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Anti-cancerous efficacy and pharmacokinetics of 6-mercaptopurine loaded chitosan nanoparticles

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ABSTRACT

6-Mercaptopurine is a cytotoxic and immunosuppressant drug. The use of this drug is limited due to its poor bioavailability and short plasma half-life. In order to nullify these drawbacks, 6-mercaptopurine-chitosan nanoparticles (6-MP-CNPs) were prepared and evaluated to study the influence of preparation conditions on the physicochemical properties by using DLS, SEM, XRD and FTIR. The *in vitro* drug release profile at pH 4.8 and 7.4 revealed sustained release patterns for a period of 2 days. The nanoformulations showed enhanced *in vitro* anti-cancer activities (MTT assay, apoptosis assay, cell cycle arrest and ROS indices) on HT-1080 and MCF-7 cells. *In vivo* pharmacokinetics profiles of 6-MP-CNPs showed improved bioavailability. Thus, the results of the present study revealed that, the prepared 6-MP-CNPs have a significant role in increasing anti-cancer efficacy, bioavailability and *in vivo* pharmacokinetics profiles.
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1. Introduction

6-Mercaptopurine (6-MP), is derived from the prodrugazathioprine that is non-enzymatically converted to 6-MP in tissues, is a purine analogue which has antineoplastic and immunosuppressant properties [1]. Since its approval as an antitumour drug from Food and Drug Administration (FDA) in 1953 [2], it has been widely used in the treatment of acute lymphoblastic leukemia (ALL), rheumatological disorders, prevention of rejection following organ transplantation and inflammatory diseases [3]. 6-MP can also be used in combination with other drugs to treat diseases like systemic lupus erythematosus, non-Hodgkin-lymphoma, polycythemia vera, inflammatory diseases (Crohn's syndrome and ulcerative colitis) [4,5].

Indeed, 6-MP is first metabolized to an active form, methylated thioinosinic acid (MeTIMP) by hypoxanthine phosphoribosyltransferase (HPRT) within cell to inhibit the de novo purine synthesis [6] and later converted to 6-thioguanine (6-TG), which is a DNA intercalating agent. For instance, incorporation of 6-TG induces cytotoxicity mediated via G2/M and/or S phase arrest [7,8]. However, 6-MP undergoes a complex biotransformation by XO (Xanthine oxidase) and TPMT (thiopurine *S* methyl transferase) which limits its bioavailability and leads to incomplete and variable bioavailability (about 16–50%) [1,9], short plasma half-life (0.5–1.5 h) [10], moderate plasma protein binding (19–30%) [11] and narrows therapeutic index which in turn results in plummeting chemotherapeutic effect and life-threatening toxicity, mainly in the form of myelosuppression [12,3].

Most importantly, polymeric nanocarriers (liposomes, niosomes, dendrimers, nanofibers, nanotubes and nanoparticles, *etc.*), proficient of sustained release with low toxicity and are essentially required for providing and maintaining desired concentration with minimal toxicity [13]. In order to justify the above mentioned issues, nanotechnology has been mainly deal with the synthesis of nanomaterials of variable's size, shape, surface charge and narrow size distribution, which has been typically explored, as it represents excellent carriers of the integration of hydrophobic 6-MP [14,4].

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However, there are several limitations on these carrier systems *viz.*, expensive or conservative synthesis procedure, poor ability to control the size distribution, instability in biological fluids, inadequate tissue distribution, low drug loading efficiency, lack of bioavailability and biodegradation with precursor material toxicity [15,5].

In order to address the afore mentioned disadvantages and to improve the oral bioavailability, the present study selected chitosan as a natural, biodegradable, biocompatible, non-toxic, less or non-immunogenic and inexpensive biopolymer [16]. Chitosan is a mucoadhesive polymer which has affinity to bind with intestinal mucosa and, thus to improve the residence time of drugs in the intestinal lumen. Consequently, it enhances their bioavailability [17]. Recently, chitosan nanoparticles were found to be promising carriers for controlled-release drug delivery systems [18]. There are varieties of methods that are used to prepare chitosan nanoparticles. The ionic gelation technique has attracted considerable attention due to its non-toxic, organic solvent free, convenient and controllable process [19]. This technique is based on the ionic interactions between the positively charged primary amino groups of chitosan and the negatively charged groups of polyanion, such as sodium tripolyphosphate (TPP) [20]. This physical cross-linking process, not only avoids the use of chemical cross-linking agents and emulsifying agents which are often toxic to organisms, but also prevents the possibility of damage to drugs, particularly biological agents [21]. In the present study, owing to their increasing pharmaceutical and biomedical applications, 6-MP-chitosan nanoparticles (6-MP-CNPs) are synthesised, characterised and evaluated for their anticancer activities in human breast cancer (MCF-7) and fibrosarcoma (HT-1080) cell lines and also *in vivo* pharmacokinetic profiles were studied to investigate the anticancer efficacy.

