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Research Article

PREPARATION AND CHARACTERIZATION OF PACLITAXEL-FOLIC ACID CONJUGATES TO TARGET CANCER CELLS

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ABSTRACT

The targeted drug delivery system improves the delivery of drugs right to the specific site, without affecting any healthy tissues and organs. It enhances the efficacy of drug at desired site by modifying the carrier to the target site and also improves patient compliance due to decreased dosing frequency. In this study, we developed a new multi-small molecule conjugated Paclitaxel (PTX) delivery system for targeted tumor therapy. Water-soluble small molecules, amino acids (Glu), FA, and fluorescent dye were successfully conjugated to PTX. Conjugation of paclitaxel to the small-molecules was further confirmed by UV, IR, NMR spectroscopic analysis and XRD analysis. In the present investigation, an attempt was made to develop, and characterize paclitaxel-Folic acid conjugates to target cancer cells through the drug-ligand for achieving controlled release. The linkage can be designed to improve water solubility, to enhance presentation of the folate to its receptor, or to release the attached drug in unmodified form only after uptake into the target cell. It also enhances the solubility of synthesized FA-Glu(OtBu)-PTX conjugate. Results of the study therefore concluded that FA-Glu(OtBu)-PTX conjugate has been effectively synthesized which could be used for formulation development like transferosomes and might be useful for targeted delivery system in cancer treatment.

Keywords: Paclitaxel, Folic acid, Paclitaxel-Folic Acid Conjugates, Transferosomes, Targeted Delivery System

1. INTRODUCTION

Breast cancer is the most commonly occurring cancer in women, comprising almost one third of all malignancies in females. It is a second leading cause of cancer. The 95% of breast cancers are carcinomas, i.e. they arise from cells in the breast and the most common sites for the spread of breast cancer are skin and distant lymph nodes [1, 2]. Surgery, radiotherapy, immunotherapy and chemotherapy are the common strategies used to treat breast cancer. American Cancer Society provides an overview of female breast cancer statistics in the United States, including data on incidence, mortality, survival, and screening. A total number of 1,658,370 new cancer cases and 589,430 cancer deaths were projected in 2015. Breast cancer alone accounts for 29% of all new cancers in women. According to the American cancer society, current year data for breast cancer in the United States are about 246,660 new cases of invasive breast cancer and 61,000 new cases of carcinoma in-situ (DCIS) will be diagnosed in women. The chance of death due to breast cancer in women is 1 in 36 [3, 4].

In conventional chemotherapy, most of the anti-cancer drugs are administered systemically using I.V. injection after which drugs are distributed between normal and cancer cells leading to unacceptable side effects [5]. Excipients present in conventional formulation containing anticancer drug may produce severe side effects like vasodilation, labored breathing, lethargy, hypersensitivity, cardio-toxicity and life threatening anaphylaxis, aggregation of erythrocytes, nephrotoxicity and neurotoxicity in many patients. To overcome these drawbacks, an alternative non-parenteral route of administration can be used to treat breast cancer patients. The targeted drug delivery system overcome these shortcomings and deliver the drugs right to the specific organ, without affecting any healthy organs and tissues. It enhances the efficacy of drug at desired site by modifying the carrier to the target site and also improves patient compliance due to decreased dosing frequency [6, 7]. It improved chemotherapy of disease due to decreased fluctuation in blood vessel [8]. Transferosomes have become a prominent novel drug delivery system in recent years due to their advantages such as continuous, non-invasive and painless administration, sustained and prolonged drug action, improved patience compliance and reduced dosing frequency in comparison to

conventional drug delivery systems [9]. During the past few years, various experimental methodologies have been successfully developed for facilitating transdermal delivery of paclitaxel but well known carriers such as liposomes and niosomes fail to do this owing to their large size and stability problems [10].

