Research paper

Erlotinib-loaded albumin nanoparticles: A novel injectable form of erlotinib and its in vivo efficacy against pancreatic adenocarcinoma ASPC-1 and PANC-1 cell lines

M. Noorani\textsuperscript{a,b}, N. Azarpia\textsuperscript{c}, K. Karimian\textsuperscript{d}, H. Heli\textsuperscript{b,⁎}

\textsuperscript{a} Department of Nanomedicine, School of Advanced Medical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran
\textsuperscript{b} Nanomedicine and Nanobiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
\textsuperscript{c} Arato Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
\textsuperscript{d} Arato Pharmaceutical Chemicals Inc., Yoosefabad, Jahanazar Avenue, Tehran, Iran

ARTICLE INFO

Keywords:
Erlotinib
Bovine serum albumin
Pancreatic cancer
Cytotoxicity

ABSTRACT

Erlotinib was loaded on albumin nanoparticles for the first time and the cytotoxic effect of the resulting nanoparticles against ASPC-1 and PANC-1 pancreatic adenocarcinoma cell lines was evaluated. The carrier (albumin nanoparticles, ANPs) was synthesized by desolvation method using a mixed solvent followed by thermal crosslinking for stabilization. ANPs and the drug-loaded ANPs were characterized by field emission scanning and transmission electron microscopies, particle size analysis and Fourier transform infrared spectroscopy. The nanof ormulation had a size of < 14 nm with a good monodispersity. Drug loading and encapsulation efficiencies were evaluated as 27 and 44%. Cytotoxicity assays after 72 h revealed the potential of ANPs to improve erlotinib toxicity (54% against 34% of free drug toward ASPC-1 cell line, and 52% against 30% toward PANC-1 cell line). Values of IC\textsubscript{50} were obtained for both cell lines and indicated significant reduction in the erlotinib dose necessary for killing the cells, while, ANPs were completely safe. The results demonstrated that erlotinib-loaded ANPs had a remarkable potential for pancreatic cancer drug delivery.

1. Introduction

Pancreatic cancer with an invasive nature is one of the most virulent cancers (Makohon-Moore and Iacobuzio-Donahue, 2016). In recent years, extensive efforts have been directed to improve therapy with disappointing outcomes for pancreatic cancer patients (Gharwan and Groninger, 2016). Encouraging results of erlotinib studies drew attentions to this drug as an emerging therapy for this cancer (Gharwan and Groninger, 2016). The results of different studies revealed the role of erlotinib as a multi-prong chemotherapeutic agent that is involved in impediment of cell cycle progression (Torres et al., 2016), induction of apoptosis as a result of up regulation of cell cycle inhibitor p27 (Torres et al., 2016) and Caspase-3 (Huether et al., 2005), inhibition of phosphorylation of EGFR and its downstream molecules that leads to inhibition of DNA synthesis (Miyabayashi et al., 2013), cell growth (Li et al., 2009) and angiogenesis in pancreatic cancer cell lines (Lu et al., 2008). Unlike its advantage over other chemotherapeutic agents (Torres et al., 2016), resistance to this medication has been reported in some patients (Suda et al., 2011). Moreover, as a result of nonspecific targeting of EGFR in normal tissues, patients taking erlotinib tablets suffer from gastrointestinal (Gordon et al., 2005), ocular (Celik and Kosker, 2015) and skin disorders (Kiyohara et al., 2013), limiting its use in some patients (Marslin et al., 2009). Furthermore, oral application of erlotinib shows a high first pass effect causing its limited bioavailability and usefulness in patients with severe hepatic dysfunctions (Saiï, 2008).

Nanotechnology has been developed in different area of medicine including theranostics, antibacterial materials, imaging, biomarkers detection, pathogens identification and drug delivery (Sattarahmady et al., 2016a), (Yang et al., 2017), (Bakhtiary et al., 2017), (Kim et al., 2017), (Khurana et al., 2014), (Sattarahmady et al., 2013), (Sattarahmady et al., 2017), (Heli et al., 2017), (Sattarahmady et al., 2015), (Negahdary et al., 2017), (Sattarahmady et al., 2016b). As for drug nanoformulations, carriers such as nanoparticles of biological macromolecules, nanoemulsions, nanogels, and nanoniosomes have been developed (Sattarahmady et al., 2016a; Nazari-Vanani et al., 2017; Numata et al., 2014; Khazaee et al., 2014). Each of the delivery systems have their specific advantages and disadvantages arising from biocompatibility, stability loading and release capacities and cost. Among these, natural hydrophilic nanocarriers can improve drug concentration in target tissue by decreasing macrophage identification

