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Original Article

DETERMINATION OF *IN VITRO* CYTOTOXICITY OF ENTRECTINIB AND PEMIGATINIB NANOSPONGES TABLETS ON A 498, MCF-7 AND PANC-1 CELL LINES

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ABSTRACT

Objective: The aim of this study was to improve the oral solubility of Pemigatinib and Entrectinib through incorporation into nanosponges (NSs), and further the cytotoxic potential of optimized formulations of NSs on A498, MCF-7, and PANC-1 cell lines in the MTT based Cell proliferation assay was analyzed.

Methods: In the current study Pemigatinib and Entrectinib were formulated in to NS tablets and cytotoxicity was determined by using A498, MCF-7, and PANC-1 cell lines. The optimized NS formulation was determined prepared into a tablet dosage form, which further was evaluated for physical parameters and *in vitro* drug release study. For cytotoxicity studies, MTT assay was conducted for these formulations, IC_{50} values were calculated for the tested compound and compared with 5-Fluorouracil.

Results: The optimized formulation was evaluated for physical parameters and *in vitro* drug release study, the results were satisfactory. The IC50 of Entrectinib NS, Pemigatinib NS and 5-Fluorouracil, against A498 cell line was 26.34, 85.24 and 15.24 µg/ml, respectively. The IC50 of Entrectinib NS, Pemigatinib NS and 5-Fluorouracil, against MCF-7 cell line was 71.54, 35.48 and 24.56 µg/ml, respectively. The IC50 of Entrectinib NS, Pemigatinib NS and 5-Fluorouracil, against PANC-1 cell line was 35.14, 22.54 and 22.54 µg/ml, respectively. It was observed that the IC_{50} of drug-loaded NS was higher than the comparator drug and these enter the cells by active transport and induce cytotoxicity to the cells.

Conclusion: The overall results from the studies suggest that Entrectinib NS and Pemigatinib NS provided efficient cytotoxic effects, which could play a significant role in the percentage cell death.

Keywords: Pemigatinib, Entrectinib, Biliary tract cancer, Nanosponges, Cytotoxicity, Cell lines

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INTRODUCTION

Biliary tract cancers (BTC)/cholangiocarcinoma represent a diverse group of epithelial cancers characterized by aggressive and chemoresistant tumors with poor long-term survival [1]. Surgery remains the only curative treatment; however, only 35% of patients can undergo curative surgery [2]. Often, limitation of surgical resection includes the presence of vascular involvement and the presence of metastatic spread to regional lymph nodes, which are often evident at time of diagnosis, given the frequent asymptomatic status of early disease. Systemic therapy for cholangiocarcinoma represents the only feasible option for patients with locally advanced or metastatic cholangiocarcinoma.

In recent years, advancements in gene sequencing have better highlighted the genetic landscape of BTC and have shown that molecular profiles segregate with anatomical location. Numerous agents have been developed to target FGFR inhibition in this clinical context. Initial agents acquiring FDA approval included Pemigatinib in April 2020. Pemigatinib is a highly potent and selective inhibitor of FGFR1, FGFR2, and FGFR3; it is a BCS class-II compound and exhibits BCS class I properties in acidic media.

The water solubility is about 0.144 mg/ml with log P value of 2.26. It is diprotic basic compound with pKa values of 3.1 and 5.7. It displays pH-dependent solubility (1.2 to 7.4), which decreases with increasing pH [3]. The other drug tested was Entrectinib which is a lipophilic, basic, moderately permeable molecule with strongly pH-dependent solubility [4]. It is crystalline solid and is a BCS class II chemical with limited solubility and intermediate permeability. Entrectinib has a solubility of 40 mg/ml in 0.07 M HCl (pH 1.2), 0.03 mg/ml at pH 5.4, and 0.002 mg/ml at pH 6.4. Its solubility is much greater in the fed condition than in the fasted state. Peak plasma concentrations were discovered to be 2-4 h in the fasted condition versus 5-7 h in the fed state [5, 6].

