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Research paper

Nanosized complexation assemblies housed inside reverse micelles churn out monocytic delivery cores for bendamustine hydrochloride

Yuvraj Singh, Anumandla Chandrashekhar, Jaya Gopal Meher, K.K. Durga Rao Viswanadham, Vivek K. Pawar, Kavit Raval, Komal Sharma, Pankaj K. Singh, Animesh Kumar, Manish K. Chourasia *

Pharmaceutics Division, CSIR-Central Drug Research Institute, Lucknow 226031, India

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ABSTRACT

Objective: We explore a plausible method of targeting bendamustine hydrochloride (BM) to circulatory monocytes by exploiting their intrinsic endocytic/phagocytic capability.
Methods: We do so by complexation of sodium alginate and chitosan inside dioctyl sulfo succinate sodium (AOT) reverse micelles to form bendamustine hydrochloride loaded nanoparticles (CANPs). Dynamic light scattering, electrophoretic mobility and UV spectroscopy were used to detail intra-micellar complexation dynamics and to prove that drug was co-captured during interaction of carbohydrate polymers. A fluorescent conjugate of drug (RBM) was used to trace its intracellular fate after its loading into nanoparticles.
Results: CANPs were sized below 150 nm, had 75% drug entrapment and negative zeta potential (−30 mV). Confocal microscopy demonstrated that developed chitosan alginate nanoparticles had the unique capability to carry BM specifically to its site of action. Quantitative and mechanism based cell uptake studies revealed that monocytes had voracious capacity to internalize CANPs via simultaneous scavenger receptor based endocytic and phagocytic mechanism. Comparative *in vitro* pharmacokinetic studies revealed obtainment of significantly greater intracellular drug levels when cells were treated with CANPs. This caused reduction in IC_{50} (22.5 ± 2.1 μg/mL), enhancement in G_2M cell cycle arrest, greater intracellular reactive oxygen species generation, and increased apopotic potential of bendamustine hydrochloride in THP-1 cells.
Conclusion: Selective monocytic targeting of bendamustine hydrochloride using carbohydrate constructs can prove advantageous in case of leukemic disorders displaying overabundance of such cells.

1. Introduction

Bendamustine hydrochloride (BM) is used for treatment of different hematological malignancies, especially leukemia [1]. It is a zwitter ionic molecule, highly unstable in water and undergoes hydrolysis to monohydroxy and dihydroxy derivative [2]. It has been reported that intravenously administered BM elicits a half-life of less than 30 min. Plasma levels follow a biphasic exponential fall with primary metabolism in liver followed by renal elimination. Due to rapid degradation in serum, its cytostatic potential is lost within a short period of time and application of relatively high doses is necessitated [3]. It is therefore being proposed that if we deliver BM directly inside circulatory leukocytes, the drug levels attained in plasma would become inconsequential and an improved therapeutic effect might be obtained. Herein, by means of this manuscript we purport to investigate whether entrapment of BM within a carbohydrate nano-structure could deliver the drug directly to its site of action.

Nanoparticles were preferred over other colloidal systems (micro particles, coarse emulsions) due to their amiability towards intravenous administration [4]; however BM's salt nature meant that it was highly improbable to ensure adequate incorporation in mainstream hydrophobic polymeric systems made of PLGA [5], PCL [6], or oil based systems such as nanoemulsions [7] and it was concurred as per literature that self-assembly type structure would be a better alternative [8]. We therefore preceded by utilizing ionic complexation of hydrophilic natural carbohydrates, sodium alginate (SA) and chitosan (CS) to capture BM. The selection of carbohydrates was done with the intention of exploiting scavenger receptors expression on monocytes [9], which respond specifically to carbohydrates [10]. Anecdotally tailoring size of cross linked drug carriers has always been difficult. Simplistic addition of chitosan to alginate solution results in formation of visually

* Corresponding author.
E-mail address: manish_chourasia@cdri.res.in (M.K. Chourasia).

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noticeable macro sized beads [11]. To circumvent this discrepancy a dispersion consisting of specialized reverse micellar structures made of dioctyl sulfo succinate sodium (AOT) was utilized.

Reverse micelles can act as nanoreactor assemblies facilitating intramicellar complexation to ensure nanoscopic size of the formed chitosan alginate nanoparticles (CANPs). Micellar size varies with location of entrapped molecule as well as water content of the polar core and was approximated using dynamic light scattering (DLS) [12]. Establishing precise location of BM was relevant to the success of professed hypothesis as it was anticipated that during the event of ionic cross linking of positively charged chitosan and negatively charged sodium alginate, the drug would be strangled by oppositely charged polysaccharides within reverse micellar core providing the sought protection. Fate of drug after repurposing into CANPs was confirmed by conjugating it with a fluorescent probe Rhodamine 123(Rh123) to form fluorescent red bendamustine (RBM). Extensive micellar elucidation, physicochemical characterization, morphological analysis via transmission electron microscopy and drug release studies was also done. Since formulation was intended for intravenous administration, it was subjected to comparative hemolytic testing. Any favorable modification in anticancer activity which might have been obtained due to incorporation of drug inside chitosan alginate nanoparticles was established on the basis of cell uptake, cell viability assay, cell cycle analysis along with extent and timing of apoptosis induction in THP-1 leukemic cell line. A comparative pharmacokinetic evaluation was also carried *in vitro* in THP-1 cells, to gauge probable utility of CANPs.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan (75–85% deacetylated), sodium alginate and fluorescein isothiocyanate (FITC) were purchased from Sigma (St. Louis, MO, USA). Bendamustine was a generous gift from Fresenius Kabi, Gurgaon, India. Coupling reagents hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCl) were procured from Fischer Scientific, India. For cell culture studies, RPMI 1640 medium, heat-inactivated fetal bovine serum (FBS), antibiotic solution (penicillin/streptomycin, 0.1% v/v), 3-[4, 5-dimethylthiazol-2-yl]-2, 5- diphenyltetrazolium bromide (MTT) were also bought from Sigma (St. Louis, MO, USA). Rhodamine 123 (Rh123) was bought from Thermo Fischer Scientific, India. Well plates and culture flasks were procured from BD Biosciences (CA, USA). Triple distilled ultra-pure water was used for all the experiments (Milli-Q plus 185 purification system; Bedford, MA, US). All ingredients were used as received. All other chemicals and solvents were of analytical grade.

