Mechanism of Action of NB2001 and NB2030, Novel Antibacterial Agents Activated by β-Lactamases

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Two potent antibacterial agents designed to undergo enzyme-catalyzed therapeutic activation were evaluated for their mechanisms of action. The compounds, NB2001 and NB2030, contain a cephalosporin with a thienyl (NB2001) or a tetrazole (NB2030) ring at the C-7 position and are linked to the antibacterial triclosan at the C-3 position. The compounds exploit β-lactamases to release triclosan through hydrolysis of the β-lactam ring. Like cephalothin, NB2001 and NB2030 were hydrolyzed by class A β-lactamases (Escherichia coli TEM-1 and, to a lesser degree, Staphylococcus aureus PC1) and class C β-lactamases (Enterobacter cloacae P99 and E. coli AmpC) with comparable catalytic efficiencies ($k_{cat}/k_m$). They also bound to the penicillin-binding proteins of S. aureus and E. coli, but with reduced affinities relative to that of cephalothin. Accordingly, they produced a cell morphology in E. coli consistent with the toxophore rather than the β-lactam being responsible for antibacterial activity. In biochemical assays, they inhibited the triclosan target enolyl reductase (FabI), with 50% inhibitory concentrations being markedly reduced relative to that of free triclosan. The transport of NB2001, NB2030, and triclosan was rapid, with significant accumulation of triclosan in both S. aureus and E. coli. Taken together, the results suggest that NB2001 and NB2030 act primarily as triclosan produgs in S. aureus and E. coli.

The ubiquitous occurrence of β-lactamases in bacteria and their association with clinical resistance to β-lactams have allowed strong interest in these enzymes to be sustained (3, 26). β-Lactamases are periplasmic in gram-negative bacteria, while they are exocellular in gram-positive bacteria. They are conveniently divided into four classes (classes A, B, C, and D) on the basis of amino acid sequence homologies (Ambler classification) (1, 15). Of these classes, classes A and C are of the greatest clinical significance. β-Lactamase inhibitors, such as clavulanic acid and the penicillanic acid sulfones, mostly target class A enzymes; and their use in combination with older compounds has restored the broad-spectrum activities of older -lactamases to generate novel antibacterial drugs with different C-3 linkers that affect chemical stability, and different toxophores that are released upon opening of the -lactam ring. Two of these compounds, NB2001 and NB2030, contain cephalothin (thienyl) and cefazolin (tetrazole) side chains at position C-7, respectively and the enolyl reductase inhibitor triclosan at the C-3 position of the cephem nucleus (Fig. 1). Triclosan is designed to act as a toxophore that is released upon ß-lactam hydrolysis in ß-lactamase-positive bacteria. Here we describe studies aimed at elucidating the mechanisms of action of NB2001 and NB2030 in Escherichia coli and Staphylococcus aureus. In E. coli, the overall mechanisms of action of these compounds were deduced from the cell morphology observed after treatment of the cells with the compounds. Their levels of transport into bacterial cells, their levels of penicillin-binding protein (PBP) binding, their activities as β-lactamase substrates, and the activity of the bound triclosan moiety relative to that of free triclosan against the enol reductase target were also determined.

MATERIALS AND METHODS

Chemicals. Unless otherwise noted, biochemicals were obtained from Sigma (St. Louis, Mo.) and culture media were obtained from Difco (Sparks, Md.). Triclosan was kindly provided by KIC Chemicals (Armonk, N.Y.), anhydrous solvents were purchased from Aldrich Chemicals (Milwaukee, Wis.), and nitrocefin was from Calbiochem (San Diego, Calif.). Benzyl-[1-14C]penicillin (58 mCi/mmol) and α-[1,14C]aminobutyric acid (50 mCi/mmol) were purchased from Amersham (Piscataway, N.J.) and ICN Pharmaceuticals (Irvine, Calif.), respectively.

Organisms. Unless stated otherwise, experiments were performed with standard susceptible strains obtained from the American Type Culture Collection and subsequently by Mobashery and Johnston (20) and other investigators (6, 10, 16). It has been part of NewBiotics' general enzyme-catalyzed therapeutic activation (ECTA) prodrug approach that harnesses unique enzymes in bacteria to achieve selective release of cytotoxic agents from substrate-like molecules (16; M. V. Sergeeva, G. H. Khabbata, B. E. Cathers, R. S. Castillo, V. R. Doppalapudi, H. H. Bendall, A. R. Bueno, J. Y. Lee, Q. Li, and N. H. Georgopapadakou, Abstr. 102nd Gen. Meet. Am. Soc. Microbiol., abstr. A-113, 2002). These antibacterial agents developed by the ECTA approach are designed so that neither they nor their products inactivate the enzymes that activate them.