Recently, the vesicular drug-carrier systems such as transferosomes have been reported to enhance the transdermal delivery of drugs, when applied onto the skin non-occlusively. Transferosomes are artificial vesicles having extraordinary feature of deformability with several orders of higher magnitude than standard liposomes [9]. The deformability of transferosomes for improved skin permeation of drug molecules can be achieved by using surfactants in appropriate ratio. Transferosomes have the ability to overcome the permeation difficulty by squeezing themselves along the inter-cellular sealing lipid of the stratum corneum i.e. paracellular passage. The resulting flexibility of transferosomes membrane minimizes the risk of complete vesicle rupture in the skin and allows transferosomes to follow the natural water gradient across the epidermis, after application onto the skin [11]. So, it is expected that the skin penetration of drug will be increased with the help of transferosomes. The entrapped drug in transferosomes can facilitate localized delivery of the drug and improve availability by means of a controlled release pattern for better treatment of breast cancer [12, 13].

Paclitaxel (PTX) is a potential chemotherapeutic agent used in the treatment of a variety of cancers. However, the current available formulations have severe side effects related to low water solubility [14]. To improve its water solubility, decrease its toxicity, increase its bioavailability, and achieve a better therapeutic effect, nanobased delivery systems have been developed for PTX, using liposomes, micelles, polymers, inorganic nanoparticles, human serum albumin, and polyethylene glycol–polylactic acid [15, 16]. Further, an albumin-bound PTX nanoparticle formulation (Abraxane®) has been approved by the US Food and Drug Administration to treat metastatic breast cancer and non-small-cell lung cancer [17, 18]. In clinical practice, some patients also experience significant undesirable side effects because of the emulsifier. Whether Abraxane® could improve survival and overcome P-glycoprotein-mediated drug resistance is still unclear. While it has been previously developed protein-carrier prodrug formulations to enhance the solubility and targeting ability of PTX, some problems have remained unresolved, such as the low PTX content and high immunotoxicity [19]. Therefore, there is still a pressing need to develop alternative PTX formulations. Esterification with amino acids has been shown to be a useful approach for increasing aqueous solubility and bioavailability [20]. In this work, PTX was derivatized at its 2′-hydroxy function by esterification with amino acids.

Folic acid (FA) has been identified as a ligand for selective delivery of therapeutic agents to cancer cells. Uptake of FA into cells is mediated by the FA receptor [21]. The human folate receptor alpha (FR-α) has high affinity for the FA ligand, and is currently considered an essential component in the cellular accumulation of FA used in chemotherapy [22]. FR-α expression is very low in most normal cells and tissues or not detectable, but is upregulated and detectable in ovarian, breast, brain, lung, and colorectal cancers. In previous study, an FA-modified drug delivery system that covalently attached FA can increase the ability to target FR-positive tumors [23].

To improve targetability of the drug to breast cancer cells, suitable ligand can be attached to the drug molecule and molecular complex using carbodiimide chemistry. The linkage can be designed to improve water solubility, to enhance presentation of the folate to its receptor, or to release the attached drug in unmodified form only after uptake into the target cell.

In the present investigation, an attempt was made to develop, and characterize paclitaxel-Folic acid conjugates through the drug-ligand interaction. This would be achieving controlled release in the extracellular tumor microenvironment and intracellular acidic compartments such as endosomes and lysosomes.

2. MATERIAL AND METHODS

2.1. Materials

Sigma Aldrich purchased folic acid (molecular weight 441 .4), dicyclohexylcarbodiimide, N-hydroxysuccinimide (NHS), fmoc-L-glutamic acid 5-tert-butyl ester (Fmoc-Glu[OtBu]-OH, molecular weight 425.49).

All other solvents and reagents used in this research were analytical grade of analytical reagent and from sigma chemicals.

2.2. Synthesis of the targeted complex of multi-small molecule-conjugated PTX (FA-Glu (OtBu)-PTX):

FA-Glu(OtBu) TX was synthesized in four steps as shown in figure 1.