2.2. Cell culture and differentiation

THP-1 cells, representative of human immortalized leukemia cells were indented form institutional cell repository. Cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine/lt, 100 U/ml penicillin and 100 μg/mL streptomycin at 37 °C in humidified atmosphere of 5% (v/v) CO_2 /air mixture. THP-1 cells (10^5/ml) were differentiated into monocyte-derived macrophages using 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 36 h. Differentiation of PMA treated cells was further enhanced after initial 36 h stimulus by removing PMA containing media and then re-incubating cells in fresh RPMI 1640 (10% FBS) for further 24 h [13].

2.3. Synthesis of fluorescent probe RBM

To a solution of BM (4-(5-(bis(2-chloroethyl)amino)-1-methyl-1H-benzo[d]imidazol-2-yl)butanoic acid), 1a, (0.077 g, 0.11 mmol), in dry dichloromethane/DMF 1:1 (4 mL), EDCL (0.025 mg,0.13 mmol), HOBt (0.017 mg, 0.11 mmol), triethylamine (0.016 μL 0.108 mmol) was added and the reaction mixture was stirred at 25 °C under nitrogen atmosphere for 1 h. After that Rh123 (methyl 2-(6-amino-3-imino-10-oxo-3,10-dihydroanthracen-9-yl benzoate), 1b, (0.040 mg, 0.11 mmol) was added to above mixture and stirred for 24 h. Reaction was monitored by TLC using (5% methanol/dichloromethane) as a solvent system. End product was washed with water (2 × 8 ml), followed by brine solution (8 mL) and the organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. Residue was purified by flash column chromatography on silica gel (60–120 mesh) (eluent: methanol/dichloromethane = 0.2% volume ratio) to afford pure compound (R_f 0.6 in 5% methanol in dichloromethane) as a reddish brown product, 2a. The scheme is depicted in Fig. 1A. Characterization of the conjugated fluorescent probe RBM was done by 1H NMR on a Bruker AC 200P, 400 MHz spectrometer, with tetramethylsilane as internal standard and molecular weight of conjugate was measured by mass spectroscopy.

2.4. UV–visible spectroscopic investigation

All investigated systems were prepared at ambient conditions. Critical micelle concentration (CMC) for AOT reverse micelle formation in ethanol and dichloromethane were obtained using conductometry as 0.2 mM and 0.24 mM, respectively. Each experimental deliberation described henceforth, irrespective of solvent used had an AOT concentration above CMC. Briefly, fixed strength RBM solutions (5 μg/mL) were dissolved in ethanol. To each of these solutions, differing weights of AOT were added followed by vortexing to arrive at aliquots of AOT (0%, 0.2%, 0.5%, 0.75% and 1% w/v, respectively) dispersed in (5 μg/mL) RBM solution. These dispersions were then scanned using a UV-1800 UV–Vis Spectrophotometer (Shimadzu), with ethanol acting as blank. Extracted spectroscopic data was plotted using Graph Pad prism 5.

2.5. Reverse micelle development

AOT content (1% w/v) was dispersed in dichloromethane to form reverse micellar dispersion using probe sonication. Depending upon composition of final dispersion, prescribed amount of water, or drug and aqueous dispersion of SA (as per Table 1) was added to the initial dispersion under continued sonication to form different batches of reverse micelles. Water content defined by R parameter according to relation: R = $[H_2O]$/[AOT] was varied as in Table 1.

2.6. Micellar elucidation by determining diffusion coefficient

DLS experiments were carried out using Zetasizer nano ZS (Malvern Instruments, Malvern, Worcestershire, United Kingdom) which uses a 633 nm He-Ne laser maxing out at 4mW. The back scattered light was collected at an angle of 173° with an Avalanche photodiode array detector. Procedurally, sample was held in a quartz cuvette and decay in magnitude of correlation coefficient (normalized $G1(\tau)$) was gauged as a function of time, which was used to excavate diffusion coefficient of analysed entities [14]. Since micellar systems are susceptible to global dilutions, the samples were analysed in their original state. However in concentrated solutions diffusion of micelles is curtailed and they can suffer restricted maneuverability. Particle size can consequently be wrongly estimated due to introduction of multiple light scattering