2. Materials and methods

2.1. Materials

In this study, 6-mercaptopurine monohydrate (Assay purity 98.0%), Clozapine, Chitosan low molecular weight (deacetylation ≥75.0%), Sodium tripolyphosphate (TPP) (purity: 85%), glacial acetic acid (≥99.85%), Dimethylformamide (DMF) and dialysis tubing cellulose membrane (Sigma–Aldrich Chemical Co., Ltd.), Formic acid, Methanol and Acetonitrile (E-merck, Germany), Monobasic sodium phosphate (NaH_2PO_4) and dibasic sodium phosphate (Na_2HPO_4) (Emplura®, Merck Specialities Private Limited, Bangalore, India) were used. Besides, Trypan blue, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetra-zolium bromide), trypsin, streptomycin, penicillin, amphotericin, Fetal Bovine Serum (FBS), Absolute ethanol, Dulbecco's Phosphate Buffered Saline (DPBS), Propidium Iodide (PI), RNase A, Triton-X 100, Annexin V-FITC (Fluorescein isothiocyanate) and Annexin V binding buffer (HiMedia Labs, Mumbai, India) and other reagents of analytical grade were also used in the study.

2.2. Preparation of 6-MP-CNPs (6-mercaptopurine chitosan nanoparticles)

Accordingly, 6-MP-CNPs were produced based on ionic gelation of TPP with chitosan as described by Aydin and Pulat [23] with slight modification and the formulations (F1–F5) were shown in Table 1. Briefly, different concentrations of chitosan were dissolved in 1% (v/v) acetic acid and mixed with 6-MP solutions (6-MP dissolved in DMF with final concentration of 1 mg/mL). Tween 80 (0.5% v/v) was added to chitosan solutions and pH maintained within a range of 4.2–4.6. Thus, prepared 6-MP-containing chitosan solutions were mixed with respective concentrations of TPP solutions [(2:1) (v/v) (chitosan: TPP)]. Finally, the nanoparticle suspension was gently stirred for 30 min at room temperature, centrifuged at 10,000 × *g* for 30 min, washed with Milli-Q-water and freeze-dried (ScanVacCoolSafe Freeze Drying).

2.3. Characterization of nanoparticles

2.3.1. Dynamic light scattering (DLS) and scanning electron microscopy (SEM)

Particle size and surface morphology of the freshly prepared 6-MP-CNPs were analysed using DLS (ZetasizerNano ZS90, Malvern, UK) and SEM (XL 30; Philips, Eindhoven, The Netherlands). Specifically, nanoparticles were sputter coated with gold (SCD 005; Bal-Tec, Balzers, Liechtenstein) prior to SEM observation. The colloidal stability and surface charge of the developed nanoparticles were analysed using zeta potential measurements (ZetasizerNano ZS90, Malvern, UK) using a fold capillary cuvette (Folded Capillary Cell-DTS1060, Malvern, UK) [23]. All measurements were conducted in triplicate.

2.3.2. Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) analysis

FTIR analysis was performed by following the method of Stuart [24]. Briefly, IR spectra of 6-MP, chitosan, and 6-MP-CNPs were recorded using FTIR Nicolet 6700 (Thermo Fisher Scientific, Madisson, WI, USA) operated by Omnic software 8.1. XRD patterns were recorded using a Philips PW 3050/10 model. The samples were recorded on a computer controlled Philips X-Pert MMP diffractometer by using the P Rofit programs and a MoK source with wavelength 0.70930 and operating with Mo-tube radiation at 50 kV and 40 Ma [22].

2.3.3. Determination of loading efficiency of 6-MP-CNPs

6-MP-CNPs were dissolved in Methanol: Water (50:50% v/v), sonicated for 15 min to obtain free 6-MP. The amount of 6-MP in nanoparticles was analysed by LCMS/MS system [MDS SCIEX Q-TRAP API 3200 mass spectrometer (Foster City, CA, USA) and Agilent HPLC system].

$$\text{Drug Loading}\,(\%) = \text{Weight of 6-Mercaptopurine}/$$
$$\times\ \text{Weight of Nanoparticles} \times 100\,[25]$$

2.4. *In vitro release profiles of 6-MP-CNPs*

In vitro drug release studies were performed using USP XXIV Type-I at 50 rpm rotation speed with 37 ± 0.5 °C temperature using 600 mL of PBS (pH 4.8 and 7.4). A calculated 10 mg (concentration 1 mg/mL) of 6-MP and 6-MP-CNPs were separately placed in dialysis tube and immersed in PBS at above mentioned pH. At predetermined time intervals, an aliquot of 3 mL of the release media was withdrawn and the dissolution medium was replaced with fresh buffer (3 mL) to maintain constant volume. The concentration of the 6-MP in release media was estimated by using a double beam UV–vis spectrophotometer (Evolution 300; Thermo Fisher Scientific, Madisson, WI, USA) at 322 nm [26,16].

2.5. Cell culture

The *in vitro* cytotoxicity study was carried out on human breast cancer (MCF-7) and fibrosarcoma (HT-1080) cell lines (National Centre for Cell Science, Pune, India). Cells were maintained in Dulbecco's Modified Eagle's Medium (4.5 g/L Glucose), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 units/mL Penicillin, 100 μg/mL Streptomycin and 2.5 μg/mL Amphotericin-B solution (HiMedia Labs, Mumbai, India). Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

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