The poor solubility of the chosen drug substances was the main inherent factor that influences the oral absorption of the drug [4]. In order to improve intrinsic solubility and to reduce the high pharmacokinetic variability observed with the existing tablet formulation, it is essential to develop an alternative formulation of Pemigatinib and Entrectinib with improved characteristics.

Various formulation strategies have been used in recent years to improve the oral bioavailability of poorly soluble medicines. To boost oral bioavailability, various classical approaches such as complexation, co-solvency, salt formation, micronization, and the use of permeation enhancers have been tested [5]. All of these approaches, however, have proven limited efficacy in drug delivery. Among the different techniques, nano-based drug delivery systems (NBDDS) have enormous potential to improve the bioavailability of poorly soluble medicines [6]. NBDDS have sparked a lot of research interest in recent years because of the potential benefits, such as improving lipophilic drug solubility, increasing permeability, improving drug stability, controlling drug distribution and elimination, and targeting drug delivery to a specific site. Several NBDDS have been produced, including nanocrystals, nanoemulsions, nanosponges (NSs), nanobubbles, liposomes, polymeric micelles, polymeric nanoparticles, and inorganic nanocarriers [7, 8].

Many studies have shown that NS dosage forms can increase the solubility and, thus oral bioavailability of poorly soluble medicines [9]. They shield molecular compounds from degradation and these have high selectivity, biocompatibility, degradability, and extended-release behaviour, which are used in cancer therapy [10]. The sponge functions as a three-dimensional scaffold or network. Polyester is used for the backbone. To produce the polymer, it is combined in solution with cross-linkers. The end result is spherically shaped particles with cavities where drug molecules can be housed [11]. Because polyester is biodegradable, it degrades gradually in the body. Its drug payload is released in a predictable manner as it degrades. By adjusting the amounts of crosslinker to polymer, the NSs can be synthesised to be a certain size

and to release medications over time. For oral administration, these may be dispersed in a matrix of excipients, diluents, lubricants, and anti-caking agents suitable for the preparation of tablets or capsules, and the major advantages of these capsules or tablets are reduced total dose, retention of dosage form, reduced toxicity, and improved patient compliance through prolonged release [12].

The aim of this study was to improve the oral solubility of Pemigatinib and Entrectinib through incorporation into NSs. This research was designed to prepare NS formulations of Pemigatinib and Entrectinib and prepare tablet dosage form for the easy administration. The formulations and end dosage form was planned for qualitative and quantitative evaluations. This paper mainly covers a comparative study of the cytotoxic potential of optimized formulations of NSs on A498, MCF-7 and PANC-1 cell lines in the MTT-based Cell proliferation assay.

MATERIALS AND METHODS

Materials

Pemigatinib was procured from Aelida Pharmaceuticals, Haryana, India. Entrectinib was obtained from Hetero Drugs Pvt Ltd, Hyderabad. ROZLYTREK was acquired from South Delhi Pharma, Delhi, India. β-Cyclodextrin was from Gangwal Chemicals Pvt. Ltd. Mumbai, India. Hyper cross-linked Polystyrenes was obtained from Gangwal Chemicals Pvt. Ltd. Mumbai, India. Diaryl carbonate and diphenyl carbonate were obtained from Euclid Pharmaceuticals Limited, Mumbai. Dimethyl formamide was from Qualigens, Thermo Fisher Scientific India Ltd, Mumbai. Fetal Bovine Serum [#RM10432] and D-PBS [#TL1006], DMEM [#AL007A], EMEM [#AL047S] were from HiMedia. MTT Reagent [# M5655] and DMSO [#PHR1309] were from Sigma. 96-well plate for culturing cells was from Corning, USA.

Preparation of pemigatinib NSs

Polystyrene-based NSs were prepared in our laboratory using diaryl carbonate for cross-linking and for the fabrication of Pemigatinib [16,17]. Polystyrene was dissolved in dimethyl formamide, to which diaryl carbonate was added and was left for the completion of reaction and formation of NSs.

Preparation of entrectinib NSs

β-Cyclodextrin (β-CD; polymer) based NSs were prepared in our laboratory by using diphenyl carbonate as a cross-linking by ultrasound-assisted method for fabrication of Entrectinib. β-CD was dissolved in dimethyl formamide, to which diaryl carbonate was added and was left for completion of the reaction and formation of NSs [13].

