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Targeting efficacy and anticancer activity of polymeric nanoparticles of SN-38 on colon cancer cell lines

Shilpi Prasad[1*] and J. S. Dangi[2]

Abstract

Background Colorectal cancer is the third most prevailing cancer in the whole world. Chemotherapeutic agents which are used for treatment have severe side effects and also have unwanted exposure to healthy cells. In the present study, polymeric nanoparticles of SN-38 were prepared (using cationic and anionic polymers). They were optimized by Box Behnken design and characterized for its physicochemical properties and in vitro drug release. Optimized formulation (CsENP) was evaluated for its targeting efficacy by Gamma Scintigraphy studies on Swiss Albino mice and in vitro Cytotoxic assay against colon cancer cell line, viz. HT-29.

Results The images of Whole body gamma scintigraphy imaging of Swiss Albino mice show that CsENP remained intact till 2 h and after that at 4 h imaging it started dispersing and releasing drug which continued till 20 h. In Organ distribution studies, no radioactivity was traced in heart from the formulation. Even in liver, spleen, kidney and lung trace radioactivity was seen after 6 h. In case of CsENP radioactivity was seen in small intestine after 2 h and maximum (87.8% radioactivity) is seen in colon and rectum area after 4 h. At equivalent concentrations, the in vitro cell viability of HT-29 cells after 72 h incubation time showed that CsENP have enhanced cytotoxicity.

Conclusions The results obtained of Whole body gamma scintigraphy imaging and organ distribution of Swiss Albino mice show that CsENP is Colon targeting and was found to be effective against colon cancer cell lines.

Keywords Polymeric nanoparticles, Colorectal cancer, Cell line, Gamma Scintigraphy, Colon targeting, Camptothecin

Background

Cancer is a major burden on society in the whole world. Colorectal cancer rates are increasing worldwide [1]. It may be a cancer, growth, lump, tumour of the colon and the rectum. The United Kingdom National Health Service says colorectal cancer is the most common cancer globally today. However, it is the third most common cancer globally [2, 3]. Current lifestyle changes have known to raise the risk of Colorectal Cancer (such as dietary changes, obesity, sedentary habits, alcohol and cigarette use), its incidence has been rising in recent years [3]. Normally chemotherapy distributes anticancer drugs arbitrarily throughout the body; randomly killing both tumour cells and healthy cells and causes several toxic and adverse effects because of the high dose given by parenteral route. One of the most recent cancer treatment options is cancer immunotherapy. Unlike the conventional approach, this approach manipulates and activates the patient's immune system to fight the cancer cells [4]. New therapeutic agents are introduced which can improve the therapy using different delivery strategies and which is providing significant clinical benefits but still oral chemotherapy targeting to the colon area will not only ensure direct

*Correspondence:
Shilpi Prasad
shilpipharma24@gmail.com
[1] Department of Pharmaceutics, Siddhi Vinayaka Institute of Technology and Sciences, Mangla, Bilaspur, C.G. 495001, India
[2] Department of Pharmaceutics, S.L.T Institute of Pharmaceutical Sciences, GGV, Bilaspur, C.G., India

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treatment of that area and will minimize its exposure to other organs or tissues causing side effects, but it will also increase patient compliance [5–8].

7-ethyl-10-hydroxycamptothecin (SN-38) is an active metabolite of Irinotecan (CPT-11), a derivative of Camptothecin [9–11]. The cytotoxicity of SN-38 is due to double strand DNA damage during DNA synthesis [12]. CPT-11, the prodrug of SN-38 was approved in the USA in 1996 and is commercially available as Camptosar® (Pharmacia Corp., Peapack, NJ) for use in the treatment of metastatic colorectal cancer [6]. It is reported that only a small fraction (2–8%) of CPT-11 is ultimately converted to the active form of SN-38 [13]. In addition, the conversion of CPT-11 from micromolar concentrations in plasma to SN-38 in nanomolar concentrations in plasma is highly variable [14]. The variability and unpredictability in the metabolic conversion rates of CPT-11 to SN-38 create significant life-threatening toxicity risks and make difficult clinical management of patients [13]. Apart from that, SN-38 is poorly soluble in aqueous solutions, and is practically insoluble in most physiologically compatible and pharmaceutically acceptable solvents [13, 15, 16]. So, formulating SN-38 in pharmaceutical parenteral delivery systems is very difficult. SN-38 does not require activation by the liver [13, 17–19]. This property makes it more advantageous than its other Camptothecin precursors, thus eliminates the interpatient variability. In addition to it, SN-38 is nearly 1000 times more potent than CPT-11 as a topoisomerase I inhibitor purified from human and rodent tumour cell lines [20–22]. Moreover, the elimination half-life of SN-38 is much longer as compared to CPT-11, thereby proving it to be a potentially highly effective antineoplastic agent [23].

