynamic parameter to compare the results of different
research groups.

PAE and CERT determinations in vitro cannot reflect
the situation in vivo. In vitro the antibiotic is eliminated
almost instantaneously whereas in vivo there is a much
more gradual decrease in antibiotic levels depending on
the elimination half-life of the antibiotic. Once levels drop
below the MIC, sub-inhibitory levels persist and this is
more accurately reflected by measuring the PA-SME rather
than the PAE. Five of the ten test isolates were
investigated for PA-SME by IMP/VC. Sub-MIC levels
which persisted beyond the 2 h 10 × MIC exposure sig-
ificantly increased the time taken for the cultures to
regrow such that the PA-SME values were significantly
longer than the PAE values. The effect was concentration
dependent. In vivo, ceftibuten reaches maximal plasma
concentrations within 2 h of oral dosing (15 mg/L after a
400 mg dose) and persists at detectable levels for >16 h
(0.4 mg/L). Thus, PA-SME values are likely to be
clinically relevant for many pathogens and confirm the
appropriateness of the current once or twice a day dosing
schedules despite the lack of PAE.

In conclusion, concentration-dependent differences in
post-antibiotic exposure counts by bioluminescence and
viable counting were found after exposure of the test
Enterobacteriaceae to ceftibuten. These may form a direct
relationship with morphological forms present in each
culture. PAE, PA-SME and kill were found to be method
dependent whereas CERT was not. Generally neither
PAE nor CERT was concentration dependent above
1 × MIC, although different strains of E. coli and K.
pneumoniae responded to ceftibuten differently with
respect to PAE and CERT. The results of the PA-SME
studies, which may be the most clinically relevant
pharmacodynamic parameter, confirm the appropriate-
ness of the current once or twice a day dosing schedules
despite the lack of PAE.

Acknowledgements

We are grateful to Schering-Plough Ltd for financial
support. These data were presented in part at the Thirty-
Response of Enterobacteriaceae to ceftibuten

Seventh Interscience Conference on Antimicrobial Agents and Chemotherapy, 28 September–1 October 1997, Toronto, Canada (Abstract A79).

References


Received 4 March 1998; returned 27 April 1998; revised 11 May 1998; accepted 6 August 1998