Pancreatic cancer is the most dangerous form of adenocarcinoma because of its difficult treatment (1). Gemcitabine is the first known chemotherapeutic drug which is available in the market for the treatment of pancreatic cancer (2). Although gemcitabine is a very effective drug for the treatment of adenocarcinoma, it has many side effects i.e. very short circulation in plasma and it accumulates in other body organs very quickly (3). So it requires in high amounts but it can lead to damage of other body organs. Many researchers have been working on the pegylation of gemcitabine (4) and making different types of nanoparticles and nanovesicles (5). The plasma stability has also been increased by polypeptide conjugation of gemcitabine (6). Another approach by scientists in nanotechnology is the formation of herceptin HER2-conjugated gemcitabine- nanoparticles which are loaded with chitosan for adenocarcinoma (7).

Methotrexate is another anti-cancer drug which inhibits the cell replication and cell proliferation (8). However, methotrexate also exhibits many drawbacks like gemcitabine, like rapid metabolism and fast excretion from the body and harmful biodistribution. The solution of the side effects is the attachment of drug with a molecule which enhances its pharmacological properties. Many researchers worked for the methotrexate pegylated nanoparticles which contained chitosan and methotrexate loaded chitosan nanoparticles which contained sodium alginate (9). Nanoparticles which loaded with human serum albumin also synthesized which showed better results as compared to the methotrexate alone (10) but these reactions are much complex.

In the present study, we synthesized covalent conjugates of gemcitabine and methotrexate with polyethylene glycol derivatives (Fig. 1), investigated the in vitro cytotoxic activity towards HCT116 (human colon cancer cell) line also in vivo analysis was performed for drug release profiling to assess their enhanced blood circulation. To avoid any carrier or nanomaterial for drug delivery, polyethylene glycol was used for pegylation because these are biocompatible and approved by the Food and Drug Administration (FDA-USA).
EXPERIMENTAL

Chemistry
In the present research, analytical reagent grade (AR) chemicals were used and purchased from Falcon Scientific, Lahore originate to Merck (Germany), Sigma Aldrich (USA) and BDH (UK) to synthesize the desired compounds. A simple method is adopted for pegylation of gemcitabine and methotrexate. 1 mmol of gemcitabine was dissolved in 20 mL of methanol and allow to stir for 30 min. 1 mmol each of O-[(N-Succinimidyl)succinyl-
PEGylated methotrexate (MTHX-PEG-01)

IR ν max (cm−1): 3244 (OH), 3340 (NH amide, stretching), 1610 (C=O carbonyl), 1122, 731 (C-H aromatic); 1HNMR (MeOD, 500 MHz): 11.01 (s, 2H, 2 OH), 9.16 (H, d, J = 10.5, ArH), 8.56 (H, s, ArH), 8.04 (s, 2H, 2 × NH), 7.14 (2H, dd, J = 7.5, 1.5, ArH), 6.94 (2H, dd, J = 7.5, 1.5, ArH), 6.36 (s, H, CH), 5.45 (H, d, J = 10.5, ArH), 4.39 (H, q, J = 7.5, CH), 4.11 (s, H, CH), 3.79 (2H, d, J = 7.1, CH2), 3.79 (2H, t, J = 7.1, CH3), 3.29 (2H, t, J = 7.1, CH3), 2.49 (s, 8H, 4 × CH2).

PEGylated methotrexate (MTHX-PEG-02)

IR ν max (cm−1): 3254 (OH), 3343 (NH amide, stretching), 1017 (C-N amine), 1683 (N-H amide, bending), 1600, 1560 (C=O aromatic), 1768 (C=O carbonyl), 1128, 731 (C-H aromatic); 1HNMR (MeOD, 500 MHz): 11.01 (s, 2H, 2 × OH), 9.16 (H, d, J = 10.5, ArH), 8.56 (H, s, ArH), 8.04 (s, 2H, 2 × NH), 7.14 (2H, dd, J = 7.5, 1.5, ArH), 6.94 (2H, dd, J = 7.5, 1.5, ArH), 6.36 (s, H, CH), 5.45 (H, d, J = 10.5, ArH), 4.39 (H, q, J = 7.5, CH), 4.11 (s, H, CH), 3.79 (2H, d, J = 7.1, CH2), 3.79 (2H, t, J = 7.1, CH3), 3.29 (2H, t, J = 7.1, CH3), 2.09 (s, 2H, CH2).