Numerous nanomaterials, including polymeric nanoparticles, micelles, liposomes, mesoporous inorganic nanoparticles, metal oxides, noble materials, and carbon nanomaterials, have been proposed and are being researched for use in cancer therapy [24]. Polymeric nanoparticles offer advantage over other delivery systems by incorporating both lipophilic and hydrophilic drug. Polymeric nanoparticles are among the most popular organic nanocarriers employed in cancer therapy and promises efficient treatment of several malignancies, including Colorectal Cancer [24]. It can release the drug at a specific site. It can be employed for improving oral delivery of drugs with poor chemical, enzymatic or metabolic stability and permeability [15, 25–28].

In the present study we have developed and characterized an oral and pharmaceutically acceptable polymeric nanoparticles of SN-38 and evaluated the targeting efficacy of the drug for colorectal site thus avoiding other areas from exposure to the highly cytotoxic drug and evaluated the therapeutic efficacy of the formulation against colon cancer cell lines.

Methods

Chemicals and reagents

7-Ethyl-10-hydroxycamptothecin was gifted by Avra Labs (Hyderabad, India). Eudragit-S100® was obtained from Evonik Industries (Mumbai, India). Pluronic F68, Chitosan (deacetylation degree-75%), Acetone and HPLC grade acetonitrile were purchased from Hi Media Labs (Mumbai, India). All other chemicals and solvents were of analytical grade.

Preparation and Characterization of CsENP

Chitosan and EudragitS100® nanoparticles (CsENP) were developed by modified ionic gelation/polyelectrolyte complexation method [18, 29]. The formulations were optimized by Box Benhken Design® and it was further characterized for the physicochemical properties and in vitro drug release [18]. The in vitro drug release and Transmission electron microscopic images are shown in Fig. 1 and the optimized formulation particle size and other parameters are given in Table 1.

Biodistribution studies

Whole body Gamma Scintillography studies on Swiss albino mice

Biodistribution of nanoparticles and their location at different time is difficult to detect in vivo after oral delivery. Detection methods based on radionuclide are non-invasive and gives reproducible results and also provides information about the location, magnitude and kinetics of the biodistribution [30]. These approaches can be easily carried out and are also fast to perform.

Labelling of nanoparticles with ^{99m}Tc

Radiolabelling of nanoparticles with ^{99m}Tc was done by a method as described by Geskovski et al. [31]. 0.5 ml of ^{99m}Tc (1.97 mCi) and 25 µl (1 mg/ml and 0.132 µmol) $SnCl_2$ dissolved in 0.1 M Hcl. The pH was adjusted to 7.0 ± 0.2 using 0.5% sodium bicarbonate and polymeric nanoparticles (CsENP) were mixed just before incubation [32]. They were incubated for 30 min in a water bath at 37 °C. The radiolabelled drug was stored in airtight container inside the lead chamber.

