Original article

Toxicity evaluation of 6-mercaptopurine-Chitosan nanoparticles in rats

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Background: The 6-mercaptopurine (6-MP) is an effective immunosuppressant and anti-cancer drug. However, the usage of 6-MP is limited due to its well-known side effects, such as myelotoxicity and hepato-renal toxicity. To curtail the potential toxic effects, we have used chitosan as a natural biodegradable and biocompatible polysaccharide to synthesize 6-Mercaptopurine-Chitosan Nanoparticles (6-MP-CNPs).

Methods: The 6-MP-CNPs size, morphology, physicochemical interactions, and thermal stability were characterized using Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and Differential Scanning Calorimetry (DSC), respectively. The loading efficiency of the 6-MP in CNPs was estimated using LCMS/MS. Then, the 6-MP-CNPs were subjected to in vivo acute and sub-acute oral toxicity evaluations.

Results: The DLS and SEM analysis respectively indicated size (70.0 nm to 400.0 nm), polydispersity index (0.462), and zeta potential (54.9 mV) with improved morphology of 6-MP-CNPs. The FTIR and DSC results showed the efficient interactive and stable nature of the 6-MP-CNPs, which sustained the drug-delivery process. The loading efficiency of 6-MP-CNPs was found to be 25.23%. The chitosan improved the lethal dose (LD50 cut off) of 6-MP-CNPs (1000 mg/kg b.w) against 6-MP (500 mg/kg b.w) and also significantly (p < 0.05) reduces the toxic adverse effect (28-day repeated oral dose) on hemato-biochemical and hepato-renal histological profiles.

Conclusion: The findings suggest that chitosan, as a prime drug-delivery carrier, significantly alleviates the acute and sub-acute toxic effects of 6-MP.

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reverse micellization (Tang et al., 2007), ionic gelation (Kumar et al., 2015), polyelectrolyte complexation (Yeh et al., 2011), modified ionic gelation with radical polymerization (Sajesh and Sharma, 2006), and desolvation (Atyabi et al., 2009). Among these methods, the ionic gelation technique has received much attention due to its non-toxic, convenient, and controllable process (Agnihotri et al., 2004).

Chitosan [poly β(1, 4)-2-amino-2-deoxy-D-glucose] is a natural biocompatible polymer with excellent encapsulation efficiencies and sustained release properties, ensuring efficiency in the drug-delivery system (Mohammed et al., 2017). Being a cationic polysaccharide, chitosan interacts with sodium triplyphosphate (TPP) by electrostatic forces (Shu and Zhu, 2002). This important cross-linking mechanism not only helps to encapsulate target drugs/biological samples but also avoids the use of harsh chemicals for cross-linking and emulsifying processes, which are often toxic to organisms (Berger et al., 2004).

In this study, we have used chitosan as a natural polysaccharide to reduce the potential side effects of 6-mercaptopurine nanoparticles. The 6-mercaptopurine-chitosan nanoparticles (6-MP-CNPs) were synthesized, characterized, and evaluated for their effects on the hematological, biochemical, and hepato-renal histopathological changes in the toxicity studies.

2. Materials and methods

2.1. Materials

The 6-mercaptopurine monohydrate (assay purity 98.0%), chitosan low-molecular-weight (deacetylation ≥ 75.0%), TPP (purity: 85%), glacial acetic acid (≥ 99.85%), and dimethylformamide (DMF) were purchased from Sigma-Aldrich.

2.2. Methods

2.2.1. Synthesis of 6-MP-CNPs

The 6-MP-CNPs were synthesized by the ionic gelation technique using chitosan and TPP as described in the literature with slight modification (Gan and Wang, 2007; Kumar et al., 2015). Briefly, a known concentration of chitosan was dissolved in 1% (v/v) acetic acid and mixed with the 6-MP solution (1 mg/mL of DMF). Tween 80 (0.5% v/v) was added to the chitosan solutions, and pH was maintained at ~4.5. The 6-MP containing chitosan solutions was then stirred with TPP solution (2:1 v/v) for 30 min and centrifuged at 10,000 g for 30 min. The pellet was resuspended with Milli-Q-water and freeze-dried in a vacuum (ScanVac CoolSafe Freeze Drying, CoolSafe 110-4).

