Activity of ketolide ABT-773 (cethromycin) against erythromycin-resistant Streptococcus pneumoniae: correlation with extended MLSK phenotypes

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Objectives: (i) To determine the inhibitory and bactericidal activities of ABT-773, a novel ketolide, against sensitive and erythromycin-resistant pneumococci; (ii) to subdivide erythromycin-resistant pneumococci into resistance phenotypes, more extensive than the conventional M and MLSB groups, by assessing susceptibilities to, and interactions between, erythromycin (14-membered macrolide), clindamycin (lincosamide), rokitamycin (16-membered macrolide), ABT-773 (ketolide), quinupristin (streptogramin B) and dalfopristin (streptogramin A).

Methods: MICs and MBCs of ABT-773 were determined for 165 strains of pneumococci (113 resistant to erythromycin). Extended phenotypes for the erythromycin-resistant strains were described in terms of intrinsic susceptibility to, and induction of resistance by, the antibiotics listed above.

Results: Erythromycin-resistant strains could be divided into 10 extended phenotypes (designated II–XI), two of which (II and IX) predominated. ABT-773 at 0.12 mg/L inhibited 109 strains (median 0.03 mg/L). MICs for the other four strains (of phenotypes X and XI) were 0.25–1 mg/L. MICs were only slightly higher when measured on agar in CO2 than by the NCCLS method (in broth in air). MBCs were usually ≤2 × MIC, but for 10 strains (eight of phenotype X, one each of types IX and XI) MBCs were >1 mg/L, and three of the latter (all type X) were tolerant. Clones of reduced susceptibility (MICs 1–8 mg/L, increased by up to 32-fold) could be isolated from some strains of phenotypes VII, IX and X, but not from those of type II (efflux mechanism) or from erythromycin-sensitive strains.

Conclusions: ABT-773 was active against all 113 erythromycin-resistant pneumococci tested, which belonged to 10 phenotypes. Extended phenotyping of pneumococci revealed interesting and potentially useful subdivisions of the classical phenotypes.

Introduction

Infections of the respiratory tract continue to be a major cause of morbidity and mortality, and the emergence of aetiological agents (especially pneumococci) resistant to conventional antibiotics has been a spur to the development of novel agents to treat such infections. In particular, increasing resistance to macrolides has reduced the therapeutic value of both older (e.g. erythromycin) and newer (e.g. azithromycin) members of this family. However, novel chemical manipulation of the macrolide structure has resulted in a new class, the ketolides, that are active against many erythromycin-resistant strains.1 Ketolides lack the 3-cladinose moiety characteristic of other macrolides; examples are telithromycin (licensed in several countries) and ABT-773 (presently in phase III clinical trials); these differ in the nature of the chemical substitutions at the 6 and 11/12 positions of the 14-membered ring.

We report here the activity of the ketolide ABT-773 against a collection of erythromycin-resistant pneumococci, extended phenotypes of which were determined by a simple disc method2 using various members of the macrolide-lincosamide-streptogramin (MLS) group.

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Materials and methods

Antibiotics

Pure compounds were obtained as follows: erythromycin BP free base (Lilly Industries, Basingstoke, Hants, UK); dalfopristin and quinupristin (Rhône Poulenc Rorer, Collegeville, PA, USA); rokitamycin (ISF SpA, Milan, Italy); ABT-773 (Abbott Laboratories, Abbott Park, IL, USA).

Antibiotic-containing discs were obtained as follows: 15 µg erythromycin, 2 µg clindamycin, 15 µg quinupristin/dalfopristin (Synercid) from Oxoid (Basingstoke, Hants, UK); 15 µg ABT-773 from Abbott Laboratories; discs containing 15 µg quinupristin, dalfopristin or rokitamycin were made by imbibing 6 mm Whatman AA discs (A1 Lab Supplies, London, UK).

Media

Mueller–Hinton agar (MHA) and broth (MHB), brain–heart infusion (BHI) broth and Columbia agar were from Oxoid. Blood agar (BA) was made by adding 5% whole horse blood to Columbia agar.

