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In vivo pharmacokinetic studies and intracellular delivery of methotrexate by means of glycine-tethered PLGA-based polymeric micelles

Madhwi[a], Rajendra Kumar[b], Pramod Kumar[a], Bhupinder Singh[b,c], Gajanand Sharma[c], Om Prakash Katare[c], Kaisar Raza[a,*]

[a] Department of Pharmacy, School of Chemical Sciences and Pharmacy, Central University of Rajasthan, Bandar Sindri, Dist. Ajmer, Rajasthan, 305 817, India
[b] UGC-Centre of Excellence in Applications of Nanomaterials, Nanoparticles and Nanocomposites, Panjab University, Chandigarh, 160 014, India
[c] Division of Pharmaceutics, University Institute of Pharmaceutical Sciences, UGC-Centre of Advanced Studies, Panjab University, Chandigarh, 160 014, India

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ABSTRACT

Methotrexate (MTX) is a widely used drug for the management of various kinds of cancers. However, numerous challenges are associated with MTX like poor aqueous solubility, dose-dependent side effects and poor-bioavailability. With an aim to explore the potential benefits in drug delivery of MTX, it was intended to fabricate glycine-PLGA-based polymeric micelles. Glycine was chemically linked to PLGA and the linkage was confirmed by FT-IR, and NMR-Spectroscopy. The developed polymeric micelles offered substantial loading to MTX with a pH-dependent drug release profile. The drug was released maximally at the cancer cell pH *vis-à-vis* blood plasma pH. The cytotoxicity of drug against MDA-MB-231 cell lines was enhanced by approx. 100% and the confocal laser scanning microscopy confirmed the localization of dye-tagged nanocarriers in the interiors of cancer cells. The bioavailable fraction of the drug was increased by approx. 4-folds, whereas elimination half-life was enhanced by around two-folds in Wistar rats. The novel approach offers a biodegradable and promising carrier for the better delivery of anticancer agents with immense promises of efficacy enhancement, improved delivery and better pharmacokinetic profile.

1. Introduction

The concept of nanocarrier-based drug delivery is generally traced back to Nobel laureate "Sir Paul Ehrlich" (1905), as he envisioned "magic bullets" which have now been transformed to "magic guns", *i.e.*, drug delivery carriers. These specially designed drug delivery systems not only load the drugs, but possess the capability to deliver the pharmacologically active agents to the site of action in an effective manner (Raza et al., 2014; Katare et al., 2010; Kumar et al., 2016a). Since then, variety of materials have been used to explore the nanotechnology-derived potentials in delivery of variety of drugs to the target sites by numerous means including liposomes, niosomes, micelles, nanoemulsions, solid lipid nanoparticles, nano lipid carriers, carbon nanotubes, polymeric micelles and C_{60}-fullerenes (Raza et al., 2015; Gualdesi et al., 2016; Thakur et al., 2016; Kumar et al., 2016b; Kumar et al., 2016c). These nanocarriers are believed to revolutionize the medicine and related-branches towards "patient-centric" therapeutics and numerous products are now available in the market, based on these approaches (Biankin et al., 2015; Katare et al., 2010; Kumar et al., 2016a). Now-a-days, more emphasis is being paid on the development of biodegradable, biocompatible and rate-controlling nanocarriers, which can not only deliver the drug, but also get well assimilated in the biological system (Tang and Singh, 2009).

Poly (lactic-*co*-glycolic) acid (PLGA) is such a biocompatible polymer, with almost negligible systemic toxicity. It is easily hydrolyzed in the biological system to lactic acid and glycolic acid, which are utilized by Kreb's cycle (Khan et al., 2016). This polymer is extensively utilized in drug delivery, owing to the unique benefits like biodegradability, immune-neutrality, rate-controlling behavior and biocompatible nature (Danhier et al., 2012). Though offering immense promises, the polymer is tagged with some inherent concerns like low drug loading, substantially higher lipophilicity and rapid clearance from the biological system (Owensiii and Peppas, 2006; Kumari et al., 2010). With an aim

* Corresponding author.
E-mail addresses: drkaisar@curaj.ac.in, razakaisar_pharma@yahoo.co.in (K. Raza).

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to circumvent the concerns of PLGA, the polymer has been conjugated with numerous hydrophilic components like dextran, PEG and chitosan (Raza et al., 2016; Yadav et al., 2016; Zhang et al., 2014). On close scrutiny, it was observed that the polymer has not been conjugated with the simplest amino acid, *i.e.*, glycine. Henceforth, it was envisioned to conjugate the PLGA with glycine and develop a nanocarrier for exploration of the delivery benefits of methotrexate (MTX) to the cancer cells.

2. Materials and methods

2.1. Materials

Methotrexate (MTX) was provided as a gift sample by M/s Ipca Laboratories, Mumbai, India. Cell lines (MDA-MB-231 cells) were purchased from The National Centre for Cell Science, Pune, Maharashtra, India. Poly-(lactic-co-glycolic acid) (PLGA; 75:25; RESOMER RG 752S; Mol. wt. 90000 g/mol) was provided *ex-gratis* by M/s Evonik Industries AG, Kirschenalle, Germany. *N,N′*-dicyclohexylcarbodimide (DCC), dimethyl sulphoxide (DMSO) and HPLC-water were procured from M/s Spectrochem Ltd Mumbai, India. Sodium hydroxide, anhydrous dipotassium hydrogen phosphate, glycine, anhydrous potassium dihydrogen phosphate and sodium chloride were obtained from M/s CDH Co, Ltd, New Delhi, India. Whatman filter paper was procured from M/s GE Healthcare UK Ltd, Buckinghamshire, UK. Dialysis membrane was received from M/s Himedia Laboratories Co Ltd, Mumbai, India. Methanol was purchased from M/s SD Fine Chemical Ltd, Mumbai, India.

