CHAPTER 4

BIOLOGICALLY ACTIVE FULLERENE DERIVATIVES

(A) FULLERENE DERIVATIZED S-TRIAZINE ANALOGUES AS ANTIMICROBIAL AGENTS

(B) FULLERENE-ISONIAZID CONJUGATE AS A NOVEL ANTIMYCOBACTERIAL

(C) PHOTOINDUCED DNA CLEAVAGE BY FULLERENE-LYSINE CONJUGATE
ABSTRACT

This chapter has been divided into 3 sections. In section A, the novel s-triazme derivatized fulleropyrrolidine derivatives (STFPY1- STFPY6) synthesized and characterized (Chapter 2) were tested for antibacterial activity. The series of novel fullerene derivatives bearing s-triazine moiety (STFPY1- STFPY6) synthesized were screened for their antibacterial activity against both gram positive bacteria (S.aureas, B.subtilis, B.pumilis) and gram negative bacteria (E.coli, pseudomonas aeruginosas and klebsiella pneumoniae) by disc diffusion method and were found to be active against these strains at very low concentration with MIC’s (minimum inhibitory concentration) comparable to standard drug ciprofloxacin.

In section B, Fullerene – isoniazid conjugate INHFPY synthesized and characterized (Chapter 2) was successfully tested for antimycobacterial activity against M. Tuberculosis and M Avium.

In section C, the novel water soluble fullerene lysine conjugate LYFPY synthesized and characterized (Chapter 2), was tested for photocleavage of DNA by gel electrophoresis.
4.1 Introduction

Ever since its discovery, C\textsubscript{60} has become a topic of considerable interest in medicinal chemistry as a potential biologically active compound. Its spherical shape together with its amazing physical and chemical properties has prompted scientist world over to exploit it in many areas ranging from material chemistry to biological sciences.\textsuperscript{1-4} Interest in using C\textsubscript{60} for diagnostic and therapeutic medicine has been accelerated ever since it was discovered that C\textsubscript{60} derivatives could cross cell membranes.\textsuperscript{5} Fullerene derivatives have found application as neuroprotective agents,\textsuperscript{6} anti-HIV agents,\textsuperscript{7,8} antimycobacterials,\textsuperscript{9} antibacterials,\textsuperscript{10-12} bone-disorder drugs,\textsuperscript{13} X-ray contrast agents,\textsuperscript{14} transfection vectors,\textsuperscript{15} photodynamic therapy agents,\textsuperscript{16} antiproliferative agents,\textsuperscript{17} drug delivery systems,\textsuperscript{18-19} inhibitors of DNA enzymes,\textsuperscript{20} free radical sponge,\textsuperscript{21} anti-inflammatory agents,\textsuperscript{22} anti-apoptosis agents,\textsuperscript{23} immunostimulatory agents,\textsuperscript{24} vaccine against human papilloma virus,\textsuperscript{25} anticancer agents,\textsuperscript{26} radiopharmaceuticals\textsuperscript{27} and MRI contrast agents.\textsuperscript{28} One of the difficulties faced in using fullerene derivatives for biological application is its low solubility in water or water miscible solvents. This problem can be solved by introduction of (a) hydrophilic group or (b) ionic substituents in the molecule. In the present investigation three of the most important biological activities of fullerene has been explored.

4.1.1 Antibacterials

There are many reports wherein fullerene derivatives have been used as antibacterials.\textsuperscript{10-12} These derivatives were having either hydrophilic functional group or ionic part along with hydrophobic fullerene moiety so that the dual purpose of interaction with cell wall
of bacteria as well as their biological activity studies in water or water miscible solvents becomes viable. Derivatized s-triazine molecules had been proved efficient in inhibiting the growth of several strains of bacteria.\textsuperscript{29-30} Because of the increased bacterial resistance to conventional antibiotics, there is an urgent need to design and develop new antibiotics based on new chemical entities. Amphiphilic peptides have shown great activity as antibacterials showing activity below \( \mu \text{M} \) range.\textsuperscript{31-33} Activity of such class of compounds has been attributed to electrostatic and hydrophobic interaction with the bacterial membrane. Hence the presence of both hydrophobic and hydrophilic group is favorable for developing new chemical scaffolds as antibacterials. Moreover the presence of Schiff base has resulted in various compounds being applicable to pharmaceutical and medicinal chemistry. Such compounds had shown antibacterial, antifungal and antitumour activity.\textsuperscript{34-36} Some of the Schiff base derivatives with appreciable cell membrane permeability were studied in cancer multidrug resistance studies. With these in view, we designed and derivatized fullerene with substituted s-triazine having Schiff base group between them so that the purpose of solubility for biological studies as well as proper interaction with cell wall of bacteria could be achieved efficiently.