Bacterial strains

*Streptococcus pneumoniae* strains were identified by colonial appearance, Gram’s stain and sensitivity to optochin. The test group comprised 113 erythromycin-resistant strains, all isolated from clinical material. Seventy-eight were from the UK, 35 from Belgium; all were recent isolates, and comprised all the erythromycin-resistant strains in our laboratory collection. A control group of 52 erythromycin-sensitive strains was selected at random from the collection.

Sensitivity testing

MICs were determined according to the NCCLS guidelines, i.e. by microdilution in MHB + 5% lysed horse blood with an inoculum of 10^4 cfu, incubated in air, and also by agar dilution on MHA + 5% whole sheep blood with an inoculum of 10^4 cfu in 5% CO_2. MBCs were determined by subculturing on to BA 0.01 mL from each well showing no growth in the microdilution test.

Susceptibility to other antibiotics was determined by the disc method, following NCCLS guidelines. Breakpoints were as given in the latter guidelines; for ABT-773 the suggested value of ≤1 mg/L was taken, and for rokitamycin the value for erythromycin was used.

Determination of extended phenotype

Conventional testing for macrolide resistance phenotype, involving disc testing with erythromycin and clindamycin, alone and in combination, allows a maximum of five classical phenotypes to be discerned, of which four (sensitive; ‘M’, associated with efflux; ‘inducible’ MLS_R; ‘constitutive’ MLS_B) are found in pneumococci. This analysis can be considerably augmented by including results of testing a ketolide, rokitamycin (a 16-membered macrolide) and the streptogramin A and B components dalfopristin and quinupristin, again alone and in combination, and thus determining the extended phenotype, as done previously with staphylococci.

Bacterial growth was harvested from BA, suspended in water to McFarland 0.5 and spread with a swab on MHA + 5% horse blood. Each plate was set with discs (2 cm apart) arranged as shown in Figure 1. Sizes and shapes of zones were recorded after overnight incubation in air. If the nature of a particular interaction was unclear, the individual test was set up again with distances between the discs being varied as appropriate.

Results were interpreted and recorded as follows: strains were scored as sensitive or resistant according to zone diameter, and inducible resistance was deduced when a reduced or D-shaped zone was observed. Only compounds to which the test strain was resistant were tested as inducing agents. Due to the low intrinsic activity of the individual streptogramin components quinupristin and dalfopristin against pneumococci, results with these compounds were recorded only in terms of their ability to induce resistance.

The different phenotypes observed were described by writing their constitutive resistances followed by an oblique followed by resistances inducible by erythromycin (in almost all cases, only erythromycin acted as an inducing agent; in the rare instances where other agents acted as inducers, this is indicated separately). The antibiotics are abbreviated: M, erythromycin; L, clindamycin; K, ketolide; Mac, rokitamycin. Thus, for example, a strain resistant to erythromycin and clindamycin, and in which resistance to rokitamycin is induced by erythromycin would be designated ML/Mac. The
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different phenotypes determined are distinguished by roman numerals, I (fully sensitive; control group) to XI.

Time–kill experiments

One hundred millilitre amounts of MHB + 5% lysed horse blood in 250 mL conical flasks were inoculated with \( \sim 10^8 \) cfu (1 mL of a suspension of cells harvested from overnight cultures on BA, adjusted to McFarland 1), and various multiples (1 \( \times \), 2 \( \times \), 4 \( \times \)) of the MIC of ABT-773 were added. Flasks were incubated with rotation (50 cycles/min) at 37°C, and samples were taken at intervals for viable count determination on BA.

Selection for resistance

Cultures (10 mL in BHI broth) were spun down and resuspended in 1 mL of water. A viable count was made, and 0.1 mL (\( \sim 10^9 \) cfu) spread on MHA + 5% sheep blood containing 10 \( \times \) MIC of ABT-773. Colonies were counted after 48 h incubation, and the proportion of cells in the original inoculum able to grow was calculated. MIC of ABT-773 and extended resistance phenotype were determined for the outgrowers, as above.