2.2. Synthesis of conjugate (glycine-PLGA)

Glycine (50 mg) was initially dissolved in mixture of water (1 mL) and methanol (10 mL). PLGA (50 mg) and DCC (8 mg) were added to the glycine solution and stirred for 24 h at room temperature. After 24 h, the whole reaction mixture was subjected to evaporation using rotary evaporator (M/s Buchi Labortechnik, AG, Switzerland). The conjugate was dialyzed against pure distilled water to remove unreacted glycine. Obtained conjugate was stored under 8 °C, till further use. Fig. 1 shows the synthetic scheme for preparation of the conjugate (López Ortiz et al., 2016).

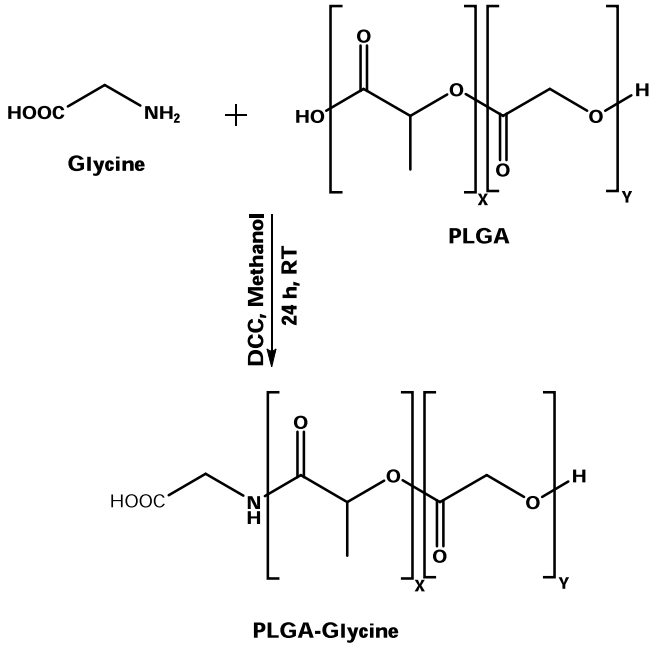

Fig. 1. Synthetic scheme for synthesis of PLGA-Glycine conjugate.

2.3. Preparation of polymeric micelles

Conjugate, 25 mg was dissolved in 1 mL of DMSO, and 10 mg of MTX was dissolved in 1 mL of methanol, separately. Both the solutions were mixed and filled in a syringe, and added drop wise into a beaker containing 10 mL of phosphate buffered solution (PBS) pH 7.4, containing 2% v/v Tween 80. The system was continuously stirred at 60 °C for 1 h (Raza et al., 2016; Yang et al., 2009).

2.4. Characterization studies

2.4.1. FT-IR spectroscopy

For the qualitative determination of various functional groups present in a given compound, Fourier Transform Infrared Spectroscopy (FT-IR) was used. Pellets were prepared using the conjugate and potassium bromide and punched with the help of hydraulic press. FT-IR Spectrometer (Spectrum two, M/s PerkinElmer Co, Walthum, Massachusetts, USA) was used to record the FT-IR data at wave number range of 4000–400 cm^{-1}.

2.4.2. NMR spectroscopy

^1H spectrum was recorded by preparing saturated solutions of the conjugate in deuterated dimethyl sulphoxide (DMSO-d_6) on Avance II 400 NMR Spectrometer (M/s Bruker Bio Spin Corporation, 500 MHz).

2.4.3. Determination of critical micellar concentration (CMC)

CMC determination was performed by well-reported Iodine method (Thotakura et al., 2016). A standard solution of iodine and potassium iodide was prepared by dissolving 250 mg of I_2 and 500 mg of KI in 25 mL of distilled water. Various dilutions of the conjugate were prepared, and 25 μL of iodine solution was added to 10 mL of each dilution. These solutions were incubated overnight, in the dark, and analyzed using UV–vis spectrophotometer at a wavelength of 366 nm. Graph between the respective absorption and concentration of polymer, was employed to determine the CMC of the conjugate.

2.4.4. Particle size distribution and zeta potential studies

Zetasizer Nano ZS (M/s Malvern, Worcestershire, UK) installed at Dr. S. S. Bhatnagar University Institute of Chemical Engineering and Technology, Panjab University, Chandigarh, India was used to determine the particle size and poly-dispersity index (PDI) values of the polymeric micellar dispersions in PBS (1 mg/mL). Zeta potential values were also determined by the same equipment.

2.4.5. Transmission electron microscopy (TEM)

Transmission electron microscope (M/s Hitachi H7000 Tokyo, Japan) installed at Central Instrumentation Laboratory, Panjab University, Chandigarh, India was used to perform the morphological studies. A drop of the micellar dispersion in PBS (1 mg/mL) was mixed with 1% w/v aqueous solution of phosphotungstic acid, and placed on the grid. Microphotographs were clicked at suitable magnification(s), after observing under the electron microscope.

2.4.6. Entrapment efficacy (EE) and extent of drug loading (DL)

Prepared micelles were filtered through 0.22 μm Whatman filter paper. After filtration, the paper was extracted in methanol, and analyzed using UV–vis spectrophotometer to determine the amount of unentrapped drug. Eqs. (1) and (2) mentioned below