We have synthesized a variety of cephalosporins with different C-7 side chains that affect enzymatic hydrolysis, cephalosporins with different C-3 linkers that affect chemical stability, and different toxophores that are released upon opening of the -lactam ring. Two of these compounds, NB2001 and NB2030, contain cephalothin (thienyl) and cefazolin (tetrazole) side chains at position C-7, respectively and the enolyl reductase inhibitor triclosan at the C-3 position of the cephem nucleus (Fig. 1). Triclosan is designed to act as a toxophore that is released upon -lactam hydrolysis in -lactamase-positive bacteria. Here we describe studies aimed at elucidating the mechanisms of action of NB2001 and NB2030 in Escherichia coli and Staphylococcus aureus. In E. coli, the overall mechanisms of action of these compounds were deduced from the cell morphologies observed after treatment of the cells with the compounds. Their levels of transport into bacterial cells, their levels of penicillin-binding protein (PBP) binding, their activities as -lactamase substrates, and the activity of the bound triclosan moiety relative to that of free triclosan against the enol reductase target were also determined.
(ATCC; Manassas, Va.): E. coli ATCC 25922, S. aureus ATCC 29213, Enterobacter cloacae ATCC 13047, Enterococcus faecalis ATCC 29212, S. aureus ATCC 33591 (mecillin resistant), Pseudomonas aeruginosa ATCC 27853, Streptococcus pneumoniae ATCC 49619, and S. pneumoniae ATCC 700671. E. coli TE18 (22) was kindly provided by H. Nikaido. E. cloacae P99 (13, 17, 31) was provided by K. Bush, and S. aureus strains RN4220 and PC1 (14, 30, 32) were provided by J. Iandolo. E. coli (TEM-1) and its parent strain, E. coli N, have been described previously (16).

MIC determinations. Antibacterial activity was determined by the broth microdilution method in Mueller-Hinton broth (Difco) according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (21).

Expression and assay of β-lactamase in whole cells. β-Lactamase expression in each strain was determined colorimetrically. A 1 ml mid-log-phase culture was grown under standard conditions for MIC determination. Nitrocefin assay. Positive fractions were pooled, concentrated to 0.5 mg/ml in dimethyl sulfoxide (DMSO; 0.25%). The membranes were collected by centrifugation at 45,000 x g for 30 min, washed with 50 mM potassium phosphate buffer (pH 7.0) and stored at −70°C if they were not used immediately. For PBP binding assays, the membranes were solubilized in 50 mM potassium phosphate buffer (pH 7.5) containing 1 M sodium chloride, 2% Triton X-100, and 1 mM β-mercaptoethanol, as described previously (9). Samples containing 100 μg of solubilized membrane protein were incubated with increasing concentrations of test compound at 30°C for 10 min. After the addition of 1 μl (0.5 μCi) of benzyl-[14C]penicillin, the incubation was continued for another 10 min. Protein was precipitated with acetone and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide and 0.1% bisacrylamide for E. coli; 10% acrylamide and 0.27% bisacrylamide for S. aureus). The gels were subsequently incubated with Enhance (New England Nuclear, North Billerica, Mass.), dried, and fluorographed at −70°C to visualize the radioactive bands.

Enol reductase cloning, purification, and assay. The fabI gene, which encodes enol reductase, of E. coli MG1655 (11) was amplified by PCR with primers EC5 (5'-CTCTCTGCTATGGTTGTTTCTTCGCGTAAG-3') and EC3 (5'-CTCTGATCTTTATGGTTGTTTCTTCGCGTAAG-3').* The PCR product was digested with NdeI and BamHI and ligated into NdeI- and BamHI-digested expression plasmid pET28b (+). The new construct was transformed into BL21(DE3) cells, and the expressed enol reductase was purified with Ni²⁺-His-binding metal chelation resin (Novagen) and Novagen His-tagged purification reagents. Protein purity was estimated to be >90% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Purified enol reductase (final concentration, 24 nm) was added to an assay mixture (100 μM NADH, 100 mM sodium phosphate buffer [pH 7.0], 4% glycerol, various concentrations of crotonyl coenzyme A [crotonyl-CoA], and DMSO [final concentration, 1%]) in a final volume of 0.12 ml and incubated at 25°C. The rate of the reaction was measured by the time to reach the NADH absorbance peak at 340 nm. The Kₘ of crotonyl-CoA was determined to be 0.5 mM.