⁎ Corresponding author at: Nanomedicine and Nanobiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
E-mail addresses: kheli67@yahoo.com, heli@sums.ac.ir (H. Heli).

http://dx.doi.org/10.1016/j.ijpharm.2017.08.102
Received 2 June 2017; Received in revised form 17 August 2017; Accepted 21 August 2017
Available online 25 August 2017
0378-5173/ © 2017 Elsevier B.V. All rights reserved.
probability after administration (Aggarwal et al., 2009). If selection criterion for choosing appropriate carrier is structural flexibility toward external condition, then albumin nanoparticles (ANPs) is one of the best options for transport of hydrophobic drug to target tissue at predictable drug destiny in the body (Sattarahmady et al., 2016a; Larsen et al., 2016; Yu et al., 2014). There are different reports concerning ANPs capacity for nasal, ocular, inner ear, brain, oral and intravenous drug delivery. ANPs have the advantages of tumor targeting through albumin-receptor-mediated active transcytosis (Khurana et al., 2014), biocompatibility, non-toxicity, non-immunogenicity, and stability. ANPs provide a high encapsulation efficiency, with significant improvement in solubility and half-life (Sattarahmady et al., 2016a; Kratz,
To the best of our knowledge, there have been no reports on erlotinib-loaded ANPs (E-ANPs), and in this study, we synthesized E-ANPs for the first time. Among various methods to synthesis ANPs, desolvation method followed by thermal cross linking to achieve stable biodegradable structure is one of the best ones (Von Storp et al., 2012). ANPs and E-ANPs were characterized by field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), particle size analysis (PSA) and Fourier transform infrared spectroscopy (FTIR). Cytotoxicity assays in ASPC-1 and PANC-1 cell lines were carried out to compare the cytotoxicity of erlotinib and E-ANPs.

2. Materials and methods

2.1. Materials and biologicals

All chemicals used for the synthesis of ANPs and E-ANPs were purchased from Scharlau (Spain). Bovine serum albumin (BSA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA). Erlotinib was obtained from Arasto Pharmaceutical Chemicals Inc. (Iran). Penicillin-streptomycin solution and fetal bovine serum (FBS) were purchased from Gibco (USA). Trypsin-ethylendiaminetetraacetic acid (EDTA) solution was purchased from Ciasson (USA). Roswell Park Memorial Institute-1640 (RPMI-1640) was obtained from Gibco (USA). Human pancreatic adenocarcinoma cell lines of ASPC-1 and PANC-1 were obtained from Pasteur Institute (Iran).

2.2. Synthesis of ANPs by the desolvation method

Dimethyl sulfoxide (DMSO), acetone and a mixture of the two solvents (acetone/DMSO of 5:1 (v/v) ratio) were used to obtain a high yield of nanoparticles by the desolvation method. Typically, BSA was dissolved in distilled water to achieve a 5% (w/v) solution and the pH was adjusted to 5.0 with HCl. The organic solvent was then added under stirring (acetone, DMSO, or a mixture of acetone/DMSO, all in four times of the volume of the BSA aqueous solution) to form ANPs. Stirring was continued for 10 min to form stable nanoparticles. Thermal cross linking was then carried out at 55 °C for acetone and the mixture of acetone/DMSO, and at 100 °C for DMSO for 30 min. The mixtures were then centrifuged at 20,000 rpm for 10 min. After removing the supernatants, ANPs were washed twice with the organic solvent by centrifugation, and the yields of the ANPs synthesis were calculated. Based on the obtained results (vide infra), the highest yield was obtained using the acetone/DMSO mixture and further synthesis was performed using this mixture.
E-ANPs were synthesized as reported above by adding 8 mg mL$^{-1}$ erlotinib to the organic solvent.

2.3. Characterization of ANPs and E-ANPs

PSA was performed using a Scatterscope, Qudix (South Korea) from 1.0 mg mL$^{-1}$ solutions of ANPs to estimate the hydrodynamic size and size distribution of ANPs, before and after erlotinib loading. A median particle size (d50) and Span value as indicator of polydispersity were obtained. The morphology and size of ANPs were also evaluated by FESEM using a TESCAN Mira 3-XMU (Czech Republic) from dried samples of ANPs suspension on a glass slide. TEM was performed by a Zeiss, EM10C (Germany) at an accelerating voltage of 80 kV. FTIR spectroscopy was done by a Bruker Tensor 27 (USA).