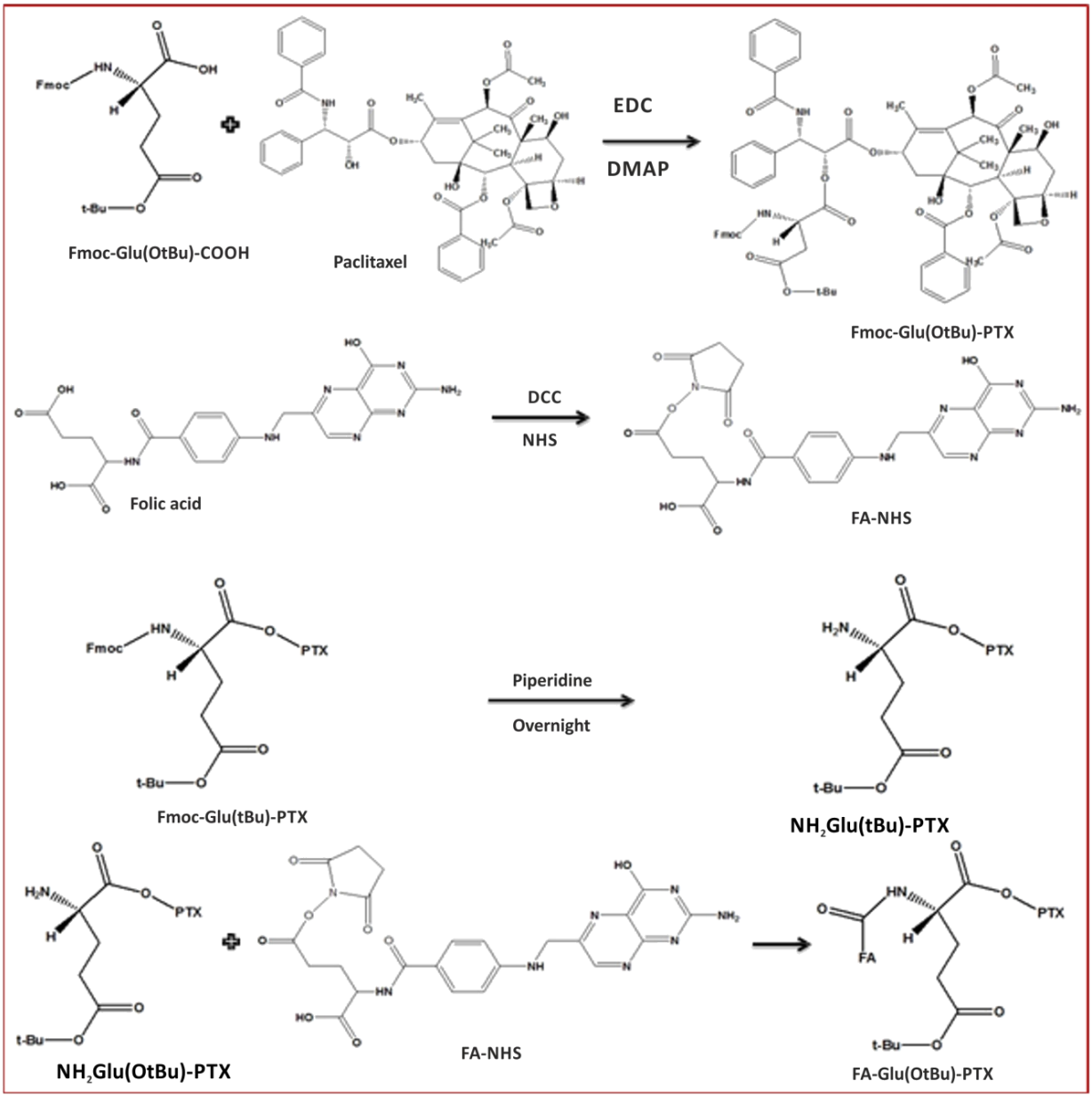

Fig. 1: Schematic representation of steps involved in synthesisofFA-Glu(OtBu)-PTX conjugate.(a) Fmoc-Glu(OtBu)-PTX; (b) FA-NHS; (c) NH_2-Glu(OtBu)-PTX; (d) FA-Glu(OtBu)-PTX.