The 50% inhibitory concentrations (IC₅₀) of clofibrate, NB2001, and NB2030 were determined by incubating the assay mixture with 0.50 mM crotonyl-CoA and increasing concentrations of inhibitor. The rates of the reaction with various inhibitor concentrations were plotted, and IC₅₀ was determined by plotting percent activity (relative to that of the control without an inhibitor) against the inhibitor concentration. To determine the IC₅₀ of each inhibitor, data were fitted to the equation percent activity = 100[(1 + [protein]/IC₅₀). ]

To determine the IC₅₀ in the presence of NAD⁺, enol reductase was pre-incubated with 100 μM NAD⁺, test compound, and the reaction mixture (without crotonyl-CoA) at 25°C for 30 min; and then the reaction was initiated by the addition of crotonyl-CoA.

Membrane integrity. Membrane integrity was measured by determination of the release of [α-¹⁴C]aminoacetic acid, a radiolabeled nonmetabolizable amino acid (7). Briefly, the assay involves feeding of the labeled amino acid to the cells for 30 min, treatment of the cells with the test compound for 60 min, and measurement of the radioactivity remaining in the cells. The cationic detergent hexadecyltrimethyl ammonium bromide was used as a positive control treatment;
when it was used at a concentration of 200 μg/ml (0.02%; wt/vol), it released 90% of the cell-associated radioactivity.

Cell morphology. E. coli ATCC 25922 was grown to mid-log phase in antibiotic medium 3 (Difco) by previously published procedures (5). Cells were diluted 1/100 in 1 ml of antibiotic medium 3 containing one of the test compounds at 0.2, 1, and 5 times the MIC. At 1 and 2 h after dilution, 5-l samples were taken,

\[ \frac{1}{100} \text{in 1 ml of antibiotic medium 3 containing one of the test compounds at 0.2, 1, and 5 times the MIC.} \]

the cells were fixed on the slide with 4',6-diamidino-2-phenylindole (DAPI) solution (5 g/ml), color change within 30 min; NT, not tested. b MRSA, methicillin-resistant S. aureus.}

### RESULTS

**In vitro antibacterial activity.** As expected, cephalothin and cefazolin showed decreased activities against all strains expressing β-lactamases: E. coli TEM-1, E. coli TE18, E. cloacae P99, and methicillin-resistant S. aureus (Table 1). They did not show decreased activity against S. aureus PC1, although the strain was resistant to benzylpenicillin (MIC > 32 μg/ml). Triclosan was highly active against all organisms (MIC < 1 μg/ml) except P. aeruginosa, against which it was inactive (MIC > 128 μg/ml). The lamactecins NB2001 and NB2030 generally had reduced activities relative to that of triclosan, but they were up to 1,000-fold more active against β-lactamase-positive strains than against β-lactamase negative strains, suggesting the β-lactamase-mediated release of triclosan. The exception was against β-lactamase-producing strain S. aureus PC1, which was reproducibly as susceptible as or less susceptible than the non-β-lactamase producer S. aureus ATCC 29213 to the two lamactecins.

### β-Lactamase kinetics.** For cephalothin, the \( K_m \) values of all four enzymes were consistent with those reported in the literature (4, 8, 29, 32) (Table 2). For NB2001 and NB2030, the \( K_m \) values were generally similar to those for cephalothin; for the TEM-1 enzyme, however, the \( K_m \) values were significantly lower. The anomaly might be related to enzyme inhibition at higher concentrations of NB2001 and NB2030. The relative catalytic efficiencies of NB2001 and NB2030 were generally within a sixfold range of those of cephalothin except in tests with strain PC1, for which they were significantly higher (Table 2).

**Binding to PBPs.** NB2001 and NB2030 had significantly reduced affinities for PBPs 1a, 3, and 4 of E. coli compared to those of cephalothin (Table 3). The two compounds also had reduced affinities for PBPs 1, 2, and 3 of S. aureus.