Optimization of radiolabelling efficiency

Various parameters (stannous chloride concentration, pH of the complex and incubation time) can influence the radiolabelling efficiency of aqueous nanodispersions. Stannous chloride concentration's effect on the labelling efficiency was studied to obtain the optimum concentration needed for maximum labelling [33]. The effect of

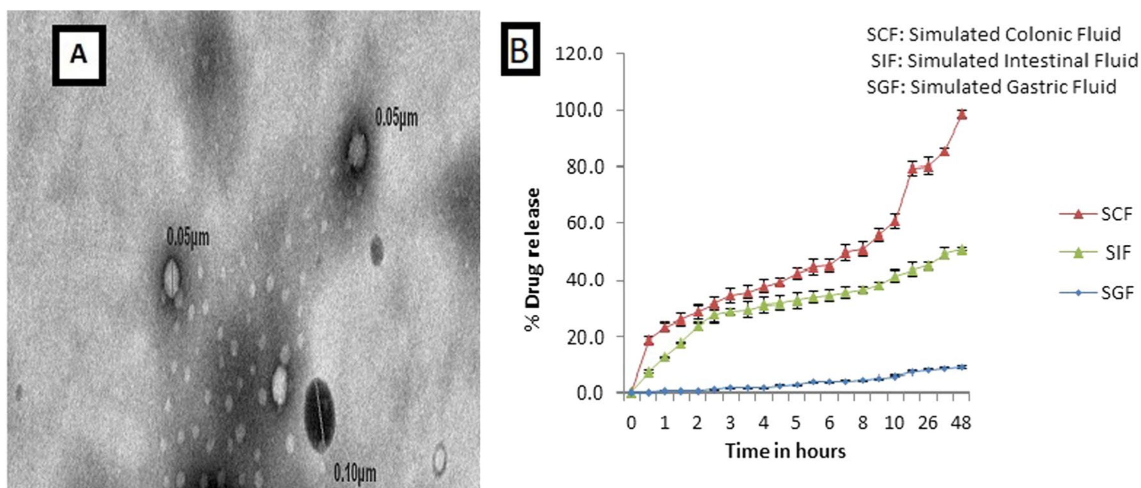

Fig. 1 **A** TEM images of CsENP **B** In vitro drug release of SN-38 from CsENP [18]

Table 1 Physicochemical Parameters of Optimized CsENP [18]

Formulation code	Parameters			
	Particle size(nm)	Entrapment efficiency (%)	Drug loading (%)	Zeta potential (mV)
CsENP	212.5 ± 1.61	75.3 ± 1.01	11.1 ± 0.6	25.6 ± 0.47

The values represent mean ± S.D (n = 3)

Table 2 Effect of change in $SnCl_2$ concentration on labelling efficiency of Nanoparticles with ^{99m}Tc

S. No	Concentration of $SnCl_2$ (µg/ml)	% Radiolabelling of CsENP
1	10	74.9 ± 2.8
2	15	76.8 ± 3.5
3	20	86.7 ± 3.3
4	25	98.7 ± 3.9
5	30	84.9 ± 3.1

The values represent mean ± S.D (n = 3)

Stannous chloride concentrations on labelling efficiency is given in Table 2. The effect of change in pH (from 4.5 to 8.5) on the labelling efficiency of nanodispersions was studied by determining the labelling efficiency while changing the pH and keeping other variables constant. The results are shown in Table 3. For studying the effect of incubation time, the radiolabelled complex was incubated for various time periods keeping other variables constant. The results are shown in Table 4.

Table 3 Effect of pH on labelling efficiency of nanoparticles with ^{99m}Tc

S. No	pH	% Radiolabelling of CsENP
1	4.5 ± 0.2	75.8 ± 3.6
2	5.0 ± 0.2	81.6 ± 3.7
3	5.5 ± 0.2	85.2 ± 2.4
4	6.0 ± 0.2	88.9 ± 3.5
5	6.5 ± 0.2	96.6 ± 3.8
6	7.0 ± 0.2	99.7 ± 3.6
7	7.5 ± 0.2	95.6 ± 4.7
8	8.0 ± 0.2	86.3 ± 3.8
9	8.5 ± 0.2	74.5 ± 3.1

The values represent mean ± S.D (n = 3)

Table 4 Effect of incubation time on labelling efficiency of nanoparticles with ^{99m}Tc

S. No	Incubation time (min)	% Radiolabelling of CsENP
1	15	78.5 ± 3.7
2	20	85.3 ± 2.6
3	25	95.4 ± 1.7
4	30	98.6 ± 3.5
5	35	96.1 ± 3.5
6	40	95.3 ± 4.3