Preparation of drug-loaded NSs tablets

The oral formulations of drug-loaded NSs were prepared by wet granulation method. The binding agent's gelatin and the polymer hydroxy propyl methyl cellulose; HPMC K4M and K100M were used to prolong the drug release up to 24 h for Entrectinib and Pemigatinib formulations, respectively. The round tablets were prepared using a single-punch tablet machine with a flat-faced single punch after addition of the rest of the formulation (Tablet Compression Machine-Single Punch, Harrisons Pharma Machinery Private Limited) [14, 15].

Characterization of prepared pemigatinib and entrectinib NSs

The particle size distribution of Pemigatinib NSs was observed by dynamic light scattering method. Polydispesity index of the particles were calculated using cumulated analysis after averaging three measurements. Zeta potential measurements were also made using an additional electrode in the same instrument (Mastersizer 2000, Malvern Instruments Ltd, Worcestershire, UK). Encapsulation efficiency was analysed by UV spectrophotometer (Labindia UV-3000+, Labindia instruments Pvt. Ltd.). The morphology of the samples was examined at magnification of 45000× by Transmission Electron Microscopy (TEM; JEM-2000 EXII; JEOL, Tokyo, Japan). Fourier Transformed Infrared (FTIR) Spectroscopy was performed using Tensor 27 FTIR Spectrophotometer in the region of 4000 to 600 cm^{-1} (Tensor 27, Bruker Optics, Germany) and Differential Scanning Calorimetry (DSC) was performed using a Perkin Elmer DSC/7 differential scanning calorimeter (Perkin-Elmer, CT-USA) equipped with a TAC 7/DX instrument controller to test the compatibility. Evaluation of tablet formulation was performed for uniformity of weight, drug content, hardness, friability, *in vitro* drug release, drug release kinetics, and stability [16-21].

In vitro cytotoxicity studies

Preparing cell line

The current investigation included A498, MCF-7 and PANC-1 cell lines [22]. These cell lines were procured from American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified Eagle's medium (PANC-1) and Eagle's Minimum Essential Medium (A498 and MCF-7). Cells were grown in 75 cm^2 bottle canted necked vented flasks (Corning) with the specified medium and the cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Cells (passages 30–50) were grown in specified medium (Gibco Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% penicillin (1000 U/ml), 1% streptomycin (1000 µg/ml) and 1% amphotericin (250 U/ml). The cells were passaged enzymatically with 0.25% trypsin-1 mmol EDTA and subcultured on 75 cm^2 plastic flasks at a density of $2.2x10^4$ cells/cm^2. Culture medium was replaced every 2 d. Cell confluence (80%) was confirmed by microscopic observance. Experiments were performed 24 h post-seeding to prevent cell differentiation. All the molecules used were of 95-97% pure and were gauged by HPLC and verified by mass spectrometry [23, 24].

MTT assay

The *in vitro* cytotoxicity assay of NS tablets of Pemigatinib and Entrectinib was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay [25]. 200 µl cell suspension (in complete culture medium with 10% FBS) was seeded in a 96-well plate (20,000 cells per well), without the test agent and allowed to grow for 24 h. After 24 h of incubation, spent media in the wells of 96-well plate were replaced with appropriate concentrations of the test compounds and incubated for 48 h at 37 °C in a 5% CO_2 atmosphere. After the incubation period, the plates were removed from the incubator; spent media was removed, followed by addition of MTT reagent to a final concentration of 0.5 mg/ml (0.2 µm filter sterilized). The plates were wrapped with aluminium foil to avoid exposure to light and placed in the incubator for 3 h. After incubation, MTT reagent was removed and 100 µl of DMSO was added. Absorbance was measured on a spectrophotometer (Tecan™ Infinite 200Pro) at 570 nm [26].