Abbreviations: PTX-Paclitaxel; FA- Folic acid; NHS: N-hydroxysuccinimide

Step1: PTX (100 mg, 0.117mmol) and Fmoc-Glu (OtBu)-OH (91.08 mg, 0.14mmol) were dissolved in 10ml CH_2Cl_2 and4-dimethylaminopyridine (14.27 mg, 0.117mmol) was subsequently introduced. Cold EDC (44.85 mg, 0.234mmol and 5mL of CH2Cl2) was added to the mixture over 20 minutes and stirred at room temperature for 22 hours.

The response combination was further diluted with 15mL of CH_2Cl_2. The organic layer was washed with water, saturated aqueous$NaHCO_3$, and dried on $MgSO_4$. The residue obtained after evaporation of the organic solvent was purified by recrystallization from diethyl ether. The purified Fmoc-Glu (OtBu)-PTX was collected in 60 percent as a white solid.

Step 2: The 44.14mg of FA (0.1mmol) in 5mL of dimethyl sulfoxide was activated with dicyclohexylcarbodiimide (DCC) and NHS (molar ratio of FA to DCC to NHS, 1:1.5:1.5) at room temperature in the dark for 5 hours. The residue was removed under decreased pressure by filtration and the activated FA was extracted with anhydrous acetone.

Step 3: Fmoc-Glu (OtBu)-PTX (150.3 mg, 0.1mmol) was dissolved in 10mL of CH_2Cl_2, 2mL of piperidine was added to the protective solvent, and the mixture was stirred at room temperature for 10 hours. The residue acquired after evaporation of the organic solvent was purified by diethyl ether recrystallization. The purified NH2-Glu (OtBu)-PTX has been achieved as a white solid.

Step 4: NH2-Glu (OtBu)-PTX (127.94 mg, 0.1mmol) was dissolved in dimethyl sulfoxide and the activated FA-NHS was added to the blend which was then stirred in the dark at room temperature for 24 hours. The reaction mixture was concentrated in a vacuum and purified on a column of silica gel. The purified FA-Glu(OtBu)-PTX was acquired as strong yellow.

2.3. Characterization of FA-Glu (OtBu)-PTX conjugate

2.3.1. UV analysis

To verify the elements of the above synthesized compounds, FA-Glu (OtBu)-PTX was assessed by Spectrophotometer (UV 1800, Shimadzu, Japan).

2.3.2. Fourier Transform Infrared Spectral (FTIR) Analysis

The sample FTIR was conducted using the FTIR tool (Bruker-Tenser, α- model) at Rajeev Gandhi Technical University, Bhopal. Any compounds infrared spectrum provides data about the organizations current in that compound. Conjugate IR spectroscopy was examined straight in strong condition without further preparing using ATR (Attenuated Total Reflection) IR spectrophotometer. The conjugate IR range was contrasted informal literature with sheer drug IR peaks.

2.3.3. ^{1}H NMR Analysis

Proton NMR of the sample was analyzed from Indian Institute of Science Education and Research, Bhopal (M.P.). ^{1}H NMR spectra were recorded on NMR instrument at room temperature with $CDCl_3$ and D_2O as solvents and TMS as internal standards.

2.3.4. X-ray Diffraction Analysis

X-ray diffraction is a popular method for studying crystal structures and atomic spacing. X-ray diffraction is based on constructive interference with monochromatic X-rays and a crystalline sample. The samples were prepared directly on the holder of the sample using the top-fill technique. XRD measures the crystallinity from maximum height or region. The sample XRD was conducted using XRD (PAN Analytical, Empyrean XRD) from the Indian Institute of Scientific Education and Research (IISER), Bhopal.

2.3.5. Physical and chemical properties of FA-Glu(OtBu)-PTX

The physical and chemical properties of the PTX formulations, including their drug loading capacity, solubility and stability were determined. The amount of conjugated PTX in FA-Glu (OtBu)-PTX was determined using HPLC method.

The stability of the ester bond in FA-Glu (OtBu)-PTX conjugate, the free PTX released from the derivatives was measured in different types of medium, including phosphate buffered saline (PBS, pH 7.4) and human plasma at 37°C for up to 12 hours. The PTX-conjugated products (5 mg each of FA-Glu (OtBu)-PTX) were dissolved in 2.5 mL of each type of medium. Next, 100 µL of each sample was taken at a designated time point. The free PTX was then extracted using 1.5 mL of ethyl acetate and measured by HPLC on a C18 column (300 mm×3.9 mm; Waters) with an acetonitrile/water gradient (45/45), 1 mL per minute at 30°C, PTX: 227 nm).