The values represent mean ± S.D (n = 3)

Radiolabelling efficiency of nanoparticles

^{99m}Tc-labelled nanoparticles (CsENP) was analysed using Instant thin layer chromatography (ITLC) system with silica gel coated fibre sheets [31, 33, 34]. Thus, the ITLC strip was spotted with the samples (2-3 μl) at 1 cm and developed up to 9 cm with acetone. Then, the strip was air dried. The strip was halved, and the radioactivity was determined in each segment with a well type gamma ray counter (Gamma ray scintillation counter, Type CRS 23C, Electronics Corporation of India Ltd; Mumbai, India). The free ^{99m}Tc which moved along with the solvent ($R_{f\,=}$ 0.9) was determined. The reduced or hydrolysed ^{99m}Tc along with the labelled nanoparticles stayed at the point of application. The amount of reduced or hydrolysed ^{99m}Tc was determined using pyridine: acetic acid: water (3:5:1.5v/v) as mobile phase. The radiolabelled nanoparticles stayed at the point of application and the free pertechnate moved along with the solvent front. The net amount of ^{99m}Tc labelled nanoparticles was calculated by subtracting the radioactivity moved with the solvent (acetone) front from that using pyridine: acetic acid: water mixture.

Stability studies of radiolabelled nanoparticles

In vitro stability studies of radiolabelled nanoparticles (CsENP) were conducted in Simulated Gastric Fluid (pH1.2) for 2 h, Simulated Intestinal Fluid (pH 6.8) for 2 h and Simulated Colonic Fluid (pH 7.0) for 1 h in order to confirm that labelled nanoparticles remain bound to sodium pertechnetate throughout the studies [35]. The labelled nanoparticles (10 mg) were placed in each of the three volumetric flasks containing 10 ml of simulated gastric fluids of pH 1.2 for 2 h, in pH 6.8 for 2 h and in pH 7.0 for 1 h. These volumetric flasks were kept in water bath at 37 ± 5 °C. At predetermined time intervals 0.2 ml of gastric fluids were pipette out and filtered. At the end of experiment the nanoparticles were recovered, washed and dried. The radioactivity of samples, nanoparticles and filtrate were counted in a well type gamma ray counter. The % radioactivity released at different time intervals of CsENP is shown in Table 5.

Table 5 In vitro stability of ^{99m}Tc labelled nanoparticles in simulated gastric fluids (SGF, SIF and SCF) at 37 ± 0.5 °C

Time (hrs)	% Radioactivity released at different time intervals from CsENP nanoparticles formulation
1	0.00
2	0.00
3	0.00
4	0.02 ± 0.001
5	2.45 ± 0.07

The values represent mean ± S.D (n = 3)

Gamma scintigraphic study (in vivo) with radiolabelled nanoparticles

All in vivo studies were performed in accordance with the study protocol as approved by the Institutional Ethical Committee, SLT Institute of Pharmaceutical Sciences, 994/a/GO/06/CPCSEA, Guru Ghasidas Vishwavidyalaya, Bilaspur, Chhattisgarh, India. The in vivo transit behaviour of the nanoparticles was carried out in male Swiss albino mice (6–7 weeks old) weighing 20–25 g that had fasted for 12 h before administration of the formulations [36]. 5 animals were taken. Each animal received single dose of radiolabelled nanoparticles (0.02 mCi/mg of nanoparticles) by oral gavage. With 2% isoflurane gas (flow of oxygen of 0.2L/min) the animals were anaesthetised and placed in prone position on the SPECT camera. Whole body Scintillography was done just after oral administration. At different time intervals, imaging studies were performed during the period of 20 h (i.e. 1, 2, 4, 6, 20 h). 0.2 mCi (10 mg nanoparticles) of dose was given and image acquisition time of 5 min using E-Cam Single Head gamma camera (Siemens, Germany) was set. Between image acquisitions, mice were free to move away from the camera. One animal was killed and dissected after 2 h, 4 h, 6 h and 20 h and its different organs (e.g. Spleen, Heart, liver, lungs, kidney, stomach, Small intestine, colon and rectum) were excised, washed quickly with cold water to remove surface blood, and placed separately on a petridish. The radioactivity was counted in a well-type gamma scintillation counter. The results are given in Table 6.