Preparation of test solutions

A vial of each cell lines was taken out from liquid nitrogen storage and thawed rapidly to room temperature. The contents in the vials were added to 9 ml of complete medium and centrifuged at 125 g for 5 min. After centrifugation, the supernatant was discarded and pellet was mixed with 10 ml of complete medium and, suspended in a T-25 flask and incubated at 37 °C with 5% CO_2. When the cell confluence reached ~80%, the cells were centrifuged at 125 g for 5 min; pellet was mixed with 15 ml of complete medium and transferred to two T-75 flasks. When the cell confluence reached around 80-90%, cells in the flask were used for the assay [27].

Data analysis

The percent viability of cells in the untreated (negative control) group was set to 100% and the % viability of cells in the treated groups was estimated relative to the negative control. The % viability was plotted against the concentration and evaluated for dose response. Based on the dose-response relationships, an appropriate model was fit to estimate the I_{max} and IC50 [28].

Percentage viability was calculated using the following formula:

$$\% \text{ Viablity} = \frac{100 \times OD_{570}\,e}{OD_{570}\,b}$$

Where,

OD570e is the mean value of the measured optical density of the dilutions of test item;

OD570b is the mean value of the measured optical density of the negative control

RESULTS AND DISCUSSION

Over the last decade, numerous efforts have been directed to the method of preparation and application of NSs. Among the numerous types of NSs, polystyrene and β-CD based NSs have gotten the most attention and are being investigated the most [29].

Characterization of prepared pemigatinib and entrectinib NSs tablets

Pemigatinib NSs and tablet formulation

The range of mean particle size was 153–316 nm, the range for encapsulation efficiency was 68.2%–91.4%, and the value for polydispersity index was 0.273-0.445. The zeta potential for the optimized formulation was found to be-29.1 mV. The drug and excipients were compatibles as confirmed by FTIR and DSC studies. SEM analysis confirmed that the Pemigatinib has successfully entrapped in the core of polymer. The weight and the thickness of pemigatinib-loaded NS tablets were within the limits of uniformity. The weight was ranged from 200±5.31 to 201±6.13 mg. Thickness ranged between 3.1±0.26 to 3.4±0.23 mm. The drug content ranged from 97.88±1.37% to 99.37±1.21%. The adequate tablet hardness is necessary requisite for consumer acceptance and handling. The measured hardness was ranged between 4.4±1.27 to 4.6±1.88 Kg/cm^2. The friability tests for all were done as per the standard procedure I. P. the results of the friability test. The data indicates that the friability was less than 1% in all formulations, ensuring that the tablets were mechanically stable [16]. *In vitro* release of the Pemigatinib loaded NSs tablets were compared with a marked product and satisfactory results were obtained (98.74±2.65% vs. 93.73±1.06%). The prepared formulations were stable during 6 mo stability study period. The complete details were reported.

Entrectinib NSs and tablet formulation

The range of mean particle size was 149-294 nm, the range for encapsulation efficiency % was 65.4%-87.3%, and the value for polydispersity index was 0.437. The zeta potential for the optimized formulation was found to be 38.1 Mv. The drug and excipients were compatibles as confirmed by FTIR and DSC studies. SEM analysis confirmed that the Entrectinib has successfully entrapped in the core of polymer. The weight and the thickness of Entrectinib loaded NSs tablets were within the limits of uniformity. The weight was ranged from 500±3.45to 501±6.73. Thickness ranged between 5.1±0.05to 5.4±0.15 mm. The drug content ranged from 98.11±1.63% to 99.34±1.55 %. The adequate tablet hardness is necessary requisite for consumer acceptance and handling. The measured hardness was ranged between 5.4 to 5.8 Kg/cm^2. The friability tests for all were done as per the standard procedure I. P. the results of the friability test. The data indicates that the friability was less than 1% in all formulations ensuring that the tablets were mechanically stable [18, 19]. *In vitro* release of the Entrectinib-loaded NSs tablets were compared with a marked product and satisfactory results were obtained (98.94±2.43% vs. 91.78±1.37%). The prepared formulations were stable during 6 mo stability study period. The complete details were reported.