### 4.1.2 Antimycobacterials

According to WHO (World Health Organization), tuberculosis is one of the leading cause of death infecting nine million people globally with three million deaths every year.\textsuperscript{37} Emergence of resistant and virulent strains have made clear the pressing need for the evolution of newer and more powerful drugs, re-examination and the re-evaluation of older ones along with detailed elucidation of the different modes of antimycobacterial activity.\textsuperscript{38} A detailed study of structure activity relationship had revealed that
hydrophobic feature was crucial for determining antimycobacterial activity and a more hydrophobic character of the compound could definitely increase the antitubercular potency. In general the logP (hydrophobicity constant) values for the most active compounds are in the range of 5.50 to 7.0. Hypothesis that a further increase of lipophilicity (higher logP values) could improve activity thus facilitating entrance through the lipid rich mycobacterial membrane could be checked by introduction of highly lipophilic moiety like fullerene (C₆₀). With a very appealing chemical as well as physical property, fullerene has been exploited in many fields ranging from biological fields to material sciences. Interaction of carboxyfullerene with proteins as well as the interaction of fullerene based nanomaterials with liquid barrier proteins have been some of the major breakthroughs of the molecule in the field of medicinal chemistry. Although there are several reports of biologically active fullerene derivatives, there is only one report where conjugation of fullerene with a well known drug has resulted in enhancement of biological activity. Apart from the ionic fullerene derivatives reported by Bosi et al, no reports have come up of fullerene derivatives as antituberculars. Hence it was felt that these are some of the areas which can be further explored in the already well established fullerene chemistry.

In parallel, isoniazid (INH) is a well known drug with pronounced activity against *M. tuberculosis*, *M. bovis* and *M. africannum* and *M. microti* at MICs ranging from 0.025-0.05 µg/ml. Several studies on the mechanism of action suggest that INH inhibits the biosynthesis of cell wall mycolic acids, thereby making the mycobacteria susceptible to reactive oxygen radicals and other environmental factors. The first line antituberculosis drug isoniazid, is a prodrug, requiring activation in the mycobacterium cell by the
catalase/peroxidase activity of katG gene product. N-Acetyl transferase (NAT) is a drug metabolizing enzyme which can acetylate the isoniazid transferring an acetyl group from acetyl coenzyme A onto the terminal N of the drug. Such acetylation greatly reduces the therapeutic activity of the drug, resulting in overdosing, decreased bioavailability and acquired drug resistance.\textsuperscript{45-46} Chemical modification of the hydrazine unit of isoniazid with a functional group that blocks acetylation while retaining the activity can be one of the possible solutions to the above mentioned problem. Many attempts made to increase the activity by bringing about chemical modification to isoniazid have not yielded desired results.\textsuperscript{47} One main reason could be the low permeability of mycobacterium cell wall towards these drugs. Isoniazid (INH) is predominantly hydrophilic and is expected to use the porin pathway to enter mycobacteria.\textsuperscript{48} The reports of (a) increased efficacy of INH as an anti-TB agent by attaching a highly lipophilic group such as cyclohexane to it by an azomethine linkage thus preventing the hydrazine moiety from acetylation, \textsuperscript{49} (b) Ionic fullerene derivatives as antimycobacterials\textsuperscript{50} and (c) Excellent antibacterial activity shown by vancomycin-fullerene conjugate,\textsuperscript{42} prompted us to design and synthesize a fullerene-isoniazid conjugate and study its antimycobacterial activity. We designed and synthesized a compound in which fullerene has been attached to isoniazid by Schiff base linkage so that the dual purpose of preventing acetylation of isoniazid plus the increase of lipophilicity could be achieved. The simultaneous presence of hydrophilic isoniazid moiety as well as hydrophobic fullerene moiety could enable the compound to cross the charged double layer and also facilitates its entry into the hydrophobic membrane of the cell wall. The main concern regarding the solubility of the fullerene derivatives for
biological studies was solved by preparing stable water suspension of the synthesized fullerene-isomazid conjugate.