In vitro cytotoxic assay on HT-29 cell line by Sulforhodamine B (SRB) assay

The human colorectal adenocarcinoma cell line HT-29 (National Cell Culture Studies, Pune, India) was maintained in a 5% CO_2 atmosphere at 37 °C in RPMI 1640 (Hi media labs, Mumbai, India) medium, supplemented with 20% fetal bovine serum (FBS), 2 μM glutamine and antibiotics. A partial monolayer is formed after 24 h, then supernatant is flicked off and the monolayer was washed once with 100 μl of the medium. The cells were sub cultured twice a week to examine the effects of SN-38 solution in Di Methyl Sulfoxide, blank nanoparticles and drug loaded nanoparticles. 10 mg of SN-38 was accurately weighed and dissolved in 10 ml of dimethyl sulfoxide so as to achieve a final concentration of 1000 μg/ml. Similarly, nanoparticles corresponding to 10 mg of drug were accurately weighed and dissolved in 10 ml of dimethyl sulfoxide to achieve a final concentration of 1000 μg/ml [2]. Range of concentrations from 10^{-3}, 10^{-2}, 10^{-1}, 1 μM of SN-38 loaded CsENP,

Table 6 Organ distribution at different time points of radiolabelled nanoparticles after oral administration (calculated as percentage of initial radioactivity)

Formulation	Time	Heart	Liver	Spleen	Kidney	Lung	Stomach	Small intestine	Colon and rectum
CsENP	After 2 h	0	0	0	0	0	0.03 ± 0.003	36.1 ± 1.4	1.3 ± 0.1
	After 4 h	0	0	0	0	0	0.01 ± 0.001	13.5 ± 2.4	87.8 ± 0.02
	After 6 h	0	1.4 ± 0.05	0	1.03 ± 0.002	0	0	0	18.2 ± 0.005
	After 20 h	0	1.6 ± 0.09	0.05 ± 0.01	2.3 ± 0.03	0	0	0	0

Data are expressed as percentage released of total radioactivity in nanoparticles administered

The values represent mean ± S.D (n = 3)

Irinotecan Hcl and pure SN-38 were taken. Adriamycin was taken as a positive control and they all were added to the culture medium containing 0.1 ml of diluted cell suspension (10,000 cells apprx.) in each well of the 96-well microtitre plate. The plates were then incubated at 37 °C for 3 days in 5% CO_2 atmosphere. After 72 h, 25 µl of 50% Tri chloro acetic acid (TCA) was added to the wells gently such that it formed a thin layer over the drug dilution to form an overall concentration of 10% [37]. The plates were incubated at 4○C for 1 h. The culture plates were washed with water (five times) to remove any traces of medium and drug left and then air dried.

In vitro cytotoxic assay by Sulphorhodamine B (SRB) assay

SRB is an aminoxanthene dye, bright-pink in colour with two sulfonic groups that under mild acidic conditions binds to basic amino-acid residues and under basic conditions gets dissociated [37]. The amount of dye which is withdrawn from stained cells is directly proportional to the cell mass. Under mild conditions, in Tri Chloro acetic acid (TCA) fixed cells, SRB binds to basic amino acid residues of protein for providing a sensitive index of cellular protein content. Colour is developed rapidly, is stable and visible which can be measured with either a spectrophotometer or a 96-well plate reader.

TCA-fixed cells were stained for 30 min with SRB 0.4% (w/v) dissolved in 1% acetic acid. TCA-fixed, SRB stained samples were air dried. Lastly, SRB was removed and cultures were quickly rinsed (four times) with 1% acetic acid to remove unbound dye [38].