The aqueous solubility of the conjugated PTX was determined using a method reported [24]. At room temperature (25°C), 100 mg of conjugated PTX was placed in 10 mL volumetric flask to which 500 µL of water was added. After saturation was reached, the aqueous solubility of conjugate was measured.

3. RESULTS AND DISCUSSION

PTX is an antitumor agent, with high clinical efficacy. However, its low aqueous solubility remains a major problem, because it can lead to high toxicity and low bioavailability [16].The currently developed formulations of PTX indicate that these problems are still unresolved [25, 26]. In present study, we developed a new multi-small molecule conjugated PTX delivery system for targeted tumor therapy. Water-soluble small molecules, amino acids (Glu), FA, and fluorescent dye were successfully conjugated to PTX, and greatly enhanced the solubility of synthesized FA-Glu (OtBu)-PTX conjugate.

Multi-small paclitaxel conjugated molecule [FA-Glu (OtBu)-PTX] was acquired as ayellow solid/powder. The solubility of paclitaxel conjugate was discovered to be comparable to the values reported. It is freely soluble in water/buffer.

On UV scanning, the FA-Glu (OtBu)-PTX conjugate absorption peak showed all the absorption peaks of the

constituent components i.e. FA, Glu, PTX at 365 nm, 220 nm and227 nm, respectively in the complex, indicating effective PTX conjugation (Figure 2).

Fig. 2: UV spectra of FA-Glu (OtBu)-PTX conjugate

Conformation of conjugate formation was performed on the grounds of relative infrared spectroscopic analysis. Infrared absorption peaks were acquired for paclitaxel, activated folic acid, NH_2-Glu (OtBu)-PTX conjugate, and FA-Glu (OtBu)-PTX conjugate.

Table1: Absorption band frequencies in IR spectrum of paclitaxel

IR Absorption Band (cm^{-1})	Assignment
3407.3	O-H Stretch
1705.6	C=O Stretch (ester)
1642.1	C=O Stretch (amide)
1243.0	C-O Stretch
1175.0	NC-O Stretch
1070.7	C-O Stretch(ketone group)
975.5	Finger print

Table 2: Absorption band frequencies in IR spectrum of FA-NHS

IR Absorption Band (cm^{-1})	Assignments
3733.90	O-H Stretch(free)
3620.52	O-H Stretch (Carboxylic acid)
3135.25	Primary Amine
1697.57	Primary Amine
1520.70	Secondary Amine
1185.09	C-O Stretch
849.48	Benzene Tri-Substitution Peak (1,2,3 Position)

Table 3: Absorption band frequencies in IR spectrum of NH_2-Glu (OtBu)-PT

IR Absorption Band (cm^{-1})	Assignments
3838.21	O-H Stretch
3747.50	Primary amine doublet
3611.45	-
2949.30	C-H Strech
2949.30	C-H Stretch
1702.11	C=O Stretch
1529.77	N-H bend
1024.83	C-N Stretch

Table 4: Absorption band frequencies in IR spectrum of FA-Glu(OtBu)-PTX

IR Absorption Band, (cm^{-1})	Assignments
3192.48	N-H Stretch of amide
2935.50	CH2 Stretching (Long chain)
2725.65	Aromatic ring breathing

In the analysis of the infrared spectrum of FA-Glu (OtBu)-PTX peaks at 943.0cm-1 (aromatic C-H bending), 1550 cm-1 (C = O stretch), 1636.8 cm-1 (C = O stretch amide), 3431.6 cm-1 (N-H stretch amide) verified the formation of amide connection and thus the development of FA-Glu (OtBu)-PTX conjugate (Figure 3; Table 1 to 4).