Results

Phenotyping

Using results obtained only from susceptibility to, and interaction between, erythromycin and clindamycin, the 113 erythromycin-resistant strains could be divided between the three classical phenotypes: M (resistant only to erythromycin), 41 (36.3%); inducible MLSB (resistant to erythromycin, which induces resistance to clindamycin), seven (6.2%); and constitutive MLSB (resistant to erythromycin and clindamycin), 65 (57.5%).

However, using the extended phenotyping scheme described above, each of the classical resistance phenotypes could be broken down further, giving a total of 10 phenotypes (II–XI; I describes erythromycin-sensitive strains), as shown in Table 1. The three most commonly found phenotypes were II, IX and X, comprising together 87.6% of the strains.

All the strains were sensitive to the ketolide ABT-773 (see below), but resistance to this antibiotic could be induced by erythromycin in 69 strains (phenotypes III, V, VII, IX and X), and by other members of the MLSB group (namely, some or all of clindamycin, rokitamycin and quinupristin) in 14 strains (phenotypes X and XI; see Table 2).

The inducibility of resistance to ABT-773 by several different MLSB antibiotics can give rise to interesting visual effects, most dramatically the formation of a square zone (Figure 2).

Antibiotic sensitivity

Antibiotics belonging to the MLSB group. All 52 of the strains in the control group (phenotype I) were by definition sensitive to erythromycin; they were also sensitive to ABT-773 (MICs 0.008–0.03 mg/L, Table 3), and by the disc method, also to clindamycin and rokitamycin. In contrast, all 113 test strains were, by definition, resistant to erythromycin; all were sensitive to ABT-773 using the suggested breakpoint of 1 mg/L (Table 3), their susceptibility usually being slightly less (two-

Table 1. Reconciliation of classical and extended macrolide-lincosamide-streptogramin-ketolide phenotypes in pneumococci

<table>
<thead>
<tr>
<th>Erythromycin resistance status</th>
<th>‘Classical’ phenotype</th>
<th>Extended phenotype</th>
<th>Constitutive/inducible resistances$^a$</th>
<th>No. of strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
<td>S</td>
<td>I</td>
<td>nil</td>
<td>52</td>
</tr>
<tr>
<td>Resistant</td>
<td></td>
<td></td>
<td></td>
<td>113</td>
</tr>
<tr>
<td>‘M’ (efflux-mediated)</td>
<td>II</td>
<td>M</td>
<td></td>
<td>38 (33.6)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>M/K</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>‘Inducible MLSB’</td>
<td>IV</td>
<td>M/Mac</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>M/K/Mac</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>‘Constitutive MLSB’</td>
<td>VI</td>
<td>M/L/Mac</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>VII</td>
<td>M/L/K/Mac</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>VIII</td>
<td>ML/Mac</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>IX</td>
<td>ML/K/Mac</td>
<td></td>
<td>48 (42.5)</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>ML/Mac/K</td>
<td></td>
<td>13 ( ^\circ ) (11.5)</td>
</tr>
<tr>
<td></td>
<td>XI</td>
<td>ML/Mac</td>
<td></td>
<td>1( ^\circ )</td>
</tr>
</tbody>
</table>

$^a$M, erythromycin; L, clindamycin; Mac, rokitamycin; K, ABT-773.

In some strains Mac and/or quinupristin are also inducers (see Table 2).

Mac and quinupristin (not M) induce resistance to K (see Table 2).

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or three-fold) than that of the erythromycin-sensitive strains. However, four strains (A13, B20, T76 and T84), belonging to phenotypes IX and X, were clearly less susceptible than the others, MICs of ABT-773 being 0.25–1 mg/L. The 113 strains were also all sensitive to the quinupristin/dalfopristin combination (Synercid), despite the poor activity of the individual components (86% and 43%, respectively) were sensitive to rokitamycin (phenotypes II–IX) and clindamycin (phenotypes II–VII).

