Anucleate Cell Blue Assay: a Useful Tool for Identifying Novel Type II Topoisomerase Inhibitors

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Received 25 July 2005/Returned for modification 17 September 2005/Accepted 30 October 2005

About 95,000 compounds were screened by the anucleate cell blue assay. Fifty-one of the hit compounds had various structures and showed inhibitory activity against DNA gyrase and/or topoisomerase IV. Moreover, the compounds exhibited antibacterial activity against a fluoroquinolone- and novobiocin-resistant strain of Staphylococcus aureus. The anucleate cell blue assay is therefore a useful tool for finding novel type II topoisomerase inhibitors.

The increase and spread of multidrug-resistant bacteria has become a serious public health problem. This critical situation necessitates the development of novel antibacterial agents with new mechanisms of action. Bacterial type II topoisomeraseases, i.e., DNA gyrase and topoisomerase IV, are essential bacterial enzymes that are known to be the target of fluoroquinolones. Since the fluoroquinolones already on the market are potent broad-spectrum antibacterial agents used to treat bacterial infections caused by both gram-positive and gram-negative bacteria, type II topoisomeraseases are still an attractive target for the development of novel antibacterial agents that can overcome bacterial resistance. However, there are no high-throughput screening systems for type II topoisomerase inhibitors.

We have previously developed a novel screening system, named anucleate cell blue assay, that detects specific inhibitors of chromosome partitioning in Escherichia coli (12). As type II topoisomeraseases, both DNA gyrase and topoisomerase IV are involved in the resolution of decatenated sister chromosome (7, 8), it is expected that our anucleate cell blue assay can help identify type II topoisomerase inhibitors. Indeed, we have previously reported that nalidixic acid, a DNA gyrase inhibitor, can be detected by the anucleate cell blue assay (12). Interestingly, it has been also reported that the anucleate cell blue assay identified A22, a MreB actin inhibitor (3, 6). In this study, we examined whether the anucleate cell blue assay is useful in the detection of other novel type II topoisomerase inhibitors.

About 95,000 commercially available chemical compounds were screened by the anucleate cell blue assay as described by Wachi et al. (12). In brief, E. coli K-12 strain SH3210 (ArpEd5 his λ pXX747) (5) was used. In nucleated cells, the expression of the repA gene under control of the λPR promoter and lacZ gene on the plasmid pXX747 was repressed by gene products from the cI gene of λ phage and the lacI gene on the chromosome of the host cells, respectively.

The plates were then incubated at 42°C for 24 h. Anucleate cell production was indicated by the development of blue zones around the growth inhibition zones in the paper disk. Blue zones around growth inhibition zones (±9 mm in diameter) were observed for 479 compounds (about 0.5%) tested by the anucleate cell blue assay. Figure 1 shows the development of blue zones (represented by gray shading) around the growth inhibition zones caused by both gram-positive and gram-negative bacteria, type II topoisomeraseases are still an attractive target for the development of novel antibacterial agents with new mechanisms of action. Bacterial type II topoisomeraseases, i.e., DNA gyrase and topoisomerase IV, are essential bacterial enzymes that are known to be the target of fluoroquinolones. Since the fluoroquinolones already on the market are potent broad-spectrum antibacterial agents used to treat bacterial infections caused by both gram-positive and gram-negative bacteria, type II topoisomeraseases are still an attractive target for the development of novel antibacterial agents that can overcome bacterial resistance. However, there are no high-throughput screening systems for type II topoisomerase inhibitors.

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FIG. 1. Development of blue zones around the growth inhibition zones by a representative compound, A8, and a reference drug, sparfloxacin (a type II topoisomerase inhibitor), in the anucleate cell blue assay. The assay was carried out as described in the text. A, A8 at 200 μg/disk; B, sparfloxacin at 1 μg/disk. The clear zones formed around the paper disks show growth inhibition zones, and the shaded zones around the growth inhibition zones indicate anucleate cell production.
hibition zones by a representative compound, A8, and a reference drug, sparfloxacin (a type II topoisomerase inhibitor). With conventional screening methods, very few compounds obtained by an in vitro enzyme inhibition assay show antibacterial activity, and a considerable amount of time is required to prepare the compounds that show antibacterial activity from these compounds. However, with the anucleate cell blue assay, it is possible to detect compounds which not only can induce the production of anucleate cells but also have whole-cell activity (namely, antibacterial activity).

From the 479 hit compounds, 138 compounds were selected by visual inspection of chemical structure, where compounds with very similar chemical structure were excluded, and the inhibitory activity of each selected compound against type II topoisomerases was examined. *E. coli* DNA gyrase supercoiling assays and topoisomerase IV decatenation assays were carried out according to the methods described by Sato et al. (11) and Peng and Marian (10), respectively. Among 138 compounds, 51 compounds (about 40%) showed inhibitory activity against DNA gyrase and/or topoisomerase IV with a concentration of a compound that inhibits 50% of enzymatic activity (IC50) of less than 100 μg/ml (data not shown). The anucleate cell blue assay can detect inhibitors of DNA synthesis, RNA synthesis, and peptidoglycan synthesis (12), but surprisingly about 40% of the selected compounds were found to be potent against type II topoisomerases. Hence, the anucleate cell blue assay is quite effective in identifying type II topoisomerase inhibitors.