In vitro cytotoxicity studies

To verify whether the Pemigatinib and Entrectinib NSs were pharmacologically active, *in vitro* cytotoxicity tests were conducted on A498, MCF-7 and PANC-1 cell lines by MTT assay. The test Pemigatinib and Entrectinib NS formulations were screened against Morphology of A498, MCF-7 and PANC-1 cell lines. The images of the cell lines treated with vehicle control, Pemigatinib NS (100 µg/ml), and Entrectinib NS (100 µg/ml) were depicted in fig. 1. The result of the screening of drug-loaded NS against morphology of A498, MCF-7 and PANC-1 cell lines shows that the NSs have shown significant cytotoxic effect, these were found effective and there was a prominent inhibition of the cancer cell proliferation [27].

The addition of one healthy cell line was conducted to determine and compare the cytotoxic effect of the Pemigatinib and Entrectinib NS optimized formulation and the standard drug (5-Fluorouracil) on normal and cancerous cells using their IC50 values [28]. The results of the cytotoxic activity of the tested compounds using MTT assay confirmed that the drug loaded into NS exhibited satisfactory cytotoxic activity in A498, MCF-7 and PANC-1 cell lines. These results are due to the advanced drug delivery system. The IC50 of Entrectinib NS, Pemigatinib NS and 5-Fluorouracil, against A498 cell line was 26.34, 85.24 and 15.24 µg/ml, respectively. The IC50 of Entrectinib NS, Pemigatinib NS and 5-Fluorouracil, against MCF-7 cell line was 71.54, 35.48 and 24.56 µg/ml, respectively. The IC50 of Entrectinib NS, Pemigatinib NS and 5-Fluorouracil, against PANC-1 cell line was 35.14, 22.54 and 22.54 µg/ml, respectively [29]. The calculated IC50 values and their standard error mean (SEM) is depicted in table 1 and fig. 2.

Cytotoxicity studies showed that Pemigatinib and Entrectinib NS exhibited a more cytotoxic effect in comparison with comparator drug. Due to slow release of drug from the NS, at the proposed concentration, the amount of free drug in NS was much lower than that of free comparator drug. Thus, the improved cytotoxic effect on the cancer cell lines may be due to higher cell penetration of the NSs [30]. This behavior could be related to the amphiphilicity of the NS for improved cell wall interaction compared with absolute hydrophobic Pemigatinib and Entrectinib. It is believed that free drug is easily available and enter the cells immediately via passive diffusion causing it to be more toxic to the cancer cells, Pemigatinib and Entrectinib that was encapsulated in the NS nanocarrier system with increase in the solubility and bioavailability must first be internalized by active transport or via endocytosis in order for it to enter the cells and induce cytotoxicity to the cells [31]. Previous studies justified that the incorporation of drug active compound into nanoparticle or a nanocarrier system did not affect or increase the efficiency of the drug on inhibiting cancer cells to proliferate. Further, the effect and function of drug compound in a nanocarrier is more effective and able to be observed better *in vivo* as the purpose of encapsulation to make the drug compound soluble and enhance the oral bioavailability might not be seen *in vitro*. The overall results from the studies suggest that Pemigatinib and Entrectnib NS formulations could play a significant role in the percentage cell death [32].

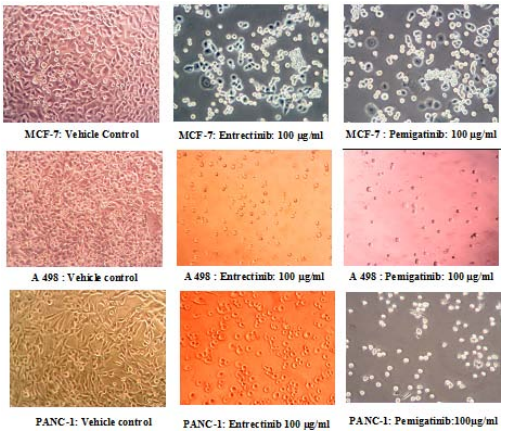

Fig. 1: Morphological screening of vehicle control, entrectinib and pemigatinib in A498, MCF-7 and PANC-1 to study the cytotoxicity

Table 1: IC50 values of pemigatinib and entrectinib NS in comparison with 5-fluorouracil