2.2.2. Characterization of nanoparticles

2.2.2.1. Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM). The size and surface morphology of 6-MP-CNPs were determined using DLS (ZetasizerNano ZS90, Malvern, UK) and SEM (XL 30, Philips, Eindhoven, The Netherlands), respectively. Nanoparticles were sputter-coated with gold (SCD005; Bal-tec, Balzers, Liechtenstein) before SEM observation. The nanoparticles’ stability and surface charge were analyzed by zeta potential measurements (ZetasizerNanoZS90, Malvern, UK) using fold capillary cuvette (Folded Capillary Cell-DTS1060, Malvern, UK).

2.2.2.2. Fourier Transform Infrared Spectroscopy (FTIR) analysis. The FTIR procedure involving sample preparations and spectral recordings were carried out by a previously described method (Moll, 1971). The IR spectra of 6-MP, chitosan nanoparticles (CNPs), and 6-MP-CNPs were recorded using FTIR Bruker optics, alpha, and operated by OPUS Spectroscopy Software. The formulations were individually placed on the sample plate of the smart plate of the IR spectra in ATR mode.

2.2.2.3. Differential Scanning Calorimetry (DSC). The DSC analysis of 6-MP and 6-MP-CNPs were performed using Auto Q20 V24.10 DSC instrument (Universal V4.5A TA Instruments). The initial and final temperatures ranged from 0 °C to 350 °C at a heating rate of 10 °C min⁻¹. Nitrogen gas (99.99% purity) atmosphere was utilized in all the cases, and the instrument was calibrated using indium as the reference material.

2.2.3. Swelling behavior study

To understand the swelling nature, CNPs were incubated in 25 mL normal saline (0.9% NaCl) at ambient temperature for 48 h. After incubation, the CNPs were processed and examined under SEM.

2.2.4. The 6-MP loading efficiency of CNPs

The 6-MP-CNPs were dissolved in methanol:water (50:50 v/v) using an ultrasonic bath sonicator for 15 min. The concentration of 6-MP was analyzed using LCMS/MS [MDS SCIEX Q-TRAP API 3200 mass spectrometer (Foster City, CA, USA) and Agilent HPLC system]. The 6-MP standards (1 to 100 ng/mL) were prepared for the analysis of samples by plotting a straight-line graph (R² = 0.9997). The drug loading rate (%) was determined using below equation.

Drug Loading (%) = Weight of 6-mercaptopurine /Weight of Nanoparticles × 100

2.2.5. In vivo toxicity studies

2.2.5.1. Animals. Healthy eight- to ten-week-old Wistar rats of both sexes weighing around 200 ± 20 g were procured from the Laboratory Animal Facility, Veterinary College, Bangalore, India. The study was carried out at the Rodent Experiment Facility, Veterinary College, Bangalore, India. Animal care and usage requirements were followed based on guidelines of the NIH Publication, National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011). The Institutional Animal Ethics Committee (IAEC) approved the experimental protocol No. 139/LPM/IAEC/2013.

2.2.5.2. Acute oral toxicity study. The acute oral toxicity evaluation of the test items (6-MP and 6-MP-CNPs) was conducted in accordance with the Organization for Economic Cooperation and Development Guideline 423 (Acute toxic class method, OECD, 2001). The experimental animals (n = 3) were fasted overnight (~12 h), and the test substance was administered in a single dose (300 mg/kg b.w) by oral gavage. The animals were individually observed after dosing at least once during the first 30 min periodically for the first 24 h. Special attention was given during the first 4 h and daily after that for a total of 14 days. The LD50 cut-off (mg/kg b.w) of test items were reported, based on mortality and necropsy findings.

