Biochemical effects of sparfloxacin on cell envelope of mycobacteria

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Sparfloxacin, a difluorinated quinolone is a potent anti-mycobacterial agent used in the treatment of mycobacterial infections. We have investigated whether sparfloxacin had other, more subtle effects on mycobacteria besides its interaction with DNA gyrase that could contribute to its therapeutic efficacy. Mycobacterium smegmatis cells grown in media with sub-inhibitory concentration of sparfloxacin were observed to have significant reduction in the biosynthesis of vital macromolecules, as shown by the incorporation of various radiolabelled precursors. The analysis of subcellular distribution of phospholipids of sparfloxacin-treated cells demonstrated an increase in the cell membrane and reduction in the cell wall, suggesting changes in the cell envelope architecture by sparfloxacin. Significant changes were also observed in other chemical constituents of the cell wall, especially in the arabinose and glucosamine contents. Mycolic acids, the major component of mycobacterial cell wall were reduced in the presence of MIC<sub>50</sub> of sparfloxacin. There was a decrease in the limiting fluorescence intensity (F<sub>max</sub>) of 1-anilinonaphthalene 8-sulfonate (ANS) indicating alterations in the organization and conformation of mycobacterial cell surface. These results suggest that the mechanism of action of anti-mycobacterial action of sparfloxacin involves mycobacterial cell envelope.

The global resurgence of tuberculosis and the emergence of multi-drug resistant (MDR) strains of <i>M. tuberculosis</i> have emphasized an urgent need for new effective anti-mycobacterial drugs<sup>1,2</sup>. Among recently developed drugs, fluoroquinolones represent an important class of therapeutic agents<sup>3</sup>. Fluoroquinolones such as ciprofloxacin and sparfloxacin have proven clinical activity in the treatment of patients with tuberculosis and leprosy<sup>4,5</sup>. Of all the fluoroquinolones evaluated in experimental studies, sparfloxacin appeared to have the greatest activity against <i>M. tuberculosis</i> including the vast majority of MDR clinical isolates<sup>6</sup>. Regarding the mechanism of action, fluoroquinolones are known to act by inhibiting the topoisomerase II enzyme, DNA gyrase and topoisomerase IV (ref. 7). However, the events that result in cell death are yet poorly defined<sup>8</sup>. In <i>E. coli</i>, substantial alterations in the integrity of cell membrane by a DNA gyrase inhibitor has also been reported, which results in to cell death<sup>9</sup>. In mycobacteria, DNA gyrase is the only target so far identified<sup>10</sup>. Although, many physiological aspects of quinolone action in mycobacteria are in parallel with earlier findings in <i>E. coli</i>, it is still important to evaluate the effect of sparfloxacin on the mycobacterial envelope, which is unusually thick and lipophilic. The aim of the present study was, therefore, to explore a possible relationship between anti-mycobacterial activity of sparfloxacin and its effect on mycobacterial cell envelope integrity.

Materials and Methods

Bacterial strain and determination of MIC and MIC<sub>50</sub>

*Mycobacterium smegmatis* MTCC6 obtained from Microbial Type Culture Collection (MTCC), Chandigarh was grown in modified Youman's medium. The MIC was determined by broth dilution method. Briefly, the drugs were added to liquid media to produce serial two-fold dilutions. These tubes and a control tube containing no drug were inoculated with a suspension of bacteria to yield a final concentration of approximately 10<sup>5</sup>-10<sup>6</sup> CFU/ml. Growth was monitored by measuring absorbance at 650 nm till the control cells reached mid-exponential phase. The MIC was defined as the minimum concentration of drug, which resulted in no visible growth and MIC<sub>50</sub> (sub-inhibitory concentration) as the concentration inhibiting growth by 50%.

Effect of sparfloxacin on DNA and protein synthesis

*M. smegmatis* cells grown in the presence of MIC<sub>50</sub> of sparfloxacin and control cells were suspended in
sterile Youman's medium. The cells were then pulsed with [3H]-thymidine and [3H]-leucine, for DNA and protein synthesis, respectively for different time intervals with constant shaking at 37°C. This was followed by the addition of 10% trichloroacetic acid and filtration on preweighed filter papers (0.45 μm), which were then dried and weighed again. The radioactivity was measured in a liquid scintillation counter, and the results were calculated as the cpm incorporated per mg dry weight of cells.