2.4. Determination of drug loading (DL) and encapsulation efficiency (EE)

DL and EE were calculated using the following equations:

\[
DL (\text{w/w}%) = \frac{\text{weight of erlotinib in E-ANPs}}{\text{weight of ANPs}} \\
EE (\text{w/w}%) = \frac{\text{weight of erlotinib in E-ANPs}}{\text{weight of initial erlotinib added}} 
\]

Weight of erlotinib in E-ANPs was calculated by subtracting weight of unloaded erlotinib from weight of initially added erlotinib. After synthesizing E-ANPs by the procedure described in section 2.2.3, the
obtained mixture was centrifuged at 20,000 rpm for 10 min, and the supernatant was used to determine the weight of unloaded erlotinib by UV–vis spectrophotometry by measuring the absorbance values at 346 nm. UV–vis spectra were recorded by a UV-2100 Beijing Rayleigh Analytical Instrument Corporation spectrophotometer (China).

2.4.1. In vitro cytotoxicity evaluation

Cytotoxicity of E-ANP was evaluated against pancreatic adenocarcinoma ASPC-1 and PANC-1 cell lines using the MTT assay. Colorimetric assay was carried out after 48 and 72 h of the cell treatments. Cell culture was developed in a medium containing 10% FBS, 1% penicillin/streptomycin in RPMI 1640, at 37 °C in an atmosphere containing 5% CO2. For ASPC-1 cell line, 15,000 cells per well, and for PANC-1 cell line, 10,000 cells per well were seeded in the plates. After 24 h, the growth medium was exchanged by different concentrations of erlotinib (dissolved in DMSO, final DMSO concentration in the wells < 0.2% (v/v)), E-ANPs or blank nanoparticles (dried samples were dissolved in the growth medium) of 1.0, 12.5, 25, 50 and 100 μg mL−1. Three untreated wells were selected as controls.

2.4.2. Statistical analysis

The ultimate MTT outcomes were evinced as mean ± standard deviation. One-way-analysis of variance (ANOVA) accompanied with Tukey’s test was used for estimating significant differences between different concentrations of each group (erlotinib, E-ANPs and ANP) and control group for each cell line after 48 and 72 h. Also, for confirming significant differences between erlotinib and E-ANPs, cytotoxicity results for each cell line and for each group were obtained in 48 and 72 h time intervals. For comparing the results, two-way ANOVA accompanied with Tukey’s test was used. All statistical analyses were performed by SPSS 22 software and statistically significant results were verified by p < 0.05.

3. Results and discussion

3.1. Synthesis conditions of ANPs by the desolvation method

In this study, desolvation method was used to synthesize ANPs and E-ANPs. Acetone, DMSO and a mixture of these two solvents in a volume of four times of the BSA aqueous solution were added. Acetone is a well-known solvent for the preparation of ANPs (Reddy and Sailaja, 2014), and DMSO is an excellent solvent for erlotinib (Torres et al., 2016; Huether et al., 2005). It has been also reported that using the acetone/DMSO mixtures of lower ratios causes an enhancement in entrapment of amphotericin B in ANPs (Italia et al., 2011). Therefore, an acetone/DMSO mixture of 5:1 (v/v) ratio was employed. On the other hand, isoelectric point of albumin is 4.7 (Carter and Ho, 1994) and pKa of erlotinib is 5.4 (Lewis, 2010). Therefore, pH = 5.0 was selected for the aqueous solution of albumin to induce opposite charges in the drug and albumin. This will cause to a primary strong drug-albumin attraction leading to a higher drug loading on ANPs with post hydrophobic, hydrogen bonding and/or electrostatic attractions.

