ABSTRACT SUMMARY
Non small cell lung cancer (NSCLC) has a grim prognosis even though there are advancements in its early diagnosis and treatment. Currently, pemetrexed (Alimta®) and gemcitabine (Gemzar®) are among the most effective chemotherapeutic drugs for the treatment of NSCLC. And in this pretest, several preclinical and clinical studies have been conducted using pemetrexed and gemcitabine combination regime for NSCLC treatment that reveal the therapeutic potential of this combination. But the other important concern is the bioavailability of the native drugs that need to be addressed for a successful optimisation of the system towards clinical applications. Polymer therapeutics is well established for the delivery of a single therapeutic agent, however in the recent years their use has been extended to the combinational drug delivery approach for better cancer management. Hence, we have developed PemPEG-Gem, a dual drug PEGylated system that carries pemetrexed and gemcitabine simultaneously for a better clinical application in the treatment of NSCLC.

INTRODUCTION
Due to the high frequency of cancer, the use of chemotherapy to evoke cure or prolongation of survival has become critically important. Research in cancer drug development achieved an experimental breakthrough in the simultaneous use of two or more chemotherapeutic agents for treating cancer, known as combination therapy. In the recent years, the introduction of two novel antimitabolites, Gemcitabine and pemetrexed in combination therapy against a broad spectrum of cancer, particularly NSCLC and ovarian cancer has shown promising results. Inhibition of de novo purine biosynthesis by pemetrexed treatment increases the activity of gemcitabine as a compensatory mechanism, thus leading to synergistic effect in combination therapy. However, the limitation associated with these antimitabolites in combination therapy is their low molecular weight (> 1 k Da) due to which they have short biological half-life. This lead to frequent administration at high doses that are escorted with myelosuppression, high levels of hepatotoxicity & renal toxicity along with toxicity towards other normal tissues or organs. Polymer therapeutics is already emerged as one of the most propitious platform for the efficient delivery of anticancer agents. The employment of polyethylene glycol (PEG) as modifying polymer for “PEGylation” has been the most successful strategy of polymer therapeutics. PEG is FDA approved for human use and is a water soluble amphiphilic polymer with high solubility and excellent biocompatibility. In recent years, PEGylated anticancer drugs are in clinical development for both single agent therapy and combination therapy. The application of PEGylation for the combination therapy ensures that such drug cocktails are truly simultaneously delivered at the target site by safeguarding their enhanced stability from rapid enzymatic degradation and increasing their bioavailability for prolonged action. Hence, we propose to develop Pem-PEG-Gem, a dual drug PEGylated system that carries pemetrexed and gemcitabine simultaneously. The FTIR and NMR studies reveal the amide bond formation between the drugs and the polymer. Besides, our pharmacokinetic studies shows Pem-PEG-Gem provide an enhanced bioavailability of these drugs in comparison to their native forms in blood serum. The in vitro studies conducted in NSCLC cell lines exhibit enhanced cytotoxicity of Pem-PEG-Gem in comparison to the native forms.

EXPERIMENTAL METHODS
Preparation of Pem-PEG-Gem:
The Pem-PEG-Gem is synthesized in presence of TEA, NHS and DCC as described by Yoo et al. The synthesis of Pem-PEG-Gem first involves the synthesis of Pem-PEG-COOH. Briefly, pemetrexed dissolved in DMSO was reacted with NHS and DCC under N2 atmosphere at room temperature for 12 h. The activated drug was then reacted with NH2–PEG-COOH (5 kDa) dissolved. The reaction was again performed under N2 atmosphere at room temperature for 4 h. The resultant solution was diluted with deionized water and dialyzed against deionized water for a period of 24 h with frequent change of dialysate in every 2 h. Then the dialyzed solution was freeze-dried to obtain the powdered form of the Pem-PEG-COOH. Subsequently, the prepared Pem-PEG-COOH is activated by NHS, DCC chemistry and then made to react with gemcitabine in presence of TEA (PEG/Gemcitabine/TEA molar ratio = 1:2:20). The reaction was performed under N2 atmosphere at room temperature for 4 h. Then the reaction mixture was dialyzed against distilled water and freeze-dried to obtain the powdered form of the conjugate.

Physico-chemical characterization of the conjugates was done by Fourier transform spectroscopy (FT IR), Proton-Nuclear magnetic resonance spectroscopy (H NMR), Reverse phase High performance liquid chromatography (RP-HPLC) and Gel permeation chromatography (GPC).

Pharmacokinetic studies were carried out to find the bioavailability of the native drugs and PEGylated drugs in animal model. The experiment on animals was performed with the permission of Institutional Animal Ethics
Committee of Institute of Life Sciences, Bhubaneswar, India.