4.1.3 Photoinduced DNA cleavage

Of all the reported activities of C₆₀, the DNA-cleaving activity and lipid peroxidation, in particular, have attracted considerable attention.⁵¹ Photoirradiation of C₆₀ result in the formation of the singlet excited state \(^1C₆₀\)*, which undergoes efficient intersystem crossing (ISC) to give the triplet excited state \(^3C₆₀\)*.⁵² There are several possible pathways for DNA cleaving process involving \(^3C₆₀\)* (a) via superoxide anion (b) via singlet oxygen or (c) direct oxidation of DNA.⁵³ Two reasons for the interest in the incorporation of fullerene into molecular structures with biological importance is the highly hydrophobic nature and unusual physicochemical properties of fullerenes, making them ideal candidates as interesting pharmocophores. Unfortunately, the direct use of fullerenes in biological applications is limited by their poor solubility in aqueous media.⁵⁴ To overcome this obstacle, two different approaches have been adopted to increase the solubility. The first strategy involves non-covalently encapsulating fullerene molecules into soluble polymeric or host molecules.⁵⁵ The second strategy relies on covalent functionalization of fullerene by introduction of hydrophilic groups by chemical modification.⁵⁶ The latter approach is attracting more interest as it can not only alter the physical and chemical properties of fullerene to readily achieve desired properties, but it can also provide useful building blocks for further molecular constructions. It is this latter approach that has piqued our interests due to the potential of using a fullerene-based amino acid derivative for the systematic creation of nano bio-conjugates with application as DNA photocleaving agent. Amino acids are the most basic and essential building unit
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for living organisms at all levels. The incorporation of fullerene-based amino acids into proteins, peptides or antibodies could lead to new applications in medicinal chemistry. To date, several approaches have been taken towards synthesizing fullerene-based amino acids.\textsuperscript{57}

In parallel L-lysine derivatives possessing a chromophore have been found to induce efficient and highly selective cleavage of double stranded DNA upon photoexcitation.\textsuperscript{58}

Previous efforts to develop intercalator based probes utilizing the diverse chemistry of amino acids for novel DNA-binding reagents have yielded compounds with nuclease activity. However, in most of the systems described to date, the intercalating moiety functions solely to deliver peptides with low intrinsic binding affinity to DNA and does not contribute to chemical reactivity. Hence if we attach fullerene to lysine, DNA cleavage activity from both the photoexcited C\textsubscript{60} and appended amino acids can be conceived. To the best our knowledge, no examination of DNA cleavage by fullerene amino acid conjugate has been performed.

With this these points in view we have synthesized a novel fullerene lysine conjugate \textbf{LYSFPY} by a simple and modular strategy.
4.2 Experimental

4.2.1 Chemicals and Reagents

All the reagents used were of analytical grade. Deionized water was used for biological studies. Agarose has been purchased from Himedia Laboratories, India.

4.2.2 Synthesis All the novel fullerene derivatives STFPY1-STFPY6, INHFPY and LFPY were synthesized by 1,3-dipolar cycloaddition reactions. Detailed synthetic procedure and systematic characterization are given in Chapter 2.

4.2.3 Apparatus and Biological Assay

All the novel fullerene derivatives synthesized STFPY1-STFPY6, INHFPY and LFPY were screened for their antibacterial activity against bacterial strains of *E.coli*, *S.aureas*, *B.subtilis*, *B.pumilis*, *pseudomonas aeruginosas* and *klebsiella pneumoniae* by disc diffusion method. A standard inoculum (1-2 × 10^7 c.f.u/ml 0.5 McFauland standards) was introduced onto the surface of sterile agar plates, and a sterile glass spreader was used for even distribution of the inoculum. The disc measuring 6.25 mm in diameter were prepared from Whatmann filter paper and sterilized by dry heat at 140° C for 1 h. The sterile plates previously soaked in a known concentration of the test compounds were placed in nutrient agar medium. Solvent and growth controls were maintained. The plates were inverted and incubated for 24 h at 37° C. The inhibition zones were measured and compared with the controls. The cultures were incubated for 24 h at 37° C and the growth was monitored visually and spectrophotometrically. Ciprofloxacin was used as the standard drug. Antimycobacterial studies were carried out on BACTEC 12B medium.
using BACTEC 460 TB, a totally automated system for culture and sensitivity of Mycobacterium by BECTON-DICKINSON USA. Antimycobacterial studies were carried out at Supratech Micropath Laboratories Ahmedabad. Supercoiled plasmid DNA was isolated from E.coli using DNA purification kit (Bangalore Genie kit). Gel electrophoresis studies were carried out on Radon electrophoresis apparatus of Biorad Lab USA.