The loading procedure must be followed by cross-linking process to prevent drug release by unraveling of the nanoparticles. Chemical cross-linkers are known to have cell cytotoxicity and can interact with the drug (Yu et al., 2014). Accordingly, thermal cross-linking was done to improve the structural integrity of the nanoparticles. For this purposes, immediately after synthesis of the nanoparticles, cross-linking was performed at 55 °C (for acetone and the mixture of acetone/DMSO), or 100 °C (for DMSO) for 30 min. The selection of the cross-linking temperatures was based on the boiling points of the solvents, keeping in the mind that erlotinib is stable under thermal stress conditions (Mahajan et al., 2015). It has been reported that multiple stages are involved in the thermal cross-linking process (Verheul et al., 1998). Upon gradual increase in temperature, side chains movements of the protein are enhanced and unfolding of the protein structure overcome the attractive forces between the protein chains, resulting in changes in the secondary and tertiary structures of the protein (Grimaldo et al., 2015; Song et al.,...
2007). Then, unfolded monomers tangle together by forming reversible and irreversible cross links and make a stable gel structure (Militello et al., 2003; Paris et al., 2012). On the other hand, there is no covalent bond in E-ANPs between erlotinib and albumin (confirmed by FTIR, vide infra), and erlotinib bond into ANPs by hydrophobic attractions and hydrogen bond. Therefore, the drug can be release from E-ANPs although at longer times. Using theses procedures, the yield of the ANPs synthesis was 65.3 ± 4.1, 88.3 ± 10.9 and 96.7 ± 4.7% using acetone, DMSO and aceton/DMSO mixture, respectively. The obtained results were consistent with those reported by other investigators (Pignatello et al., 2009). In our studies, acetone/DMSO mixture was selected as the optimum solvent for desolvation.

3.2. Characterisation of the nanoparticles

Fig. 1 shows FESEM images of ANPs and E-ANPs with different magnifications. Based on low-magnified images, both types of the nanoparticles comprised planar thin particles with a thickness of 35 ± 7 and 39 ± 7 nm for ANPs and E-ANPs, respectively. The average area of ANPs and E-ANPs were also measured as ∼400 and 485 nm², respectively. At higher magnifications, the images indicate that the platelets actually comprised of small aggregated nanoparticles of less than 9 nm. In Fig. 2, TEM images of ANPs and E-ANPs with different magnifications are presented, manifesting cross-linked nanoparticles with a gel structure arising from thermal cross-linking process (Grimaldo et al., 2015). Microscopic images of ANPs and E-ANPs also revealed that drug loading did not lead to structural changes and morphology in ANPs.

PSA was used to investigate hydrodynamic size and dispersity of the nanoparticles before and after loading with erlotinib (Fig. 3). Hydrodynamic size of ANPs and E-ANPs were obtained as 10.3 and 14.0 nm, respectively. In addition, span values (as an indicator of dispersity of the nanoparticles) were obtained as 0.9 for both ANPs and E-ANPs indicating narrow size distributions. The higher value of the hydrodynamic size of E-ANPs is related to the presence of erlotinib and intermolecular forces between the drug and albumin. Comparing these results with FESEM and TEM images indicates aggregation of the nanoparticles during sample preparations. The size of nanoparticles is an important parameter in determining their interaction with blood components after injection and as passive targeting based on the enhanced permeability and retention effect. The nanoparticles should be small enough to pass through the blood vessels of tumor cells (Jiang et al., 2008). The size of E-ANPs is suitable for passive targeting of pancreatic cancer cells.

Fig. 4 shows FTIR spectra of ANPs and E-ANPs. In the spectrum of ANPs, there are different peaks at 692, 867, 1227, 1526, 1656, 2953 and 3274 cm⁻¹. The peak at 692 cm⁻¹ is associated to N–H wagging, peak at 867 cm⁻¹ is associated to stretching of C–C, peak at 1227 cm⁻¹ is correlated with stretching C–N band of amide III region that is associated to presence of β-sheet conformation, peak at 1526 cm⁻¹ is correlated with bending vibration of N–H band and stretching vibration of C–N band of amide II region, peak at 1656 cm⁻¹ is associated with C=O stretching vibration of amide I region, peak at 2953 cm⁻¹ indicates stretching vibration of C–H band, and peak at 3274 cm⁻¹ indicates stretching vibration of OH group (Rajith and Ravindran, 2014). After loading erlotinib, shifts in amides I, II and III bands occurred due to changes in the secondary structure of the protein, and broadening of the peak at 3274 cm⁻¹ confirmed erlotinib loading (Rajith and Ravindran, 2014).