**Extraction and identification of lipids**

Lipids were extracted and purified by the method of Folch et al. Individual phospholipid components were separated by thin layer chromatography (TLC) on silica gel H plates by using the solvent system chloroform-methanol-7N ammonia (65:25:4, vol/vol/vol). Phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidylinositol were identified with specific spray reagents. For phosphatidylinositolmannosides (PIMs), TLC plates were sprayed with α-naphthol reagent (containing 10.5 ml of 15% ethanolic α-naphthol, 6.5 ml of concentrated H2SO4, 40.5 ml of ethanol and 4.0 ml of water) and then heated till the brownish purple spots appeared. Individual phospholipids were quantitated by estimating lipid phosphorus in the spots separated on TLC by the method of Bartlett as modified by Marinetti.

**Synthesis of phospholipids**

Cells were harvested in the mid-exponential phase of growth, washed with normal saline and suspended in Kreb's Ringer buffer under sterile conditions. Cells were incubated at 37°C for 1 hr under shaking conditions to obtain a homogenous suspension and [1-14C] sodium acetate (25 μCi/100 ml of medium) was added to the cells and incubation was continued for 90 min. From each culture 10 ml portions were transferred to tubes containing 0.5 ml of 1 M KCN. The tubes were centrifuged at 27,000xg for 15 min. The cell pellet was recovered and lipids were extracted. Radioactivity was counted in a liquid scintillation fluid containing 0.4% (wt/vol) PPO, (2, 5-diphenyloxazole) and 0.05% (wt/vol) POPOP [1, 4-bis (5-phenyloxazoly) benzene].

**Biochemical characterization of cell wall / cell membrane fractions**

The cell wall and cell membrane were isolated by the differential centrifugation method of Kearney and Goldman. The crude cell wall fraction thus obtained was purified by digestion with 0.2% trypsin in 0.67 M phosphate buffer (pH 8.0) for 3 hr at 37°C with continuous stirring. The sediment that was obtained by centrifugation at 10,000xg for 30 min was washed by centrifugation once with phosphate buffer, three times with 1 M NaCl solution, and then twice with distilled water. The final sediment was suspended in a small amount of distilled water and lyophilized. The purity of cell wall and cell membranes was determined by measuring the activity of ATPase, a marker of cell membrane according to the method described by Penumarti and Khuller. Total and individual phospholipids were extracted and estimated in cell wall and cell membrane fractions by the methods as described earlier. Samples of the cell wall fraction were acid hydrolyzed by the method of Takeya et al. for the quantitative determination of amino sugars and reducing sugars. Mycolic acids of the cell wall were extracted by the method of Winder and Collins dried under vacuum weighed (gravimetric method).

**Structural studies**

Structural studies with 1-anilinonaphthalene-8-sulfonate (ANS) were carried out to monitor the changes on cell surface of M. smegmatis grown in the presence of sub-inhibitory concentration of sparfloxacin. The assay mixture consisted of a total volume of 2 ml in 10 mM citrate phosphate buffer (pH 6.5) containing 0.7 M NaCl, 10 μM ANS and whole-cell protein concentration ranging from 5 to 100 μg as estimated by the method of Lowry et al. The fluorescence emission was recorded in a spectrophotometer (SFM-25; Kontron AG Zurich Switzerland) with the excitation wavelength at 390 nm and the emission wavelength of 510 nm. The fluorescence developed was recorded and the data was plotted as the reciprocal of the fluorescence signal (arbitrary unit) versus the reciprocal of the concentration of whole cell protein. A straight line was obtained whose extrapolation with the ordinate gave the reciprocal of limiting fluorescence (F_max) of ANS in the membrane.

**Results**

**Anti-mycobacterial activity of sparfloxacin**

The effect of sparfloxacin against M. smegmatis was examined by growing the cells in the presence of sparfloxacin at concentrations ranging from 0.02-
0.2 μg/ml until the mid-exponential phase. Results were expressed as the relative percent of growth as compared to that of the control, plotted against the concentration of sparfloxacin. The MIC and MIC₅₀ were found to be 0.08 μg/ml and 0.2 μg/ml, respectively and were confirmed on the basis of colony forming units.

Measurement of DNA and protein synthesis

There was 41.7% decrease in the synthesis of DNA within 5 min in the cells grown in the presence of MIC₅₀ of sparfloxacin and remained almost constant till 30 min. The effect of sub-inhibitory concentration of the drug on protein synthesis as measured by incorporation of [³H]-leucine was observed to decrease by 36% as compared to control.