4.3 Results and Discussion

4.3.1 SECTION A. Fullerene derivatized s-triazine analogues as antimicrobials agents

Bacterial inhibition zone values for all the fulleropyrrolidines are given in Table 1. The investigation of antibacterial screening revealed that all the derivatives showed moderate to good bacterial inhibition. Bacterial inhibition zones developed by fulleropyrrolidines (STFPY1 - STFPY6) were found to much bigger in size as compared to s-trazine based Schiff base precursors (Table 2) indicating the contribution of fullerene moiety towards antimicrobial activity of the synthesized fulleropyrrolidines. Moreover the zones developed by (STFPY1 - STFPY6) were higher for almost all the strains as compared to other fulleropyrrolidines (INHFYPY and LFPY). Amongst the s-triazine derivatized fulleropyrrolidines (STFPY1 - STFPY6), it was STFPY6 which was found to be most active especially against gram positive strains S.aureas and B.subtilis. The zone developed by the solution of compound STFPY6 (100 µg/ml) in the plate against S.aureas and B.subtilis is as shown in the Figure 1. The minimum inhibitory
concentrations of various fulleropyrrolidines are given in Table 3. Compound STFPY6 having ionic \(-\text{NH}_3^+\) group was found to be the most active followed by the derivative STFPY1 in which both \(-\text{NH}_2\) groups were free. The activity was found to decrease on increasing the substitution on \(\text{NH}_2\) group. This observation can be attributed to the fact that on increasing the substitution on \(\text{NH}_2\) group, the hydrophilicity of the molecule as a whole decreases which in turn decreases the disruption caused to the negatively charged bacterial cell membrane (Figure 2).

The investigation of antibacterial screening data revealed that all the fullerene derivatives bearing s-triazine moiety showed moderate to good bacterial inhibition. Although unclear, the probable mode of actions seems to be the interaction of positive charge or the hydrophilic part of the fullerene derivatives (STFPY1- STFPY6) with the negatively charged cell wall of the bacteria followed by the disruption of the cell wall caused by the hydrophobic fullerene moiety.
FIGURES

Figure 1. The zone developed by the solution of compound STFPY6 (100 μg/ml) in the plate against S.aureas(1a) and B.subtilis(1b)

Figure 2. Interaction of triazine derivatized fulleropyrrolidines with cell wall of bacteria
### Tables

#### Table 1. Zone of inhibition of fulleropyrrolidines (STFPY1-STFPY6) (conc. 100 μg/ml)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Staphylococcus Aureus</th>
<th>Bacillus Subtilis</th>
<th>Bacillus Pumilis</th>
<th>Escherichia Coli</th>
<th>Pseudomonas Aeruginosa</th>
<th>Klebsiella Pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td>STFPY1</td>
<td>20</td>
<td>28</td>
<td>28</td>
<td>25</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>STFPY2</td>
<td>19</td>
<td>27</td>
<td>24</td>
<td>24</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>STFPY3</td>
<td>16</td>
<td>23</td>
<td>20</td>
<td>21</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>STFPY4</td>
<td>15</td>
<td>21</td>
<td>18</td>
<td>20</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>STFPY5</td>
<td>13</td>
<td>15</td>
<td>13</td>
<td>14</td>
<td>15</td>
<td>.a</td>
</tr>
<tr>
<td>STFPY6</td>
<td>22</td>
<td>32</td>
<td>31</td>
<td>25</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td>Standardb</td>
<td>22</td>
<td>38</td>
<td>36</td>
<td>27</td>
<td>32</td>
<td>19</td>
</tr>
</tbody>
</table>

- a indicate that the bacteria is resistant to compound.
- b Ciprofloxacin is used as the standard drug.

#### Table 2. Zone of inhibition of intermediate Schiff bases (STHa-STHe) (conc. 100 μg/ml)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Staphylococcus Aureus</th>
<th>Bacillus Subtilis</th>
<th>Bacillus Pumilis</th>
<th>Escherichia Coli</th>
<th>Pseudomonas Aeruginosa</th>
<th>Klebsiella Pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td>STHa</td>
<td>09</td>
<td>16</td>
<td>15</td>
<td>15</td>
<td>11</td>
<td>.a</td>
</tr>
<tr>
<td>STHb</td>
<td>09</td>
<td>18</td>
<td>13</td>
<td>15</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>STHe</td>
<td>11</td>
<td>12</td>
<td>10</td>
<td>14</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>STHd</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>STHe</td>
<td>09</td>
<td>10</td>
<td>10</td>
<td>.a</td>
<td>.a</td>
<td>.a</td>
</tr>
<tr>
<td>Standardb</td>
<td>22</td>
<td>38</td>
<td>36</td>
<td>27</td>
<td>32</td>
<td>19</td>
</tr>
</tbody>
</table>