**Enoyl reductase inhibition.** Triclosan inhibited purified E. coli enoyl reductase, with IC\(_{50}\) similar to those reported previously (11) (Table 4). On the other hand, the IC\(_{50}\) of NB2001 and NB2030 were 16 and 70 times higher than those of triclosan in the absence of preincubation with NAD\(^+\). The IC\(_{50}\) of NB2030 was 160 times higher than that of triclosan when the preincubation step was included. The results can be accounted for by the amount of free triclosan detected by high-pressure liquid chromatography (HPLC) in the two compounds developed by the ECTA approach (Table 4).
Effects on membrane integrity. Both NB2001 and NB2030 produced direct effects on the membranes of \textit{E. coli} and \textit{S. aureus}, as did triclosan, but at concentrations of 50 to 100 \(\mu\)g/ml, which are much higher than their MICs or the concentrations that affect enoyl reductase, suggesting that their antibacterial activities are due to enoyl reductase inhibition rather than direct effects on the membrane. The results are consistent with the bacteriostatic effects of triclosan and NB2001 reported previously (16). Interestingly, the two compounds were toxic to \textit{S. aureus} cells, while NB2030 was detected in both organisms (Fig. 3A). In contrast, the levels of NB2001 uptake into \textit{E. coli} cells were reduced compared to those into \textit{S. aureus} cells, while NB2030 was detected in \textit{E. coli} cell extracts but not in \textit{S. aureus} cell extracts after exposure to the compound (Fig. 3B and C). Intracellular levels of NB2001 and NB2030 may also have been affected by chemical or enzymatic

### Table 2. Kinetic parameters for compounds developed by the ECTA approach and comparison with those for \(\beta\)-lactams with different \(\beta\)-lactamases

<table>
<thead>
<tr>
<th>(\beta)-Lactamase (species) and substrate</th>
<th>(K_m) ((\mu)M)</th>
<th>Relative (k_{cat})</th>
<th>Relative (k_{cat}/K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1 (\textit{E. coli})</td>
<td>46</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>100</td>
<td>61</td>
<td>28</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>14</td>
<td>20</td>
<td>67</td>
</tr>
<tr>
<td>NB2001</td>
<td>24</td>
<td>14</td>
<td>26</td>
</tr>
<tr>
<td>NB2030</td>
<td>68</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PC1 (\textit{S. aureus})</td>
<td>7.8</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>6.3</td>
<td>0.59</td>
<td>6.4</td>
</tr>
<tr>
<td>NB2001</td>
<td>20</td>
<td>18</td>
<td>61</td>
</tr>
</tbody>
</table>

\(\text{TEM-1 (E. coli) benzylpenicillin: } 46 \mu\text{M, relative } k_{\text{cat}} = 100, \text{relative } k_{\text{cat}}/K_m = 100.\)

\(\text{TEM-1 (E. coli) cephalothin: } 100 \mu\text{M, relative } k_{\text{cat}} = 61, \text{relative } k_{\text{cat}}/K_m = 28.\)

\(\text{TEM-1 (E. coli) NB2001: } 14 \mu\text{M, relative } k_{\text{cat}} = 20, \text{relative } k_{\text{cat}}/K_m = 67.\)

\(\text{TEM-1 (E. coli) NB2030: } 24 \mu\text{M, relative } k_{\text{cat}} = 14, \text{relative } k_{\text{cat}}/K_m = 26.\)

\(\text{PC1 (S. aureus) benzylpenicillin: } 68 \mu\text{M, relative } k_{\text{cat}} = 100, \text{relative } k_{\text{cat}}/K_m = 100.\)

\(\text{PC1 (S. aureus) cephalothin: } 7.8 \mu\text{M, relative } k_{\text{cat}} = <0.001, \text{relative } k_{\text{cat}}/K_m = <0.01.\)

\(\text{PC1 (S. aureus) NB2001: } 6.3 \mu\text{M, relative } k_{\text{cat}} = 0.59, \text{relative } k_{\text{cat}}/K_m = 6.4.\)

\(\text{PC1 (S. aureus) NB2030: } 20 \mu\text{M, relative } k_{\text{cat}} = 18, \text{relative } k_{\text{cat}}/K_m = 61.\)

### Table 3. PBP-binding profiles of NB2001, NB2030, and standard compounds with \textit{E. coli} ATCC 29522 and \textit{S. aureus} ATCC 29213 membranes