2.2.5.3. Sub-acute (28-day repeated dose) oral toxicity study. The sub-acute oral toxicity evaluation of the test items (6-MP and 6-MP-CNPs) was carried out in accordance with the Organization for Economic Cooperation and Development Guideline 407 (Repeated dose 28-day oral toxicity study in rodents, OECD, 2008). The experimental animals [12 animals (6 male and 6 female) per group] were randomly assigned to normal control (normal saline-2 mL/kg), 6-MP-CNPs (15 mg/kg-low dose, 30 mg/kg-mid dose, and 50 mg/kg-high dose), and 6-MP (50 mg/kg) treatment groups. These doses were selected based on the animal
equivalent dose of human dose (1 to 5 mg/kg), which are pre-
scribed for different disease conditions (acute lymphatic leukemia,
Crohn’s disease, rheumatologic disorders, etc). Also, with interest to
evaluate the marginal safety of 6-MP-CNPs, the selected low, mid,
and high dose corresponded to therapeutic, supra-therapeutic, and
toxic dose (~3 times more than therapeutic dose), respectively. All
the test items were orally administered each day for 28 days, and
the animals were monitored for clinical signs of toxicity.

2.2.5.4. Hematological and biochemical analysis. On day 28, the
experimental animals were fasted overnight. Their blood samples
were collected in anticoagulant (Calcium Disodium EDTA, Merck,
India) and non-anticoagulant tubes via retro-orbital plexus using
hematocrit capillary tubes under isoflurane anesthesia. Anticoagu-
lant blood samples were immediately processed for hematological
evaluations using automatic blood cell counter (ERMA PCE-210N),
whereas harvested serum samples were stored at −80 °C until
analysis of biochemical parameters using ErbaChem 5 Plus V2 Bio-
chemistry Analyzer.

2.2.5.5. Histopathological analysis. After collecting the blood on day
28, the experimental animals were subjected to whole-body perfu-
sion (Phosphate-buffered saline-PBS) and fixation (10% neutral
buffered formaldehyde -NBF) to harvest their liver and kidneys.
The harvested samples were stored in 10% NBF, dehydrated in
graded anhydrous ethanol, and embedded in paraffin. The fine tis-
sue sections of ~5 μm thickness were stained with hematoxylin
and eosin (H&E) and examined under an Olympus BX50 light
microscope.

2.2.6. Statistical analysis
The data were analyzed using a two-tailed student’s t-test and
two-way analysis of variance (ANOVA). All the values were pre-
sented as mean ± SEM. The probability (p) values of ≤0.05 were
considered statistically significant.

3. Results and discussion

3.1. Synthesis and characterization of 6-MP-CNPs
The synthesis of 6-MP-CNPs was optimized using the ionic gela-
technique with a constant volume ratio of chitosan: TPP (2:1) at
different concentrations (Kumar et al., 2015). The most repro-
ducible size, shape, and loading efficiency of the nanoparticles
were obtained at concentrations of chitosan (0.75 mg/mL), TPP
(0.5 mg/mL), and 6-MP (1.0 mg/mL). The DLS results of
6-MP-CNPs showed an average particle size (187.0 nm) with a
polydispersity index (0.462) and zeta-potential (54.9 mV), which
represents uniform distribution and stable nature of nanoparticles
(Fig. 1A and B) (Debnath et al., 2018). The SEM image analysis of
6-MP displayed an irregular size (~10 to 40 μm) and morphology
(Fig. 1C). However, the interactive nature of positively charged pri-
mary amino groups of chitosan and the negatively charged polyan-
ion groups of TPP modified the size (~150 to 400 nm) and shape of
the 6-MP (Fig. 1D) (Shu and Zhu, 2002). The synthesized CNPs were
incubated for 48 hr in normal saline to understand the in vitro
swelling behavioral properties and drug release process (Walke
et al., 2015). As a result, the SEM image analysis demonstrated an
increased size from ~25 to 55 nm to ~400 to 600 nm (Fig. 1E
and F). To perform an in vivo acute and subacute toxicity studies,
the loading efficiency of 6-MP-CNPs (25.23%) was quantified using
LCMS/MS.