Optical density measurement and analysis of results

200 µl of 10 mM Tris base solution (pH 10.5) was added to each well, and the plates were left for 30 min to solubilize the protein bound dye. Optical density was measured at 510 nm in a microplate reader. For primary screening a minimum of 50% cell-growth inhibition is required for proving compound toxicity against cell lines. The cell number can be obtained by determining the optical density (OD) of SRB in each well. As it is directly proportional to the cell number.

The percentage growth inhibition was calculated using the following equation:

$$\% \text{ control of cell growth} = \frac{\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{day0}}}{\text{mean OD}_{\text{negative control}} - \text{mean OD}_{\text{day0}}}$$

$$\%\text{Growth inhibition} = 100 - \%\text{of control cell growth}$$

Results

Optimization of radiolabelling of nanoparticles

Radio tagged nanoparticles were optimized for radiolabelling %age by taking varying concentration of $SnCl_2$ (10–30 µg/ml). At 25 µg/ml of $SnCl_2$ the radiolabelling efficiency of CsENP (98.7%) was maximum, so 25 µg/ml of $SnCl_2$ was taken for reduction of Technetium. At pH 7.0 ± 0.2, the radiolabelling efficiency of CsENP (98.9%) was maximum so pH 7.0 ± 0.2 was maintained during the radiolabelling process. At incubation time of 30 min, the radiolabelling efficiency of CsENP (98.6%) was found maximum. So, incubation time of 30 min was selected for radiolabelling of formulations.

Stability of radio labelled formulations

The stability studies of radio labelled nanoparticles were conducted in simulated gastric fluids and the % radioactivity released at different time intervals from radio tagged nanoparticles was found out. CsENP do not release any radioactivity till 3 h. The % radioactivity released was < 5% which shows that radio tagging of nanoparticles was stable even in the harsh environment of gastrointestinal tract. The study was conducted for 5 h as the half life of ^{99m}Tc is 6 h.

In vivo bio distribution studies

Whole body gamma scintigraphic imaging of Swiss Albino mice were taken at different time points initially and after 1 h, 2 h, 4 h, 6 h and 20 h. The images show that CsENP remain intact till 2 h and after that at 4 h imaging it started dispersing and releases till 20 h. It is due to the pH dependent property of CsENP. In 2 h CsENP reaches

the colon (pH 7.0 or more) and starts releasing the drug by burst effect and releases till 20 h. After 20 h CsENP is washed out as per the normal excretory process of the body. The Gamma Scintigraphy images of murine model at different time interval are shown in Fig. 2.

Organ distribution studies

The radioactivity in isolated organs was quantified by gamma scintillation counter at different time points (2 h, 4 h, 6 h and 20 h). No radioactivity was traced in heart from all formulations. Even in liver, spleen, kidney and lung trace radioactivity is seen after 6 h. In case of CsENP radioactivity is seen in small intestine after 2 h and maximum (87.8% radioactivity) is seen in colon and rectum area after 4 h.

In vitro cytotoxic assay on HT-29 cell line by Sulforhodamine B (SRB) assay

The SRB assay is one of the most commonly used methods for in vitro cytotoxicity studies. This method requires simple and inexpensive reagents and within a few days a large number of samples can be tested. Therefore SRB assay is an efficient and highly cost-effective method for cytotoxicity studies [37]. At equivalent concentrations, the in vitro cell viability of HT-29 cells after 72 h incubation time showed that SN-38 loaded nanoparticles (CsENP) have enhanced cytotoxicity and results were found to be statistically significant at $P \leq 0.05$ (at 95% significance level). The result is given in Table 7 and shown in Fig. 3, also proved that SN-38 is more cytotoxic than Irinotecan HCl [13, 16].