<table>
<thead>
<tr>
<th>Strain and compound</th>
<th>Conc((\mu\text{g/ml})) for complete inhibition of benzyl(\text{[14C]})penicillin binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E. coli}</td>
<td>PBP 1 (80 kDa)</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>2</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>2</td>
</tr>
<tr>
<td>NB2001</td>
<td>10</td>
</tr>
<tr>
<td>NB2030</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>\textit{S. aureus}</th>
<th>Conc((\mu\text{g/ml})) for complete inhibition of benzyl(\text{[14C]})penicillin binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylpenicillin</td>
<td>10</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>0.1</td>
</tr>
<tr>
<td>NB2001</td>
<td>100</td>
</tr>
<tr>
<td>NB2030</td>
<td>100</td>
</tr>
</tbody>
</table>

\(\text{Binding was assayed with } 0.1, 0.5, 2, 10, \text{and } 100 \mu\text{g of the compound of interest per ml as described in Materials and Methods. PBP 2 of } \textit{E. coli} \text{ is } 71 \text{ kDa, and PBP 2 of } \textit{S. aureus} \text{ is } 73 \text{ kDa. PBP 3 of } \textit{E. coli} \text{ is } 64 \text{ kDa, and PBP 3 of } \textit{S. aureus} \text{ is } 70 \text{ kDa. PBP 4 of } \textit{E. coli} \text{ is } 52 \text{ kDa, and PBP 4 of } \textit{S. aureus} \text{ is } 46 \text{ kDa.}\)

### Table 4. Inhibition of enoyl reductase (Fab\(L\)) activity by triclosan, NB2001, and NB2030

<table>
<thead>
<tr>
<th>Compound</th>
<th>Apparent IC(50) ((\mu)M)</th>
<th>Increase in IC(50) compared to that of triclosan (fold)</th>
<th>% Free triclosan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triclosan</td>
<td>1.9</td>
<td>0.26</td>
<td>1</td>
</tr>
<tr>
<td>NB2001</td>
<td>30</td>
<td>8.5</td>
<td>16</td>
</tr>
<tr>
<td>NB2030</td>
<td>130</td>
<td>42</td>
<td>70</td>
</tr>
</tbody>
</table>

\(\text{APPARENT IC}_{50} \text{ were determined as described in Materials and Methods. Values represent the results of at least three separate experiments. Standard deviations for triclosan and NB2001 assays were less than } 10\%. \text{NB2030 showed standard deviations between } 10 \text{ and } 25 \text{ of the mean values.}\)
degradation of the compounds during the assay (Fig. 3D). Free triclosan was detected in the transport assay samples with NB2030 and \textit{S. aureus} and to a lesser extent in the samples with NB2001 and \textit{E. coli}.

**DISCUSSION**

The experiments presented here suggest that the two β-lactam-containing antibacterials, NB2001 and NB2030, developed by the ECTA approach deliver their toxophores via β-lactamases since a trend of enhanced activity against organisms expressing class A or class C β-lactamases was shown for the compounds. For example, there was a 62-fold increase in the activity of NB2001 between \textit{E. coli} ATCC 25922 and TE18 and a 16-fold increase in the activity of NB2001 between \textit{E. coli} ATCC 25922 and the strain overexpressing TEM-1. The results are somewhat complicated by differences in the triclosan susceptibilities of the different strains.

Nevertheless, in a comparison of isogenic strains, the non-β-lactamase-producing strain N and strain N expressing TEM-1, there were 500- to 1,000-fold differences in the MICs of NB2001 and NB2030 and the MIC of triclosan. This suggests that β-lactamase is responsible for the increased activity against this strain and that there is minimal (0.1%) triclosan contamination and/or non-β-lactamase-mediated triclosan release. The large difference in MICs was reproducible throughout the period that the experiments described in this report were carried out, except for the biochemical experiments involving enoyl reductase (Table 4), which were performed a year later. In those experiments, NB2001 and NB2030 were analyzed directly for triclosan contamination, and the levels of contamination were found to be 4 and 1%, respectively.

NB2001 also showed at least an eightfold higher level of activity against β-lactamase-overexpressing strain \textit{E. cloacae} P99 than against \textit{E. cloacae} ATCC 13047. Again, inherent differences in triclosan susceptibilities complicate interpretation of the results. Surprisingly, NB2001 was less active against β-lactamase-overexpressing \textit{S. aureus} strain PC1 than against \textit{S. aureus} ATCC 29213. This may be due to differences in drug uptake between the two strains or enzymatic degradation of NB2001 by a non-β-lactamase-mediated mechanism during MIC determination, as was observed for NB2030 in our \textit{S. aureus} ATCC 29213 transport assays. The results of kinetic experiments support the lack of toxophore release by the PC1 β-lactamase and show that NB2001 is a poor substrate for the PC1 enzyme relative to benzylpenicillin (Table 2). This is also an issue in the nitrocefin test, since nitrocefin has the same side chain as cephalothin.