Table 4 compares MIC parameters determined by the NCCLS method (as in Table 3), with values found using the agar dilution method. The latter MICs (determined in the presence of CO₂) were usually one dilution higher. It thus appears that either method is acceptable for measuring MICs of ABT-773 for pneumococci.

MBCs were determined for all 165 strains, and parameters shown in Table 4 suggest that the ketolide was generally bactericidal at the MIC or one dilution higher. However, 10 strains had MBCs > 1 mg/L: eight of these (B20, T26, T68, T76, T77, T78, T80, T84) were phenotype X, and the others (A13 and T12) were phenotypes XI and IX, respectively. Three of these strains (T26, T68 and T80) can be regarded as tolerant, having MBC/MIC ratios of ≥8.

Other antibiotics. There were different associations in the various phenotypes between resistances to unrelated antibiotics: thus, phenotype II was more likely to be resistant to ofloxacin (45%) and less to tetracycline (26%), while phenotype IX strains were less often resistant to ofloxacin (6%) but more frequently to chloramphenicol (48%). All the type X strains were sensitive to ofloxacin and resistant to tetracycline.

Mutant selection

Seventeen strains from various phenotypes were examined for the presence of individual cells able to grow at 10 × the respective MIC of ABT-773. No mutants were found from strains of phenotypes I (erythromycin sensitive) and II (M-type, efflux). Bacteria with decreased sensitivity were
ABT-773 against erythromycin-resistant pneumococci

Table 3. MICs of ABT-773 for pneumococci of different phenotypes, determined by broth dilution

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. strains</th>
<th>0.008</th>
<th>0.015</th>
<th>0.03</th>
<th>0.06</th>
<th>0.12</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>geo. mean</th>
<th>MIC50</th>
<th>MIC90</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>52</td>
<td>11</td>
<td>36</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.014</td>
<td>0.015</td>
<td>0.015</td>
</tr>
<tr>
<td>II</td>
<td>38</td>
<td>2</td>
<td>25</td>
<td>6</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<td>IV</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.008</td>
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<td></td>
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<tr>
<td>VI</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>48</td>
<td>8</td>
<td>31</td>
<td>8</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
<td>0.06</td>
<td>0.5</td>
</tr>
<tr>
<td>X</td>
<td>13</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.13</td>
<td>0.06</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XI</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.008</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

found among strains from other phenotypes (Table 5), but MICs usually did not exceed 2 mg/L, except in the case of strain T68, where the MIC was 8 mg/L. Phenotyping of the outgrowers showed that the inducible resistance to ABT-773 present in the parent strains had become constitutive, giving a phenotype of MLMacK, except for strain D12 (phenotype VII) where resistance was still inducible against a background of reduced intrinsic susceptibility.

Time–kill

Three strains, of different phenotypes, were tested. For two (T18, type I, and T120, type IX) killing was slow (≈1 log in 6 h). However, the third strain (T34, type II) was killed more quickly, the viable count falling by 99.9% in ≈5 h.

Discussion

The incidence of resistance to erythromycin among pneumococci varies widely according to geographical location. Extreme values from the literature are 4% in Estonia7 to 90% in Taiwan (P.-R. Hsueh, unpublished results, cited in Hsueh et al.8); a recent worldwide survey9 reported an overall figure of 32%. At The Royal Free Hospital, we found an incidence of 16.4% in 2001, similar to that reported overall for the United Kingdom by others9,10 namely 11–13%. There is general agreement that such resistance is on the increase.