The XRD experiments were conducted for FA-Glu (OtBu)-PTX conjugate. PTX conjugate diffractogram showed sharp and high intensity peaks at 9.0, 18.5, 27.9, 37.0° and a very low intensity peak at 11.0, 20.1, 21.5, 23.5, 33.0, 46.0, 56.0°. The resulting diffraction patterns depict the drugs crystalline nature and the corresponding diffraction pattern is shown in (Figure 4). XRD assessment disclosed the degree of crystallinity and conjugate. ByX-ray diffraction assessment, the obtained sample of paclitaxel and small-molecule conjugated paclitaxel was discovered crystalline in nature and showed very near peaks at 2 distinct values. The maximum intensity was observed at the values 9.0, 18.5, 27.9, 37.0°.

Successful conjugation of paclitaxel to the small-molecules was further confirmed by NMR proton assessment. The protons chemical changes (ppm) on PTX outlined as follows: 3.84 & 3.58 ppm-C2′H-OH), (2.56 & 2.47 ppm (-C7H-OH), 1.98 & 1.78 ppm (C1 –OH-). By comparing the results of pure paclitaxel (PTX) 1H

NMR spectrum with that of FA-Glu (OtBu)-PTX conjugate 1H NMR spectrum to verify the effective conjugation response as all distinctive peaks of the connected molecule were detected (Figure 5).