Effect of sparfloxacin on lipids and phospholipids of M. smegmatis

Cells grown in the presence of sub-inhibitory concentration of sparfloxacin revealed significant quantitative alterations (Table 1). There was a significant reduction in the total lipids (p<0.05) and total phospholipids (p<0.01). This decrease was also reflected in the content of individual phospholipids with significant decrease (p<0.001) in the content of PIMs with no change in the levels of PE and CL.

Table 1—Total lipids, phospholipids and individual phospholipids of M. smegmatis grown at MIC₅₀ of sparfloxacin.

<table>
<thead>
<tr>
<th></th>
<th>Total lipids (mg/g dry wt. of cells)</th>
<th>Total phospholipids (mg/g dry wt. of cells)</th>
<th>Individual phospholipids (mg/g dry wt. of cells)</th>
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<tbody>
<tr>
<td>Control</td>
<td>177.9±1.63</td>
<td>32.8 ± 1.96</td>
<td>9.82 ± 0.54</td>
</tr>
<tr>
<td>SPX</td>
<td>145.3±19.0*</td>
<td>26.07 ± 1.45**</td>
<td>4.66±0.23***</td>
</tr>
</tbody>
</table>

***p<0.001; **p<0.01; *p<0.05; NS, non significant; a, sparfloxacin.

Synthesis of lipids

Since quantitative changes were observed in lipids and total phospholipids of cells grown in the presence of sub-inhibitory concentration of sparfloxacin, precursor incorporation studies were carried out to determine the rate of phospholipid synthesis. Incorporation of radioactivity into total phospholipids was found to be significantly decreased (p<0.01) (Table 2). This decrease in total phospholipids was mainly due to decrease in the synthesis of PIMs (p<0.001) with no change in the synthesis of PE and CL.

Quantitation of mycolic acids

On quantitating mycolic acids by gravimetric method (Fig. 1), sparfloxacin grown cells were found to contain reduced content (p<0.01) as compared to control. [¹⁴C] sodium acetate was also used as precursor to study the in vivo synthesis of mycolic acids. Incorporation of radioactivity into mycolic acids of cells grown in the presence of sparfloxacin revealed net reduction (p<0.01) in the synthesis of mycolic acids (Fig. 1).

Analysis of cell wall and cell membrane phospholipids

Subcellular fractions of M. smegmatis grown in the

<table>
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<th>Total lipids (cpm×10⁴/g dry wt. of cells)</th>
<th>Total phospholipids (cpm×10⁴/g dry wt. of cells)</th>
<th>Individual phospholipids (cpm×10⁴/g dry wt. of cells)</th>
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<tbody>
<tr>
<td>Control</td>
<td>33.0 ± 1.40</td>
<td>16.8 ± 0.55</td>
<td>7.7 ± 0.18</td>
</tr>
<tr>
<td>SPX</td>
<td>35.0 ± 3.74 NS</td>
<td>14.8 ± 0.73*</td>
<td>4.5 ± 0.63 NS</td>
</tr>
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</table>

**p<0.01; *p<0.05; NS, non significant.
presence of sub-inhibitory concentrations of sparfloxacin were isolated by differential centrifugation as described in Materials and Methods. The comparison of ATPase activity in the two fractions showed the membrane to have much higher activity (1.30 μmoles Pi liberated/mg protein/30 min) as compared to cell wall (0.10 μmoles Pi liberated/mg protein/30 min). This confirms the purity of these fractions. Further, total yield of purified cell wall was found to be approximately 400 mg/g dry weight of cells. Phospholipid profiles of the subcellular components viz. cell wall and cell membrane were investigated (Table 3). A significant decrease (p<0.001) in the cell wall phospholipids was observed in cells grown in the presence of MICₙ₀ of sparfloxacin. This reduction in total phospholipids was manifested in all the individual phospholipids i.e. PIMs, PE and CL. On the contrary, there was a significant increase (p<0.001) in the phospholipids of cell membrane as compared to control, which was mainly due to the accumulation of PIMs and PE (Table 3).
### Biochemical composition of cell wall

Representative constituents of cell wall viz. arabinose, galactose, glucosamine and mycolic acids were analyzed (Table 4). Similar to whole cells, cell wall of *M. smegmatis* grown in the presence of MIC$_{50}$ of sparfloxacin was found to have less amount of mycolic acids ($p<0.001$) as compared to cell wall of control cells. However, there was an apparent increase in the content of arabinose ($p<0.05$) and glucosamine ($p<0.01$) with no change in the content of galactose.