- a indicate that the bacteria is resistant to compound.
- b Ciprofloxacin is used as the standard drug.
Table 3. MIC results of Fulleropyrrolidines (STFPY1–STFPY6)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Staphylococcus</th>
<th>Bacillus Subtilis</th>
<th>Bacillus Pumilis</th>
<th>Escherichia Coli</th>
<th>Pseudomonas Aeruginosa</th>
<th>Klebsiella Pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td>STFPY1</td>
<td>10</td>
<td>0.1</td>
<td>0.5</td>
<td>10</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>STFPY2</td>
<td>12</td>
<td>0.1</td>
<td>0.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>STFPY3</td>
<td>25</td>
<td>0.5</td>
<td>1.0</td>
<td>12.5</td>
<td>25</td>
<td>12.5</td>
</tr>
<tr>
<td>STFPY4</td>
<td>25</td>
<td>0.5</td>
<td>1.0</td>
<td>12.5</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>STFPY5</td>
<td>50</td>
<td>1.0</td>
<td>5.0</td>
<td>25</td>
<td>25</td>
<td>-^</td>
</tr>
<tr>
<td>STFPY6</td>
<td>6.25</td>
<td>0.08</td>
<td>0.25</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Standard</td>
<td>6.25</td>
<td>0.06</td>
<td>0.25</td>
<td>6.25</td>
<td>6.25</td>
<td>12.5</td>
</tr>
</tbody>
</table>

^ - indicate that the bacteria is resistant to compound

b Ciprofloxacin is used as the standard drug
4.3.2 SECTION B. Fullerene-Isoniazid conjugate as a novel antimycobacterial

4.3.2.1 Preparation of water suspension (INHFPY)

The water suspension of fullerene – isoniazid conjugate INHFPY was prepared by a method similar to that used by Lyon et al.\(^6\) 50 mg of compound INHFPY in 50 ml of Milli-Q water was stirred over low heat (40\(^\circ\)C) for 2 weeks. The brown suspension was then filtered sequentially through a Whatman filter, a 0.45 \(\mu\)m Osmonics nylon membrane and a 0.22 \(\mu\)m nylon membrane to remove aggregates larger than 200 nm. The water suspension obtained was then concentrated by evaporating excess water by vacuum evaporation to get different concentrations ranging from 0.1 to 50 \(\mu\)g/ml.

4.3.2.2 Size and concentration of the suspended particles

The size of suspended particles of compound INHFPY in water was determined by transmission electron microscopy (TEM) using ZEISS EM-900 at 85,000 magnifications. TEM samples were prepared by depositing a drop of dispersed compound INHFPY particles in water on the Formvar-coated copper grids (200 mesh) with electron microscope operated at 100 keV. Figure 3 shows transmission electron micrograph of aggregated nanoparticles of compound INHFPY. The size of the aggregates obtained from TEM electro micrographs were found to be between 50 to 70 nm. The concentration of suspended aggregates in water was determined spectrophotometrically. The particles in suspended form in water were extracted using chloroform. 5 ml of chloroform was added to 5 ml of compound suspension in water and the mixture was then stirred for 2.5 hours. The compound INHFPY in chloroform gave characteristic peak of fullerene.
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moiety at 337 nm from which a standard curve was plotted (Figure 4). The concentration of compound INHFPY in the extracted chloroform was found by comparing the absorbance from the standard plot.

4.3.2.3 Biological studies

The preliminary screening of the antimycobacterial activity of fullerene-isoniazid conjugate (INHFPY) suspension in water and intermediate compounds IAD and IAH in DMSO, were conducted in vitro at a concentration of 10 µg/mL against *M. tuberculosis* and *M. avium*. Compounds effecting > 90% inhibition of growth were retested at lower concentration to determine their minimum inhibitory concentration. Anti-Mycobacterium activity was studied using *M. avium*, 2 strains of *M. tuberculosis* - H37Rv and H6/99. Fullerene-isoniazid conjugate INHFPY showed excellent activity against all these strains as compared to isoniazid as well as the intermediates IAD and IAH. Comparison of the activity with ionic fullerene derivatives showed that they are much more efficient as antimycobacterials and the role of fullerene as well as isoniazid moiety in the molecule was justified. Table 4 summarizes the minimum inhibitory concentration of fullerene – INH conjugate INHFPY and intermediates IAD and IAH as compared to standard drug (isoniazid). Cytotoxic activity assays were performed in Vero cells to determine the maximum nontoxic dose (MNTD expressed as µg/ml). It indicates the drug concentration that decreases cell multiplication less than 50% of the control. Cytotoxicity studies on Vero cells revealed a MNTD value of 30 µg/ml. Protection index obtained as ratio of cytotoxicity (MNTD) and activity (MIC) for compound INHFPY is given in Table 5. In principle, compound INHFPY showed a very good in vitro biological profile.
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It had an impressive activity towards all three tested strains along with poor cytotoxicity resulting in high protection index. Its activity towards \( M. tuberculosis \) H\(_{37}\)Rv was found to be 0.50 \( \mu \)g/ml which was comparable to isoniazid. Moreover the activity towards the resistant strains was excellent when compared to the reported ionic fullerene derivatives as well as intermediate IAD and IAH, highlighting the importance of both the fullerene and the isoniazid moiety in the compound. The same studies were carried out with STFPY1-STFPY6 and LYFPY. The antimycobacterial activities of all these compounds were negligible as compared to INHFPY.