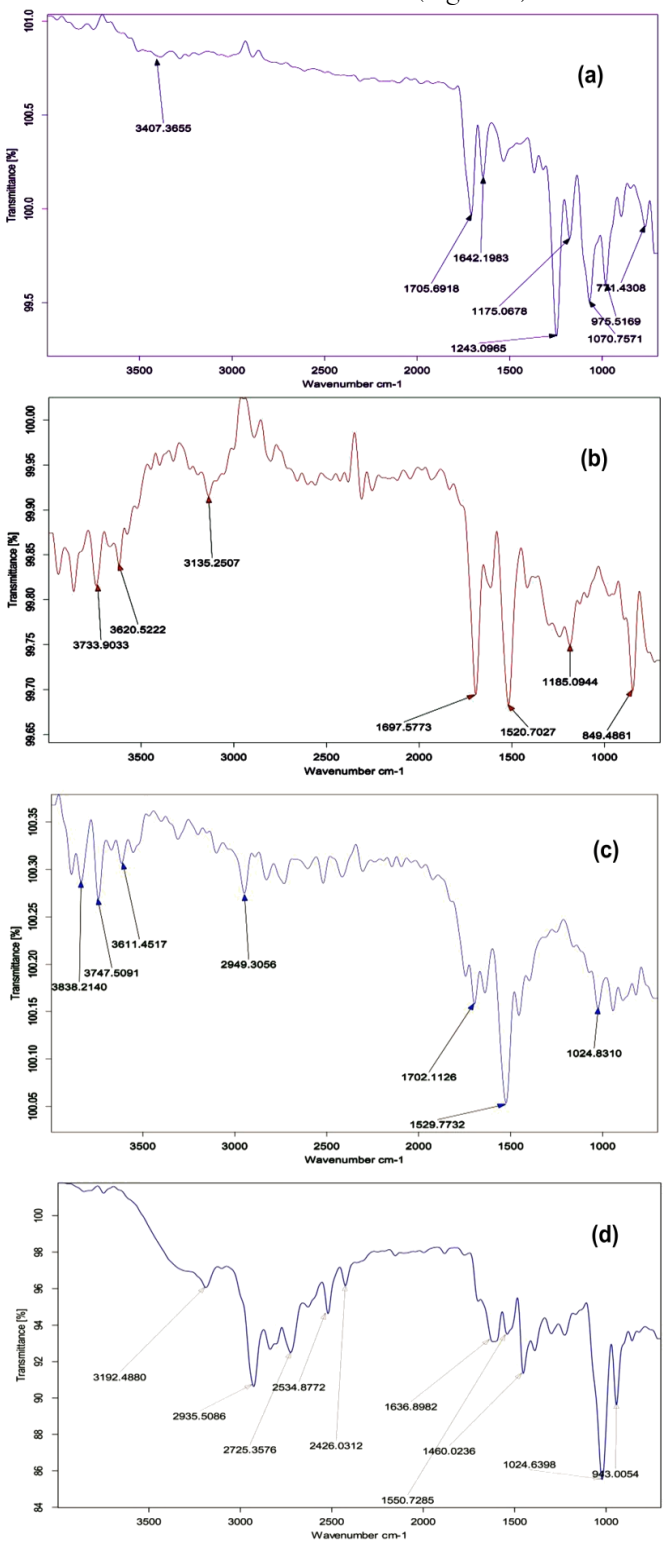

Fig. 3: Infrared spectrum of (a) paclitaxel, (b) FA-NHS, (c) NH_2-Glu(OtBu)-PTX, (d) FA-Glu(OtBu)-PTX

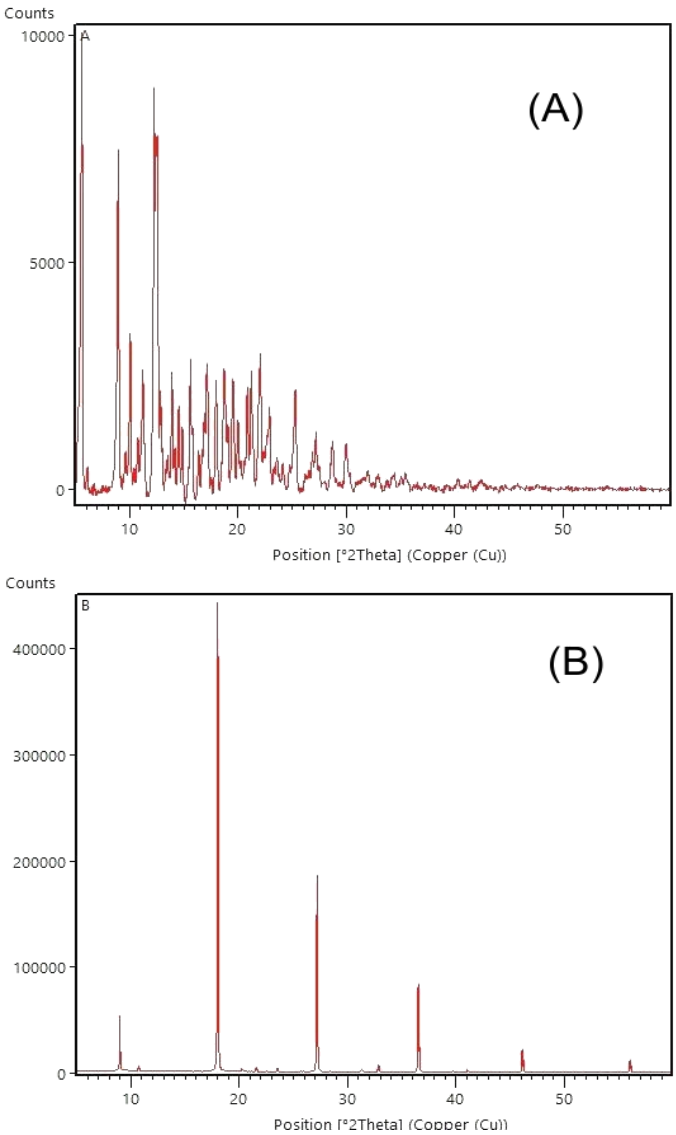

Fig. 4: XRD spectra of (A) PTX (B)FA-Glu(OtBu)-PTX conjugate

PTX loading amount in the conjugate was 38.84%±1.84%. Aqueous solubility of the multi-small molecule-conjugated PTX formulations, observed by the direct observations as, FA-Glu(OtBu)-PTX(8.85±0.02 mg/mL). Investigation on the cleavage of the linkers in the products was measured as the amount of free PTX released from the conjugate after incubation in PBS (pH 7.4) and human plasma, respectively, at 37°C. The FA-Glu (OtBu)-PTX formulation released PTX more rapidly in human plasma (48.64% per 4 hours) and released less PTX in PBS (28.1% per 4 hours).