Discussions

Eudragit® S 100 polymer is a pH sensitive polymer and it is solubilized at pH 7[39]. Thus, it prevents the radioactivity release for 3 h. The % radioactivity was less than 5%, which shows that radio tagging of nanoparticles was stable even in the acidic environment of gastrointestinal tract. As half life of ^{99m}Tc is 6 h so the study was conducted for 5 h. In vivo biodistribution

Table 7 Percent growth inhibition at different concentrations of SN-38, IHCl, drug loaded nanoparticles at various concentrations for HT-29 cell line

Drugs/formulation	Concentration μM			
	10^{-3}	10^{-2}	10^{-1}	1
SN-38	67.4 ± 3.6	49.6 ± 4.1	36.7 ± 2.9	21.5 ± 2.6
I Hcl	93.6 ± 3.8	84.6 ± 3.9	75.8 ± 2.8	69.5 ± 2.4
CsENP	72.3 ± 4.2	57.8 ± 3.5	45.8 ± 2.1	28.7 ± 4.1
Adriamycin	10.6 ± 0.1	2.3 ± 0.24	0.6 ± 0.02	− 1.6 ± 0.04

The values represent mean ± S.D (n = 3)

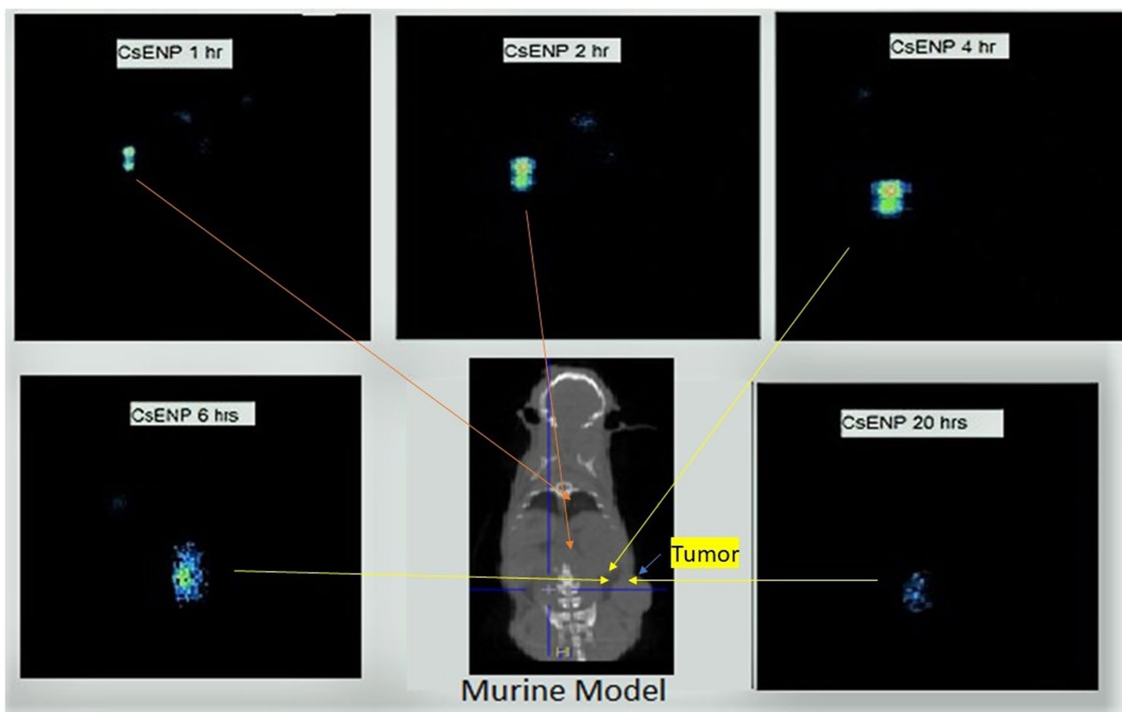

Fig. 2 Gamma Scintigraphic images of CsENP in mice at different time intervals

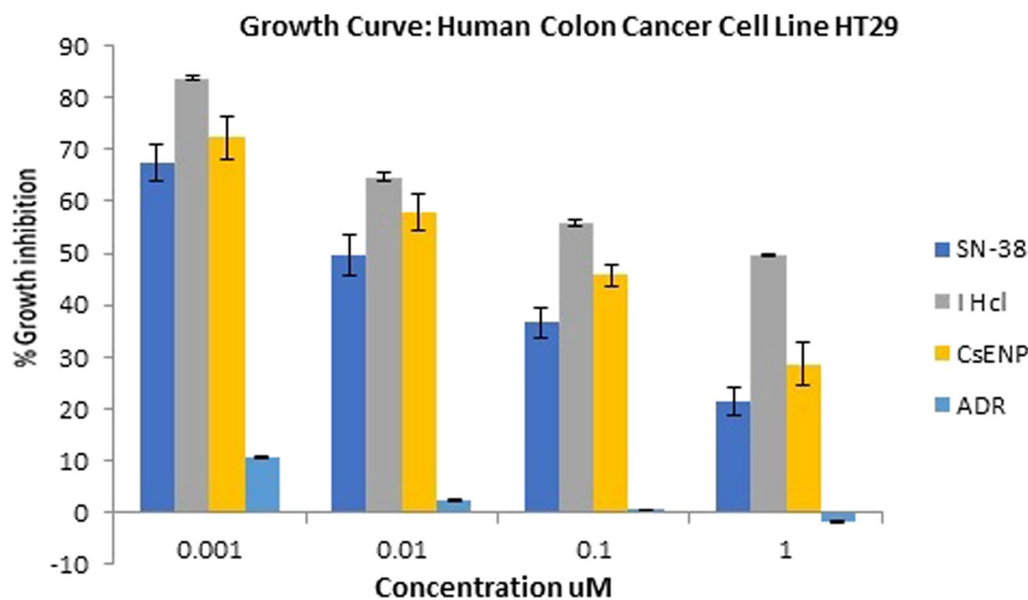

Fig. 3 % Growth inhibition at various concentrations of drugs and formulations for HT-29 cells

studies in murine model revealed that CsENP remained intact till 2 h and it started releasing the drug at 4 h study. It is due to the pH dependent property of CsENP. Organ distribution studies shows the targeting efficacy of CsENP for colon and rectum area. It also revealed that the vital organs of the body are not exposed to the cytotoxic effect of SN-38 and are safe. In vitro cytotoxic assay on HT-29 cell line shows that CsENP has statistically significant cytotoxicity on human colon cancer cells HT-29 as SN-38 at $P \leq 0.05$. Since SN-38 is not soluble in any pharmaceutically acceptable solvents so its absorption in physiological fluid is a problem. Formulating it in the form of polymeric nanoparticles not only maintain its cytotoxicity but also make it suitable for administration in colorectal cancer patients.