Since these active compounds exhibited antibacterial activity against a *Staphylococcus aureus* strain which is resistant to most antibacterial agents including fluoroquinolones and novobiocin (data not shown) and could be classified into eight groups on the basis of their structures, one compound was selected from each group as a representative for further studies. As shown in Fig. 2, the chemical structures of the representative compounds varied and were obviously different from those of the known topoisomerase inhibitors, i.e., sparfloxacin and novobiocin. In order to examine whether each representative compound can inhibit type II topoisomerases in bacterial cells, *E. coli* cells treated with each compound were observed with a

![FIG. 2. Chemical structures of representative compounds detected by the anucleate cell blue assay. The structures of sparfloxacin and novobiocin are shown for reference.](image1)

![FIG. 3. Morphological changes in *E. coli* induced by the representative compounds found in this study. *E. coli* M101 cells were incubated at 37°C with the compounds at concentrations of 1 MIC for 2 h. A, A8; B, A9; C, A11; D, A36; E, A106; F, A185; G, A188; H, A199; I, control (no treatment).](image2)
phase-contrast microscope. Inhibition of type II topoisomerases in E. coli cells is indicated by the presence of elongated cells with damaged DNA (1). E. coli M101 cells were incubated at 37°C with each representative compound at 1 MIC for 2 h. As expected, 60 to 80% of the cells became about five times longer than nontreated cells in each preparation, except for the A36-treated cells (Fig. 3).

It is well known that the fluoroquinolones form a ternary complex with topoisomerase in the presence of DNA, resulting in lethal double-stranded DNA breaks (4). On the other hand, novobiocin inhibits ATPase activity of DNA gyrase by competing with ATP for binding to the DNA gyrase B subunit (9). In this study, MICs of the representative compounds for the fluoroquinolone-resistant strain S. aureus G2/pRK3 (13) and the novobiocin-resistant strain S. aureus N742 (2) were almost the same as those for the susceptible strain S. aureus RN4220 (Table 1). In addition, the antibacterial activity of the representative compounds against the multidrug-resistant strain S. aureus KMP9 was similar to that against S. aureus RN4220. Although the effects of the representative compounds on S. aureus enzymes in vitro and in vivo were not examined, it can be assumed from the results above and from microscopic observations of E. coli cells that the representative compounds have novel mechanisms of type II topoisomerase inhibition.

Using the anucleate cell blue assay, we have identified in this study 51 compounds which have various structures and show in vitro inhibitory activity against bacterial DNA gyrase and/or topoisomerase IV. Some of these compounds might have novel mechanisms of action to inhibit bacterial type II topoisomerases. The anucleate cell blue assay is therefore a useful tool for finding novel type II topoisomerase inhibitors.

We thank Hiroaki Yoshida for his critical reading of the manuscript and useful discussion.

### REFERENCES


### TABLE 1. Antibacterial activity and inhibitory activity against type II topoisomerases of representative compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>S. aureus RN4220 MIC (µg/ml)</th>
<th>S. aureus G2/pRK3 MIC (µg/ml)</th>
<th>S. aureus N742 MIC (µg/ml)</th>
<th>S. aureus KMP9 MIC (µg/ml)</th>
<th>E. coli NIHJ JC-2 MIC (µg/ml)</th>
<th>E. coli M101 MIC (µg/ml)</th>
<th>Gyrase IC50 (µg/ml)</th>
<th>Topoisomerase IV IC50 (µg/ml)</th>
</tr>
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<tr>
<td>A8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>64</td>
<td>4</td>
<td>450</td>
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<tr>
<td>A9</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>64</td>
<td>64</td>
<td>12.5</td>
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<tr>
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<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>32</td>
<td>2</td>
<td>100</td>
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<td>A36</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
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<td>16</td>
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<td>2</td>
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<td>4</td>
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<tr>
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<td>32</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>256</td>
<td>128</td>
<td>&gt;100</td>
<td>50</td>
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<td>Sparfloxacin</td>
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<td>Novobiocin</td>
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<td>64</td>
<td>0.5</td>
<td>0.25</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* a: S. aureus RN4220, a susceptible laboratory strain; S. aureus RN4220-G2/pRK3, a derivative of S. aureus RN4220 which shows high-level fluoroquinolone resistance; S. aureus N742, a derivative of S. aureus RN4220 which shows high-level novobiocin resistance; S. aureus KMP9, a multidrug-resistant strain; E. coli NIHJ JC-2, a susceptible strain; E. coli M101, a multidrug efflux pump-deficient mutant (ΔacrAB).

b: DNA gyrase, E. coli DNA gyrase supercoiling activity; topoisomerase IV, E. coli topoisomerase IV decatenation activity.