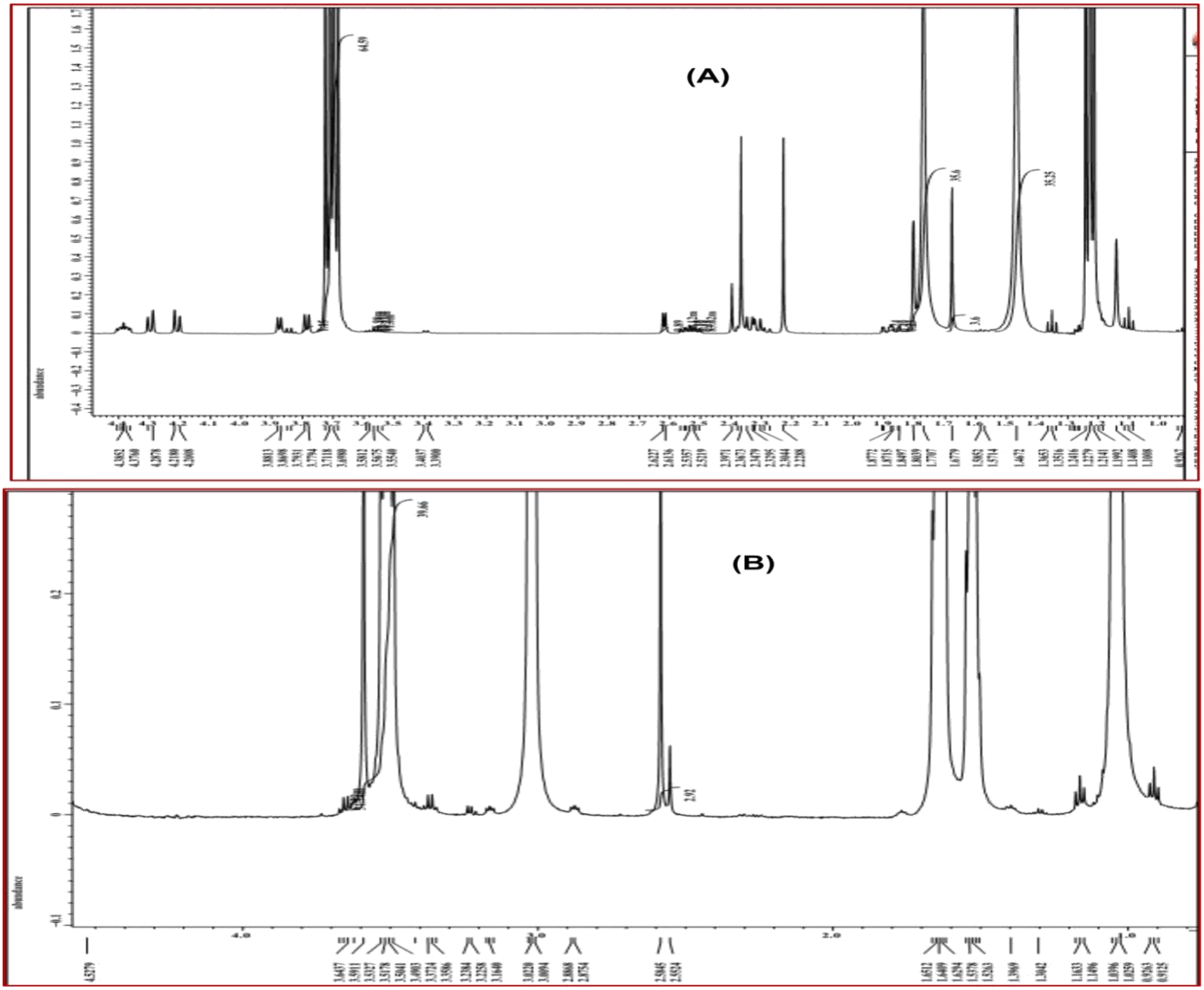

Fig. 5: ^{1}H-NMR spectra of (A) PTX (B) FA-Glu (OtBu)-PTX

4. CONCLUSION

It is therefore concluded that FA-Glu (OtBu)-PTX conjugate has been effectively synthesized which could be used for formulation development of transferosomes and might be useful for targeted delivery system for cancer treatment.

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