Compound	IC50 value (µg/ml)		
	A 498	MCF-7	PANC-1
Entrectinib NS	26.34±0.16	71.54±0.3	35.14±0.21
Pemigatinib NS	85.24±0.06	35.48±0.2	22.54±0.13
5-Fluorouracil	15.24±0.04	24.56±0.03	22.54±0.15

Data is given in mean±SD, n=3

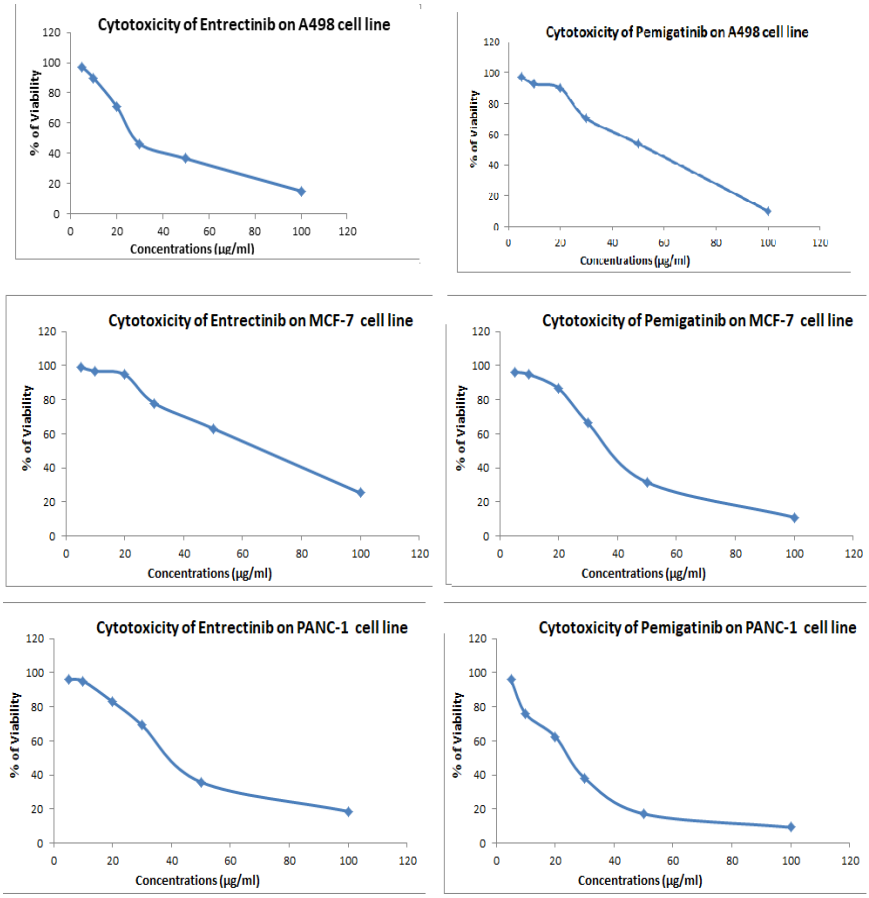

Fig. 2: Cytotoxicity studies of pemigatinib and entrectininb NSs

CONCLUSION

The Pemigatinib NS and Entrectinib NS formulation was successfully developed and was optimized. *In vitro* release of the NSs tablets were compared with a marked product and satisfactory results were obtained, confirming the efficiency of NS for improving the solubility and dissolution rate of poorly water-soluble drugs. Further the cytotoxicity was confirmed by cell line studies using A498, MCF-7 and PANC-1 cell lines. The inhibitory effect of the comparator drug and drug-loaded NS formulations on cells lines was examined by MTT assay. It was observed that the IC_{50} of Pemigatinib NS and Entrectinib NS was higher than pure comparator drug. Pemigatinib and Entrectinib were encapsulated in the NS nanocarrier system, to increase the solubility and bioavailability must first be internalized by active transport for it to enter the cells and induce cytotoxicity to the cells. In conclusion, our results show that Pemigatinib NS and Entrectinib NS formulation significantly reduced the cell viability and altered the cellular morphology of A498, MCF-7 and PANC-1 cell lines.

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Nil

AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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