### Structural studies

Alterations in the cell envelope and the effect of altered lipid composition on membrane structure was studied by using a fluorescent probe, ANS, which is known to bind both intact cells and cell membrane. Limiting fluorescence ($F_{\text{max}}$) values of ANS bound were calculated from double reciprocal plot of relative fluorescence intensity versus protein concentration. On incubating ANS with intact cells, a significant decrease in $F_{\text{max}}$ (54 ± 4.64) was seen in the cells grown in the presence of MIC$_{50}$ of sparfloxacin as compared to control (303 ± 43.2).

### Statistical analysis

The results of the present study were analyzed by applying student's 't' test.

### Discussion

The major anti-bacterial effects of fluoroquinolones are considered to be mediated via binding to DNA gyrase or topoisomerase IV. However, the biochemical events associated with anti-mycobacterial activity of fluoroquinolones are still poorly understood. In the present study, a wide range of biochemical effects of sparfloxacin have been elucidated in *M. smegmatis*, which is a fast growing analogue of *M. tuberculosis*.

*M. smegmatis* cells grown in the presence of sub-inhibitory concentration of sparfloxacin showed a significant inhibition (41.7%) of DNA synthesis within 5 min. In *E.coli*, quinolones are known to inhibit DNA synthesis rapidly, if gyrase is the target and slowly if topoisomerase is the target$^{20,21}$. Our observations are consistent with these reports indicating interaction of sparfloxacin with DNA gyrase in mycobacteria. Earlier, Drlica et al.$^{10}$ also reported a decrease in DNA synthesis in *M. smegmatis* cells treated with ciprofloxacin.

Besides interaction of fluoroquinolones with the enzymes of DNA synthesis, there are conflicting reports suggesting their effect on bacterial envelope$^{22,23}$. Due to unusually thick and lipid-rich mycobacterial envelope, it is important to investigate the effect of fluoroquinolones on this component. In the present study, analysis of total lipids and total phospholipids revealed a significant decrease in these components of whole cells and cell wall of *M. smegmatis* grown in the presence of MIC$_{50}$ of sparfloxacin which is in accordance with the findings of Verma et al.$^{24}$. Mycobacterial cell envelope is also characterized by the presence of exceptionally high molecular weight α-akyl, β-hydroxy acids, the mycolic acids$^{25}$. A significant reduction in mycolic acid content in cells grown in the presence of sparfloxacin further confirmed the cell envelope alterations. In addition, the analysis of other major constituents of mycobacterial cell wall viz. arabinose, galactose and glucosamine also revealed significant changes. The data presented here is consistent with the concept that the synthesis of entire cell wall core is dependent on the proper formation of mycolyl arabinogalactan complex. Cell wall biosynthesis is a dynamic process in which change in one component may lead to quantitative alterations in others.

In addition to cell wall changes, an increase in the phospholipid content of cell membrane was also

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**Table 4—Biochemical composition of cell wall of *M. smegmatis* grown in the presence of MIC$_{50}$ of sparfloxacin**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Control</th>
<th>SPX</th>
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<tbody>
<tr>
<td>Arabinose (mg/g dry wt cell wall)</td>
<td>35.7 ± 5.2</td>
<td>56.7 ± 3.98*</td>
</tr>
<tr>
<td>Galactose (mg/g dry wt cell wall)</td>
<td>38.1 ± 7.7</td>
<td>34.7 ± 2.99 NS</td>
</tr>
<tr>
<td>Glucosamine (mg/g dry wt cell wall)</td>
<td>11.7 ± 1.0</td>
<td>14.8 ± 0.5**</td>
</tr>
<tr>
<td>Mycolic acid (mg/g dry wt cell wall)</td>
<td>911±33.0</td>
<td>718±14.3***</td>
</tr>
</tbody>
</table>

***p<0.001; **p<0.01; *p<0.05; NS, non significant.
observed in the presence of sparfloxacin. The various biochemical alterations in cell wall and cell membrane suggested a changed organization of cell envelope. The fluorescent probe ANS has been widely used to study the structural changes induced by a large variety of compounds in natural and artificial membranes due to its binding to both lipids and proteins. In the present study, a significant reduction in the limiting fluorescence intensity of ANS further confirmed the surface alterations in the presence of sparfloxacin, which could be attributed to changed lipid composition of the cell envelope.

Thus, the results of present study suggest that in mycobacteria, inhibition of DNA replication in sparfloxacin-treated cells leads to a cascade of physiological events culminating in cell envelope changes, which may lead to lethal events in the cells.

Acknowledgement

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References