The cytotoxicity assay was done by MTT assay for analyzing the potential of cytotoxicity of Pem-PEG-Gem formulation in comparison to those of (dose-equivalent) combination regimens of native gemcitabine plus pemetrexed, and the native drugs in various NSCLC cancer cell lines.

RESULTS AND DISCUSSION

Pem-PEG-Gem is synthesized by a two step process. Initially, pemetrexed conjugated to PEG. Next, Pem-PEG-COOH synthesized is conjugated to gemcitabine. For further confirmation of the Pem-PEG-Gem, various analytical techniques were used. The NMR signals of Pem-PEG-Gem at δ = 8.7 ppm and 10.7 ppm correspond to amide signals. (Fig. 1a) Thus, NMR confirms the covalent bond formation in Pem-PEG-Gem.

Figure 1: (a) 1H NMR spectra, (b) FT IR spectra and (c) GPC analysis of Pem-PEG-Gem.

As shown in figure 1(b), Pem-PEG-Gem displayed the characteristic peaks of amide bond at 1628 cm\(^{-1}\), 1575 cm\(^{-1}\), 1537 cm\(^{-1}\) and 1450 cm\(^{-1}\). Hence, this confirms the amide bond formation between Pem-PEG-Gem. The characteristic peak of COOH-PEG-NH\(_2\) was obtained at 18.5 ml retention volume. (Fig. 1c) In addition, a dominant peak at a lower retention volume appeared at 14 ml owing to higher molecular weight. This clearly indicates the formation of Pem-PEG-Gem.

The amount of drug conjugated to PEG was analyzed by RP-HPLC system. The amount of gemcitabine in 1 mg of Pem-PEG-Gem was 20 µg and amount of pemetrexed in 1 mg of Pem-PEG-Gem was 25 µg.

Figure 2: The in vivo bioavailability study of native pemetrexed (10mg/kg), native gemcitabine (10 mg/kg) and Pem-PEG-Gem in normal BALC/c mice were evaluated by RP-HPLC.

The graph in the figure 2 depicts the decreasing plasma levels of native pemetrexed and gemcitabine compared to Pem-PEG-Gem with increasing time points. The concentration of pemetrexed and gemcitabine in plasma was evaluated in mice for the period of 72 h. PEGylation allows a higher bioavailability of pemetrexed and gemcitabine in the in vivo condition in comparison to the native form of the pemetrexed and gemcitabine.

Table 1: The IC50 values (µg/ml) of the MTT assay performed for various treatmentS for 5 days in A549 and NCI-H460 cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Parameter</th>
<th>Gemcitabine equivalent</th>
<th>Pemetrexed equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H460</td>
<td>Native drug (P/G)</td>
<td>0.2257 µg/ml</td>
<td>0.1441 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Native (Pem+Gem)</td>
<td>0.0036 µg/ml</td>
<td>0.0022 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Pem-PEG-Gem</td>
<td>0.0009 µg/ml</td>
<td>0.0004 µg/ml</td>
</tr>
<tr>
<td>A549</td>
<td>Native drug (P/G)</td>
<td>1.4446 µg/ml</td>
<td>0.1283 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Native (Pem+Gem)</td>
<td>0.1281 µg/ml</td>
<td>0.0006 µg/ml</td>
</tr>
<tr>
<td></td>
<td>Pem-PEG-Gem</td>
<td>0.03426 µg/ml</td>
<td>0.0001 µg/ml</td>
</tr>
</tbody>
</table>

The cytotoxicity parameter of Pem-PEG-Gem in comparison to native drugs was assessed in A549 and NCI-H460 cells by MTT assay. In this regard, we have calculated the concentration which causes 50 % inhibition of control growth rate i.e., IC50 value as shown in Table 1. The enhanced cytotoxicity was observed in both the cell lines after 5 days treatment of Pem-PEG-Gem in comparison to native drugs alone and in combination.

CONCLUSION

The present study demonstrates Pem-PEG-Gem has an amide bond formation between the drugs and the polymer. Further, bioavailability study showed prolonged circulation time of gemcitabine and pemetrexed in Pem-PEG-Gem in plasma of mice. And, the simultaneous additive action of pemetrexed and gemcitabine in Pem-PEG-Gem lead to the increased cytotoxicity in the cancer cells. Thus, Pem-PEG-Gem is a suitable candidate for clinical application in cancer therapy.

REFERENCES


ACKNOWLEDGMENTS

M. Vandana would like to thank “Council of Scientific and Industrial Research”, Government of India, for providing a senior research fellowship.