An analysis of the lipophilic character showed that the calculated logP \(^{64}\) value for fullerene – Isoniazid conjugate INHFPY (21.03) is much higher as compared to IAD (0.68) and IAH (1.12) indicating that it is much more capable of interacting with the hydrophobic cell wall of mycobacteria. These results in which the antimycobacterial activity was improved by increase in lipophilicity, provided support for the hypothesis that an increase of lipophilicity (by introduction of fullerene) could improve antimycobacterial activity.

Hence, from the lipophilicity point of view and also from the pharmacological studies it became clear that the synthesized fullerene-isoniazid conjugate can be an ideal drug candidate for the development of anti-TB drugs. Since the Schiff base precursors IAD and IAH were not found to be as active against the strains tested as compared to fullerene-INH conjugate, we can attribute a major role in the activity to the fullerene moiety. On comparison of activity with ionic fullerene derivatives, the presence of isoniazid moiety was also justified. Moreover the cytotoxicity for fullerene-isoniazid conjugate INHFPY (\( \text{MNTD}_{50} = 30 \mu \)g/ml) was found to be much better as compared to
intermediates **IAD** (MNTD$_{50}$ = 10 µg/ml) and **IAH** (MNTD$_{50}$ = 15 µg/ml). The presence of Schiff base linkage prevents the acetylation of the hydrazine moiety and since the compound has a hydrophilic as well as hydrophobic group it can easily enter the hydrophobic membrane of the cell wall and rupture it. It is generally expected that entry of the drug in the mycobacterium may be either through porins or through crossing hydrophobic core of the outer membrane. Porin pathway is viable for small hydrophilic molecules, whereas for more bigger and hydrophobic molecules like fullerene-INH conjugate, membrane pathway seems to be the most probable route. Although this is supposed to be the mode of entry, the exact process by which the compound acts as well as different ways to enhance its activity is currently being investigated in our laboratory.
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FIGURES

Figure 3. TEM micrograph of INHFPY aggregates in water

Figure 4. Plot of Absorbance vs conc. of INHFPY in chloroform
### Table 4. Minimum Inhibitory concentration (MIC) of compounds IAD, IAH and INHFPY as compared to standard drug Isoniazid and ionic fullerene derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>M. tuberculosis H$_{37}$Rv ATCC 27294 MIC (µg/ml)</th>
<th>M. tuberculosis H6/99 MIC (µg/ml)</th>
<th>M. avium ATCC 27291 MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAD$^a$</td>
<td>5</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>IAH$^a$</td>
<td>5</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>INHFPY$^b$</td>
<td>0.50</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.25</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>Most active ionic fullerene derivative (Ref No. 52)</td>
<td>50</td>
<td>5</td>
<td>50</td>
</tr>
</tbody>
</table>

$^a$ DMSO solution $^b$ Water suspension

### Table 5. In vitro activity, cytotoxicity and protection index (PI) of INHFPY against the strains of mycobacterium

<table>
<thead>
<tr>
<th></th>
<th>M. tuberculosis H$_{37}$Rv</th>
<th>M. tuberculosis H6/99</th>
<th>M. avium</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^a$MIC</td>
<td>0.50</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>$^b$PI</td>
<td>60</td>
<td>12</td>
<td>6</td>
</tr>
</tbody>
</table>

$^a$MIC (µg/ml) MNTD$_{50}$ (µg/ml) toward Vero cells= 30 µg/ml $^b$PI= protection index as the ratio of cytotoxicity (MNTD$_{50}$) to in vitro activity (MIC)
4.3.3 SECTION C. Photoinduced DNA cleavage by Lysine derivatized fulleropyrrolidine (LYFPY)