PEGylated gemcitabine (GMCT-PEG-01)

IR ν max (cm−1): 3244 (OH), 3340 (NH amide, stretching), 1010 (C-N amine), 1689 (N-H amide, bending), 1602, 1562 (C=O aromatic), 1764 (C=O carbonyl), 1124, 733 (C-H aromatic); 1HNMR (MeOD, 500 MHz): 9.16 (H, d, J = 10.5, ArH), 8.04 (s, 2H, 2 × NH), 6.36 (s, H, CH), 5.45 (H, d, J = 10.5, ArH), 4.39 (H, q, J = 7.5, CH), 4.11 (s, H, CH), 3.79 (2H, d, J = 7.1, CH2), 3.79 (2H, t, J = 7.1, CH3), 3.61 (s, 2H, 2 × OH), 3.29 (2H, t, J = 7.1, CH3), 2.49 (s, 4H, 2 × CH2).

PEGylated gemcitabine (GMCT-PEG-02)

IR ν max (cm−1): 3241 (OH), 3332 (NH amide, stretching), 1014 (C-N amine), 1683 (N-H amide, bending), 1600, 1560 (C=O aromatic), 1761 (C=O carbonyl), 1122, 731 (C-H aromatic); 1HNMR (MeOD, 500 MHz): 9.16 (H, d, J = 10.5, ArH), 8.04 (s, 2H, 2 × NH), 6.36 (s, H, CH), 5.45 (H, d, J = 10.5, ArH), 4.39 (H, q, J = 7.5, CH), 4.11 (s, H, CH), 3.79 (2H, d, J = 7.1, CH2), 3.79 (2H, t, J = 7.1, CH3), 3.61 (s, 2H, 2 × OH), 3.29 (2H, t, J = 7.1, CH3).

PEGylated methotrexate (MTHX-PEG-03)

IR ν max (cm−1): 3246 (OH), 3338 (NH amide, stretching), 1014 (C-N amine), 1683 (N-H amide, bending), 1600, 1560 (C=O aromatic), 1768 (C=O carbonyl), 1122, 731 (C-H aromatic); 1HNMR (MeOD, 500 MHz): 11.01 (s, 2H, 2 × OH), 9.16 (H, d, J = 10.5, ArH), 8.56 (H, s, ArH), 8.04 (s, 2H, 2 × NH), 7.14 (2H, dd, J = 7.5, 1.5, ArH), 6.94 (2H, dd, J = 7.5, 1.5, ArH), 6.36 (s, H, CH), 5.45 (H, d, J = 10.5, ArH), 4.39 (H, q, J = 7.5, CH), 4.11 (s, H, CH), 3.79 (2H, d, J = 7.1, CH2), 3.79 (2H, t, J = 7.1, CH3), 3.29 (2H, t, J = 7.1, CH3), 2.49 (s, 8H, 4 × CH2).

Anticancer activities

In vitro anti-proliferation assay of standard and both samples was performed by CellTiter-Glo® 3D Cell viability assay against HTC116 cells. Cells
were cultured in Dulbecco modified eagle medium supplemented with 10% fetal bovine serum at 37°C with 5% CO₂. The cells HTC116 were harvested by trypsinization. Cells at the density of 5 × 10³ cells/well were seeded in 96-well plates and incubated for 24 h to allow cell attachment. After attachment spheroids formed in the plates, images of spheroids taken after 24 h and fluorescence data captured to determined cytotoxicity. The cells were incubated then with free drugs as well as all conjugated compounds at equivalent drug concentrations of 50-1,562 µM for 48 h. Next day after 48 h 25 µL of cell titer glow reagent added to each well of spheroids plate then incubated with shaking for 30 min at room temperature and captured luminescence data. After 48 h images of all spheroids were taken and IC₅₀ value was calculated after absorbance was measured at 268 nm for gemcitabine hydrochloride and 258 nm for methotrexate compounds using microplate reader (BioRad, USA). And IC₅₀ of all the compounds were determined by plotting a graph on PRISM 7.0.