FTIR analysis was performed to determine the 6-MP and CNPs
interactions. The fundamental vibrations of primary functional
groups and the frequency (cm⁻¹) of the CNPs and 6-MP-CNPs are
depicted in Fig. 2A and B, respectively. The stretching vibrations
of −OH and C=H bond of the CNPs were observed to be
3421.92 cm⁻¹ and 2935.43 cm⁻¹, respectively (Dorniani et al.,
2013; Peniche et al., 1999). The absorption peaks at
1642.23 cm⁻¹, 1536.57 cm⁻¹, 1416.54 cm⁻¹, and 1385.84 cm⁻¹
were assigned to C=O stretching of the amide I band, bending
vibrations of the N–H (N-acetylated residues, amide II band),
C–H bending, and OH bending, respectively. The peak at

Fig. 1. DLS results of 6-MP-CNPs are depicting size distribution (A) and zeta potential distribution (B). SEM analysis of 6-MP (C), 6-MP-CNPs (D), CNPs-before swelling (E), and CNPs-after swelling (F) behavior analysis.
1153.57 cm⁻¹ was assigned to the structure of saccharide, whereas 1096.82 cm⁻¹ and 1021.90 cm⁻¹ were assigned to the skeletal vibrations involving the C–O stretching (Xu and Du, 2003). The peak observed in Fig. 2B for 6-MP-CNPs at 3425.46 cm⁻¹ and 2923.64 cm⁻¹ were assigned to N–H and C–H stretching, respectively, which confirms the 6-MP complex with CNPs. Increased intensity and shifting of peaks at 1565.75 cm⁻¹ and 1410.46 cm⁻¹ of the CNPs were probably due to existing adsorption and/or encapsulation process of 6-MP and CNPs, which in turn, helped to enhance the dissolution properties of 6-MP-CNPs (Kumar et al., 2014). In other words, the decrease in intensity of the peak at 1099.66 cm⁻¹ (C=S/ring vibration) shown in Fig. 2B also suggests that an exocyclic (S) atom contributed with chitosan to elucidate the adsorption and/or encapsulation process of 6-MP with CNPs (Dorniani et al., 2013).

The DSC analysis was performed to assess the thermal behavior of the CNPs, 6-MP, and 6-MP-CNPs (Fig. 3A, B, and C). The CNPs exhibited a broad endothermic peak centered at 80.95 °C. This peak is attributed to the loss of water connected with the hydrophilic groups of the CNPs. The exothermic peak, which appeared at ~270 °C, corresponds to the decomposition of amine units of CNPs. We did not record the comprehensive decomposition spectrum. However, the results of Ferrero and Periolatto (2012) and Tamer et al. (2017) support our findings. The 6-MP exhibited a broad endothermic and a narrow exothermic peak centered at 183.34 °C and 211.17 °C, respectively (Lv et al., 2016). Also, thermograms of 6-MP-CNPs exhibited a reduced endothermic peak (as compared to 6-MP) centered at 143.16 °C, and a similar exothermic peak of chitosan centered at 302 °C. These minor changes are partially attributed to improved dissolution properties of 6-MP at the temperatures below its melting point. Finally, the observed peak of 6-MP in the DSC thermograms of 6-MP-CNPs was owing to the non-interactive nature of the 6-MP and chitosan polymer.

3.2. In vivo toxicity studies

3.2.1. Acute oral toxicity

Acute oral toxicity study of 6-MP-CNPs and 6-MP was started at a dose of 300 mg/kg bw. On day 14 of the experimental period, 6-MP-CNPs did not show any mortalities, although one animal died.
in the 6-MP group. Therefore, the dosing of three additional animals (with the same dose) was performed to confirm the mortality/survivability. The observed results were the same. Hence, dosing of three additional animals at the next higher dose (2000 mg/kg bw) was performed, and the resulting animal mortalities were two and three, respectively. No abnormalities were
found in the organs at necropsy. The toxicity of 6-MP-CNPs and 6-MP in Globally Harmonized Classification System (GHS) was categorized as Category 4 (>300–2000 mg/kg bw). Hence, the respective LD_{50} cut-off values were 1000 and 500 mg/kg bw (Clarke et al., 1953). Therefore, a lethal dose of 6-MP-CNPs was considerably more than 6-MP.