3.3. Determination of erlotinib loading and encapsulation efficiency

There are two methods of incorporation and incubation for the drug loading on the albumin nanoparticles, and using the former one, higher drug loading and encapsulation efficiency were attained. Here, DL and EE were obtained as 27% and 44%, respectively. As for compare to another carriers for erlotinib, erlotinib loading into poly(lactic-co-glycolic acid) nanoparticles had a DL and EE of 21.7% and 22.8%, respectively (Marslin et al., 2009), and erlotinib loading on lipid nanoparticles had a DL of 1.4% (Vrignaud et al., 2012). In this regard, different functional groups in albumin nanoparticles and their astonishing ligand binding behavior makes this protein as one of the best carrier for drug delivery (Chen, 2010). Compared to lipid, gelatin, synthetic polymers and chitosan nanoparticles, ANPs result in more encapsulation efficiency (Jain and Banerjee, 2008; Rafati et al., 1997).

3.4. Cytotoxicity evaluation

Cytotoxicity evaluation of erlotinib (dissolved in DMSO and diluted with the culture medium), ANPs and E-ANPs were evaluated using the MTT assay by viable cells quantitative counting after 48 h and 72 h incubating cells with different concentrations of ANPs, erlotinib and E-ANP. The results are presented in Fig. 5. Cytotoxicity profile of ANPs was independent to concentration for both cell lines, and after 72 h reached 5% and 3% for ASPC-1 and PANC-1 cell lines respectively (p > 0.05). After 48 h, cytotoxicity caused by erlotinib and E-ANPs increased by increasing concentration (p < 0.05), and this effect was more evident in E-ANPs group in both cell lines. Gradual increase in the toxicity of erlotinib and E-ANPs continued to 72 h for both cell lines (p < 0.05). The E-ANPs toxicity increased to 54% and 52% for ASPC-1 and PANC-1 cell lines respectively, while toxicity of erlotinib reached to 34% and 30% for ASPC-1 and PANC-1 cell lines, respectively. The data demonstrated that ANPs result in higher cytotoxicity than free drug in ASPC-1 and PANC-1 cell lines. This was confirmed by statistically significant differences between 48 h data (p = 0.04 for ASPC-1 and p < 0.001 for PANC-1 cell lines) and between 72 h data (p < 0.001 for both the cell lines). Also, there was statistically significant differences between 48 h and 72 h data for erlotinib and E-ANPs toward both cell lines (p < 0.05). Therefore, ANPs afford higher cytotoxic effect than the free form of the drug. The values of IC₅₀ for erlotinib toward ASPC-1 and PANC-1 cell lines were obtained as 116.9 ± 0.1 and 148.1 ± 0.1 μg mL⁻¹, respectively. In Addition, the values of IC₅₀ for E-ANPs toward ASPC-1 and PANC-1 cell lines were obtained as 38.7 ± 0.1 and 49.0 ± 0.1 μg mL⁻¹, respectively. The results of cytotoxicity evaluation indicated that a lower dose of the drug can be used by the E-ANPs formulation. This could in turn reduce unwanted side effects of erlotinib and increase the efficacy of the drug in the E-ANPs formulation. This was more pronounced in the ASPC-1 cell line.

Compared to the previously reported nanocarriers for erlotinib (Marslin et al., 2009; Vrignaud et al., 2012; Mandal et al., 2016), ANPs could significantly improve drug loading. Lipid nanoparticles were used in the only reported injectable nanof ormulation of erlotinib, which provided ~40% cytotoxicity toward pancreatic cancer cell line after 96 h (Vrignaud et al., 2012). Considering the biocompatibility, our results demonstrate a superior formulation, indicative of promising in vivo bioavailability of E-ANPs.

4. Conclusion

ANPs were synthesized as a drug carrier by desolvation method with high a yield followed by thermal cross linking. The interaction of the carrier with erlotinib was confirmed by FTIR. MTT assay confirmed potential of ANPs to improve the drug toxic effect on the pancreatic cancer cells, while reducing the concentration of drug needed to kill a 50% of cancerous cells; this is an important achievement in drug delivery. Due to the small size, high loading and encapsulation capacities and acceptable cytotoxic activity, E-ANPs showed a promising future in treating pancreatic cancer. E-ANPs gave us a clue that in vivo estimation can be the next step to evaluate another aspect of this novel nanocarrier.
Acknowledgments
This paper has been extracted from the M. Noorani M.Sc. thesis supported by the Research Council of Shiraz University of Medical Sciences (10485). We also would like to thank Center for Development of Clinical Research of Nemazee hospital and Dr. Saeed Ghanbari for statistical analysis assistance.

References