RESULTS AND DISCUSSION

Chemistry

Four new pegylated gemcitabine and methotrexate were synthesized by a simple reaction of

![Figure 2. Drug release profile (a) in vitro (b) in vivo](image)

Table 1. Physicochemical data of PEGylated gemcitabine and methotrexate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Colour</th>
<th>Yield (%)</th>
<th>&quot;LogP&quot;</th>
<th>&quot;RI&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMCT-PEG-01</td>
<td>Off white</td>
<td>84.1</td>
<td>-0.79</td>
<td>0.73</td>
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<tr>
<td>GMCT-PEG-02</td>
<td>Off white</td>
<td>88.0</td>
<td>-0.28</td>
<td>0.83</td>
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<tr>
<td>MTHX-PEG-03</td>
<td>Yellow</td>
<td>89.8</td>
<td>-1.93</td>
<td>0.65</td>
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<tr>
<td>MTHX-PEG-04</td>
<td>Yellow</td>
<td>93.1</td>
<td>0.21</td>
<td>0.78</td>
</tr>
</tbody>
</table>

*Derived from ChemDraw Ultra 12.0, *Mobile phase: chloroform and methanol (8 : 2)
Pegylated gemcitabine and methotrexate: in vitro cytotoxicity analysis...

gemcitabine and methotrexate with polyethylene glycol as explained in the experimental section. The products were recrystallized after washing with cold distilled water, recrystallized with methanol and dried over magnesium sulfate and all the pegylated drugs were obtained in good yield (84.1-93.1%). The structures of these pegylated drugs were confirmed by NMR and FT-IR. The FT-IR. The characteristics band at 3246-3254 cm⁻¹ of (O-H carboxylic), N-H amide stretching at 3332-3343 cm⁻¹ and 1010-1017 cm⁻¹ for C-N, for all compounds reveals the conjugation of polyethylene glycol and assured the completion of pegylation process. In ¹HNMR spectra, a signal at δ 8.04 ppm for NH-CO of benzene ring and 2.29 ppm of CH₂ attached to ring while signal at δ 11.01 corresponds to OH of carboxylic acid of compound MTHX-PEG-03 and MTHX-PEG-04 which further confirm the completion of pegylation process and formation of pegylated products.

Drug release profile

_in vitro_ and _in vivo_ drug release profile of all compounds showed a slow and sustained release pattern with respect to time but the release profile of free drug showed a very rapid release of drug as shown in Figure 2.

The sustained and controlled release behavior of pegylated drugs over the extended period showed a linear profile and this release pattern was not limited to diffusion process instead supporting chemically controlled release pattern which was dependent only on how fast the compound hydrolyzed in PSB. The released compounds were analyzed through reverse phase HPLC and results are presented in Table 2.

**Anticancer activities**

Anticancer activities of pegylated as well as free drugs under investigation were evaluated by CellTiter-Glo® 3D (Promega Corporation, Madison, USA) cell viability assay against HCT116 cell line. The results (Table 3) showed that pegylated drugs have slightly different IC₅₀ values (Table 3) but are

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ value (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMCT-PEG-01</td>
<td>7.6</td>
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<tr>
<td>GMCT-PEG-02</td>
<td>8.3</td>
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<td>MTHX-PEG-03</td>
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<td>MTHX-PEG-04</td>
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<tr>
<td>Gemcitabine</td>
<td>7.5</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>8.2</td>
</tr>
</tbody>
</table>
equally effective in killing the cancer cells. Pegylated methotrexate showed the lowest IC₅₀ and this antiproliferation effect might due to their cell permeability having lower cLogP values as shown in Table 1.

Since the CellTiter-Glo® 3D cell viability assay was performed which relies on the thermostable properties of a proprietary luciferase (Ultra-Glo™ Recombinant Luciferase), which generates a stable “glow-type” luminescent signal. The large size

![Figure 3. Spheroids formation after 24 h](image)

![Figure 4. Spheroids after addition of drugs at 48 h](image)
spheroids were observed when the cells were not treated with drugs as shown in Figure 3. After treating with pegylated and free drugs, small to moderate size spheroids were formed after getting the luminescence data as shown in Figure 4, greater the luminescence less will be cell viability. From this luminescence values IC50 value was calculated for each drug under investigation.

**CONCLUSION**

We have prepared pegylated gemcitabine, methotrexate and evaluated their anticancer effects against the HCT116 cell line by using CellTiter-Glo® 3D cell viability assay against. The pegylated drugs have slightly different IC50 values from each other but are equally effective in killing the cancer cells. Pegylated methotrexate showed the lowest IC50 and this antiproliferation effect might due to their cell permeability having lower cLogP comparative to others. The sustained release pattern was also assessed in vitro and in vivo, the results revealed a slow and sustained release pattern with respect to time but the release profile of free drug showed a very rapid excretion.

**Compliance with ethical standards**

**Informed consent**

Not applicable

**Conflict of interest**

We wish to confirm that there are no known conflicts of interest associated with this publication.

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**Ethical approval**

This article contains studies with animals performed by the authors and ethical approval with the document provided in the experimental section.

**REFERENCES**


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