Conclusions

Polymeric nanoparticles of SN-38 were prepared by polyelectrolyte complex method. Optimized formulation (CsENP) was characterized for shape, size, entrapment efficiency, Zeta potential and Drug loading. The Colon targeting efficacy of CsENP was due to the pH dependent properties of Eudragit® S 100 Polymer. Gamma Scintigraphy and Organ distribution studies further proved the targeting efficacy of CsENP and confirmed that the vital organs of the body are not exposed to the cytotoxic effect of SN-38. In vitro cytotoxic studies on HT-29 cells proved that SN-38 loaded nanoparticles have enhanced cytotoxic effect than its prodrug Irinotecan Hydrochloride. Thus oral administration of CsENP (polymeric nanoparticles of SN-38) was found to be effective and safe for the treatment of colon cancer.

Abbreviations

OD	Optical density
SRB	Sulforhodamine B
SN-38	7-Ethyl 10-hydroxy camptothecin
IHCL/CPT-11	Irinotecan hydrochloride
^{99m}Tc	^{99M}Technitium
TCA	Trichloroacetic acid
FBS	Fetal bovine serum
ITLC	Instant thin layer chromatography
$SnCl_2$	Stannous chloride

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Author contributions

S.P designed, analysed the data, wrote the manuscript, done data interpretation, editing, formatting, and graphical work. J.S.D was associated with supervising, advising and structuring the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate

All in vivo studies were performed in accordance with the study protocol as approved by the Institutional Ethical Committee, SLT Institute of Pharmaceutical Sciences, 994/a/GO/06/CPCSEA, Guru Ghasidas Vishwavidyalaya, Bilaspur, Chhattisgarh, India.

Consent for publication

Not applicable.

Competing interests

"The authors declare that they have no competing interests".

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