Finally, contamination of NB2001 and NB2030 with free triclosan or β-lactamase-independent hydrolysis may account for the increased activities of NB2001 and NB2030 against \textit{S. pneumoniae} ATCC 700671 and \textit{E. faecalis} ATCC 29212 relative to that of the parent compound, cephalothin. While the results of the experiments with \textit{E. coli} N support the dependence of the activities of NB2001 and NB2030 on β-lactamase release, further experiments are needed to determine whether this effect is also the case with clinical isolates. However, it is known from HPLC time course experiments of NB2001 hydro-
ysis (16) and NB2030 hydrolysis (data not shown) that compound degradation and triclosan release are dependent on the presence of \(\beta\)-lactamase.

Assays to determine the enzyme kinetics of NB2001 and NB2030 reveal that they are good substrates for both TEM-1 and AmpC \(\beta\)-lactamases. Surprisingly, the former enzyme hydrolyzes NB2030 with increased efficiency relative to its efficiency of hydrolysis of NB2001 and cephalothin, while the latter enzyme hydrolyzes NB2030 with a correspondingly decreased efficiency. Significantly, while cephalothin is a poor substrate for PC1 (\(k_{cat}/K_m\) less than 0.01% of the value for benzylpenicillin), NB2001 and NB2030 have \(k_{cat}/K_m\) values of 6.4 and 61% of the value for benzylpenicillin, respectively.

Our results also suggest that, as \(\beta\)-lactams, NB2001 and NB2030 are less active than cephalothin against \(E.\ coli\) and \(S.\ aureus\). The level of binding to the essential PBP 3 of \(E.\ coli\) is significantly (10- to more than 50-fold) reduced, and the compounds are unable to induce filamentation. According to previous work (9, 28), PBPs 1b, 2, and 3 of \(E.\ coli\) and PBPs 2 and 3 of \(S.\ aureus\) are essential and are targets of \(\beta\)-lactam antibiotics. The two lamactacins bound poorly to all essential PBPs in the two organisms.

The antibacterial activities of NB2001 and NB2030 appear to reflect the activity of the triclosan toxophore more than they reflect the activity of the \(\beta\)-lactam moiety. Consistent with their triclosan-dependent antibacterial activities, treatment of \(E.\ coli\) cells with NB2001 and NB2030 at their MICs resulted in cells with normal morphologies. It is therefore possible that bacterial resistance to NB2001 or NB2030 could occur through the loss of \(\beta\)-lactamase genes, which would then increase the susceptibility of the cell to \(\beta\)-lactams. Although triclosan has additional activity at higher concentrations due to direct effects on the cell membrane and the activity is retained in NB2001 and NB2030, this activity is associated with mammalian cytotoxicity and thus is not clinically useful.

The experiments with enoyl reductase suggest that intact NB2001 and NB2030 do not inhibit the enzyme, consistent with computer modeling of its active site (M. Hixon, unpublished data). The three-dimensional structure of enoyl reductase containing a bound NAD\(^+\) molecule and a triclosan mol-
ecule (12) suggests that steric hindrance would not allow NB2001 and NB2030 to bind within the active site of the molecule. While our enoyl reductase assay results were complicated by the residual triclosan in the preparation, the reduction in enzyme inhibition that was observed is consistent with the presence of contaminating triclosan as opposed to inhibition by NB2001 or NB2030.

In summary, the ECTA approach presented here allows clinical pathogens that express class A or class C β-lactamases to be targeted. Despite the antibacterial activity potentially stemming from the β-lactam nucleus, evidence from in vitro and in vivo studies with NB2001 and NB2030 points to toxophore release as the major contributor to antibacterial activity. Neither compound possessed sufficient activity in animal models of infection to enter clinical development, most likely because serum binding caused more than 1,000-fold shifts in the MICs. However other cephalosporin conjugates that use triclosan analogs with decreased levels of serum binding (Q. Li, V. R. Doppalapudi, R. S. Castillo, A. R. Bueno, J. Y. Lee, G. W. Stone, Q. Zhang, S.-F. Chen, H.-P. Hong, S.-F. Lin, Y.-Y. Lu, J. Macdonald, and N. H. Georgopapadakou., Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. F-2142, 2003) might eventually yield candidates for clinical development.

REFERENCES