There are also geographical differences with regard to resistance phenotypes. In the USA11 and Canada12 an efflux mechanism (the M phenotype) is at least as common as ribosomal modification (MLSb), whereas in many other countries the MLSb phenotype predominates, sometimes by 10-fold or more, as in France13 and Spain14, and in other locations in a less pronounced way, e.g. two- or four-fold in Italy15 and Germany.16

Our finding that all the erythromycin-resistant pneumococci tested, irrespective of phenotype (and thus of genotype), were sensitive to the ketolide ABT-773, taking a breakpoint of MIC ≤1 mg/L, is largely in agreement with results of others.17–21 In two of these reports17,18 ermB strains requiring 2 and 4 mg/L for inhibition were found: these would be regarded as intermediate or resistant by the suggested breakpoints.5 It is noticeable that there are quite large differences between the MIC parameters reported in these studies, e.g. MIC50 for erythromycin-sensitive strains has ranged18,21 from 0.002 to 0.06 mg/L. Telithromycin appears somewhat less active than ABT-773 against sensitive pneumococci and those of mef(A) genotype, but the two ketolides exhibit similar activity against other strains.21

Pneumococci differ from staphylococci in that isolates of the former species showing the ‘constitutive MLSB’ phenotype (VIII–XI) remain sensitive to ketolides, whereas in staphylococci this phenotype is accompanied by resistance.2,14 On the other hand, while ketolides do not themselves induce the enzyme responsible for MLSB resistance,22 resistance to ABT-773 could be induced by erythromycin or another related antibiotic in pneumococcal phenotypes III, V, VII, IX and X (61.1% of those tested). However, this does not present

Table 4. MICs of ABT-773 for 163 pneumococci

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MIC (mg/L)</th>
<th>agar dilution</th>
<th>broth dilution</th>
<th>MBC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>0.015→&lt;1</td>
<td>0.008→1</td>
<td>0.008→&lt;1</td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>0.06</td>
<td>0.03</td>
<td>0.06</td>
<td>0.5</td>
</tr>
<tr>
<td>90%</td>
<td>0.12</td>
<td>0.06</td>
<td>0.06</td>
<td>0.5</td>
</tr>
<tr>
<td>Geo. mean</td>
<td>0.055</td>
<td>0.032</td>
<td>0.057</td>
<td></td>
</tr>
</tbody>
</table>
a significant practical problem, as it seems unlikely that a patient would be treated simultaneously with a ketolide and another macrolide.

We agree with Hyde et al.\(^\text{11}\) that strains with the M phenotype (e.g. our phenotype II organisms) were less likely to be resistant to chloramphenicol or tetracycline, while those of the MLS\(_B\) phenotype (mostly phenotype IX) were more often resistant to those antibiotics.

Nilius et al.\(^\text{17}\) suggested that the difference between the activity of ABT-773 against constitutive \(ermB\) strains of \(Streptococcus pyogenes\) and pneumococci of similar genotype was due to methodological variation (broth versus agar, incubation in air versus in CO\(_2\)), but they did not investigate this phenomenon further. However, we found (Table 4) only a minor difference in the activity of ABT-773 against pneumococci when tested in parallel in broth incubated in air (NCCLS recommendations) and on agar incubated in CO\(_2\). This suggests that the influence of methodology on apparent sensitivity may be species specific, as we have reported previously.\(^\text{23}\)

In conclusion, ABT-773 is active against a wide range of resistant pneumococci, and the extended phenotyping scheme used here reveals interesting and possibly useful subdivisions of the classical phenotypes.

Strains of phenotype X (a sub-group of the classical phenotype ‘constitutive MLS\(_B\)’) appear of particular interest, as they are less sensitive to ABT-773 than other types (Table 1), they may contain individuals of decreased susceptibility (Table 5), and they seem to be more difficult to kill than other phenotypes (the MBC for eight of the 13 was >1 mg/L); fortunately they are relatively rare (11.5% of all the resistant strains tested). They are readily distinguished from the more commonly found phenotype IX (which has the same classical phenotype) by being intrinsically resistant to the 16-membered macrolide rokitamycin. Extended phenotyping may be helpful in epidemiological studies, and may also act as a springboard to further investigation of the genetic basis of pneumococcal resistance to antibiotics of the macrolide-lincosamide-streptogramin-ketolide (MLSK) group.

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## References


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