3.2.2. Sub-acute oral toxicity

3.2.2.1. Hematological and biochemical parameters. The high dose of 6-MP and 6-MP-CNPs treatment (for either sex) indicated significant (ab p < 0.001) reduction in the hematological parameters [total white blood cell count (WBC), total red blood cell count (RBC), hemoglobin (HB)], as compared to the low- and mid-dose (6-MP-CNPs) and saline treatment (Table 1). These toxic reductions could be because of an immunosuppressant activity of 6-MP. At the same time, the serum biochemical parameters [alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine, and blood urea nitrogen (BUN)] were significantly (ab p < 0.001) increased at high dose treatment of 6-MP and 6-MP-CNPs (Table 1). Likewise, these changes were in a dose-dependent manner. Based on our data and existing information, 6-MP is well-known to cause a significant toxic effect on bone marrow, liver, and kidney with reduced appetite, body weight (data not shown), and dehydration. These toxic consequences alter normal physiological profiles (Clarke et al., 1953; Philips et al., 1954). At low doses, all the hematobiochemical parameters fell within the normal physiological range and were statistically significant (ab p < 0.001) with the saline treatment group.

3.2.2.2. Histopathology. The H&E staining of the liver and kidney tissues are depicted in Fig. 4. The high dose of 6-MP treatment showed severe congestion in the central vein and sinusoidal space. The lesions of swollen hepatocytes with moderate vacuolization of cytoplasm and pyknotic nuclei were significant (Fig. 4). Pharmacologically, 6-MP metabolized into the active 6-TGN by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). However, the catabolic conversions of 6-MP by thiopurine s-methyltransferase (TPMT) drive into the 6-methyl mercaptopurine and 6-methyl mercaptopurine ribonucleotides (6-MMP), which in turn, leads to hepatotoxicity (van Asseldonk et al., 2012; Zimm et al., 1985). We assume that the mid and high dose 6-MP-CNPs with its sustained release mechanism and altered catabolic process reduced the hepatic congestion and sinusoidal space with pyknotic nuclei (Fig. 4). Interestingly, no appreciable changes were noticed at the low dose of 6-MP-CNPs. At a high dose, the renal architecture showed severe congestion, tubular epithelial degeneration, and glomerular atrophy in 6-MP treatment as compared to 6-MP-CNPs (Fig. 4). These renal architectural changes might be as a result of an accumulation of 6-MP urinary metabolites in renal tubules, which can cause internal hydronephrosis (Oikonomou et al., 2011; Philips et al., 1954; Zimm et al., 1985). However, the low dose of 6-MP-CNPs showed no appreciable changes in renal architecture, whereas mild tubular epithelial degeneration with glomerular atrophy was observed for mid and high doses (Fig. 4).

4. Conclusion

Chitosan is a natural polysaccharide used to synthesize 6-MP-CNPs as a novel drug-delivery system to reduce toxicity. The physicochemical interaction of chitosan and 6-MP was described using DLS, SEM, FTIR, and DSC. The loading efficiency of 6-MP in CNPs as a novel drug-delivery system to reduce toxic profile and to improve therapeutic dosage in the in vivo preclinical studies.

Authorship contribution

All the authors contributed to this study. P.K.G., A.R.P., J.S.S., and J.S.S., hypothesized the project and study design. P.K.G., A.R. P., and J.S.S. performed the experiments. P.K.G., D.J., and H.L.R., analyzed the data and interpreted the results. P.K.G. wrote the manuscript draft. A.R.P., D.J., and H.L.R., read the manuscript and provided critical evaluation and theoretical insights. All the authors discussed and interpreted the results. All the authors read and approved the final version of the manuscript.
Fig. 4. Representative image of histopathological evaluation (H&E) of liver and kidney after 28-day repeated oral dosing of saline (2 mL/kg), 6-MP (50 mg/kg), and 6-MP-CNPs (low dose-15 mg/kg, mid dose-30 mg/kg, and high dose-50 mg/kg), n = 6, male.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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