4.3.3.1 DNA cleavage studies general procedure

The 30 μL of aqueous solution of DNA pBR322 (0.50 μg μL⁻¹) was diluted by adding 270 μL of water. A total of eight different combinations were placed in 8 lanes of the electrophoresis apparatus to study the DNA cleavage (Figure 5). In Lane 1, only the effect of photoirradiation of DNA was analyzed. In Lane 2, the effect of photoirradiation of DNA was analyzed in presence of Nicotinamide adenine dinucleotide (NADH). In Lane 3, the effect of photoirradiation of DNA was analyzed in presence of NADH and LYFPY. Typically, 10 μL of aqueous solution of LYFPY (1.0 × 10⁻⁵ M), 10 μL of aqueous solution of DNA pBR322, NADH (5 μL, 0.126 M) and 8 μL of Tris-EDTA buffer (10 μL, 150 × TE, pH 8.0) were mixed in a micro test tube under dark conditions. Samples were irradiated with visible light for 3 h at 298 K, mixed with 10 μL of loading buffer (0.1% bromophenol blue and 30% glycerol in TBE buffer), and loaded onto a 1% agarose gel containing ethidium bromide (1 μg mL⁻¹). The gels were run at a constant voltage of 70 V for 2 h in TBE buffer, washed with distilled water, visualized under a UV transilluminator, and photographed using an instant camera. The lane 4 comprised of DNA, LYFPY and NADH but without photoirradiation. In lane 6, 7 and 8 the same set of experiments were carried out with LYFPY being replaced by intermediate Boc-lysine hydrazone (BLH).
4.3.3.2 Analysis of gel electrophoresis studies

The DNA cleavage activity of LYFPY was investigated using pBR322 supercoiled plasmid (form 1) in a LYFPY - NADH system under visible light irradiation. As shown in Figure 5, buffer solution (pH 8.0) of LYFPY was found to cleave pBR322 supercoiled DNA into form 2 (nicked DNA) after 3 hour visible light irradiation at 298 K (Lane 3). Under dark condition no DNA cleavage was observed (Lane 5). The same experiment was carried out with intermediate lysine-terephthalaldehyde conjugate BLH. No DNA cleavage was observed under both the conditions of photoirradiation and in dark (Lane 6, 7, 8).

In control experiments, it was found that no DNA cleavage was found to occur in dark or under photoirradiation in the absence of either LYFPY or NADH (Lane 1, 2, 4), showing the importance of both NADH and compound LYFPY in the cleavage activity.

Effect of visible light irradiation time on DNA cleavage was analyzed by changing the irradiation time from 1 to 6 hours. The amount of nicked DNA was found to increase with the prolongation of irradiation time. After six hours of irradiation the percentage of nicked DNA almost reaches saturation.

The concentration of LYFPY and NADH has been investigated to obtain a better understanding of the DNA cleavage by the synthesized fullerene-lysine conjugate LYFPY. The yield of nicked DNA is improved with the increase in concentration of LYFPY. At a low concentration (1 × 10^{-6} M), only a small amount of supercoiled plasmid DNA is converted to nicked DNA, while the conversion ratio of supercoiled plasmid DNA is improved dramatically as the concentration of LYFPY is increased to 1.0 × 10^{-5} M which is much lower than that reported of γ-cyclodextrin-bicapped C_{60} (CD/C_{60}) by Wang et.al.
The conversion ratio of supercoiled plasmid DNA is quite small at a low NADH concentration, while the proportion of nicked DNA was substantially increased at a high NADH concentration, consistent with previous results that NADH is an important coagent for the photoinduced cleavage of DNA by fullerenes. In order to analyze the role of positive charge on compound LYFPY in cleavage activity, the same experiments were also carried out with intermediate compound BLYFPY. Because of low solubility, a suspension of compound BLYFPY in water was prepared for biological studies. Under the same experimental conditions i.e. with photoirradiation and in presence of NADH, compound BLYFPY was also found to cleave DNA but only at concentration higher than 1mM, which is about 100 times more than that required for compound LYFPY. This difference in activity clearly indicates the better interaction of positively charged compound LYFPY with negatively charged DNA. The same experiments were carried out with triazine-fullerene conjugates (STFPY1 – STFPY6) and fullerene – isoniazid conjugate INHFPY. Only positively charged STFPY6 was found to cleave DNA but only at concentration above 1mM. All these studies showed that both fullerene as well as unprotected lysine moiety is essential for DNA cleavage.

4.3.3.3 Mode of Action

The photoinduced DNA cleavage depends greatly on the generation of reactive oxygen species (ROS), including singlet oxygen (\( {^1}O_2 \)), superoxide radical anion (\( O_2^- \)), and hydroxyl radical (\( {^*}OH \)), which are proposed to be the actual active species for the photoinduced DNA cleavage. The triplet excited state \( C_{60} \) (\( {^3}C_{60}^* \)) generated via the intersystem crossing from the singlet excited state of \( C_{60} \), which is produced upon light irradiation, is a key intermediate for generation of ROS. Either the energy transfer
process can occur, where the energy is transferred from $^3\text{C}_{60}^*$ to the oxygen molecule to generate $^1\text{O}_2$, or the electron transfer process can occur in the presence of reductant, such as NADH, where $^3\text{C}_{60}^*$ is reduced to produce $\text{C}_6\text{O}^-$ anion radical ($\text{C}_6\text{O}^-$), followed by an electron transfer from $\text{C}_6\text{O}^-$ to the oxygen molecule to give rise to $\text{O}_2^-$ or $\cdot\text{OH}$ in a further step. However, the aggregation of $\text{C}_6\text{O}$ has been shown to significantly accelerate the decay of $^3\text{C}_{60}^*$, resulting in less interaction time between $^3\text{C}_{60}^*$ and oxygen molecule, thus reducing the generation of $^1\text{O}_2$. Zhang et.al have shown that larger aggregation destabilizes $\text{C}_6\text{O}$ anion radical, suggesting that the $\text{C}_6\text{O}$ radical anion generated by reducing $^3\text{C}_{60}^*$ with NADH would be less stable in the more aggregated $\text{C}_6\text{O}$ solution, thus impairing the generation of $\text{O}_2^-$ and $\cdot\text{OH}$. Water-soluble fullerenes are found to be easily aggregated in aqueous solution, where the hydrophobic force between fullerene surfaces is proposed to be the driving force for such aggregation. Wang et.al have shown that although the hydrophobic force that drives the aggregation of $\text{C}_6\text{O}$ molecules can also enhance the interactions between $\text{C}_6\text{O}$ and DNA since DNA bases are hydrophobic, smaller the size of aggregated particles more is the efficiency in photoinduced DNA cleavage. Hence dynamic light scattering (DLS) experiment was carried out to analyze the size of the aggregated particles of LYFPY, if any. The concentration for DLS analysis was taken as $1.0 \times 10^{-5}$ M, the same that was used for DNA photocleavage. The average size of aggregated particles were found to be 20 nm which is much smaller as compared to the aggregates of γ-cyclodextrin-bicapped $\text{C}_{60}$ (CD/$\text{C}_{60}$) reported by Wang et.al. In order to understand the mode of DNA cleavage, the same experiments of gel electrophoresis were carried out in presence of sodium azide and L-histidine, singlet oxygen scavengers. No change in DNA cleavage was observed in the presence of either
of these two singlet oxygen scavengers. However the cleaving activity was clearly inhibited by the addition of superoxide dismutase (SOD), which quenches O₂⁻. This result suggested that O₂⁻ is a key intermediate for DNA-cleaving activity of LYFPY in presence of NADH (Figure 6).

4.4 Conclusion

The investigation of antibacterial screening data revealed that all the fullerene derivatives bearing s-trazine moiety (STFPY1-STFPY6) showed moderate to good bacterial inhibition as compared to INHFPY and LYFPY. STFPY6 was found to be the most active probably because of the positive charge and the triazine carried by it could have helped in better interaction with the negatively charged bacterial cell membrane. Fullerene – isoniazid conjugate INHFPY was designed, synthesized and tested to probe the influence of lipophilicity on antimycobacterial activity, based on the hypothesis that an increase of lipophilicity could improve inhibitory activity towards mycobacteria. The conjugate thus synthesized had a high logP value or lipophilicity as compared to isoniazid and previously reported ionic fullerene derivatives. The in vitro studies of the synthesized fullerene-isoniazid conjugate showed excellent activity against M. tuberculosis and M. avium with low cytotoxicity. Fullerene - lysine conjugate LYFPY was found to cleave supercoiled DNA under photoirradiation in presence of NADH. Although the mechanism of action is not very clear, superoxide radical generated on photoirradiation seems to be the reactive oxygen species (ROS) behind the DNA cleavage. This work opens up interesting prospects in the field of DNA cleavage by this novel class of fullerene based amino acids.
Figure 5. Photocleavage of pBR322 supercoiled DNA by fullerene-lysine conjugate LYFPY analyzed by agarose gel electrophoresis. \([\text{LYFPY}] = 1 \times 10^{-5} \text{ M}, [\text{BLH}] = 1 \times 10^{-5} \text{ M}, [\text{NADH}] = 1 \times 10^{-3} \text{ M}\)

Figure 6. Proposed mode of DNA cleavage by LYFPY
References


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