Toxicology and Applied Pharmacology 275 (2014) 232–243

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

# Ligand-conjugated mesoporous silica nanorattles based on enzyme targeted prodrug delivery system for effective lung cancer therapy

Shenbagamoorthy Sundarraj [a,*], Ramar Thangam [a,b], Mohanan V. Sujitha [a], Karuppaiya Vimala [a], Soundarapandian Kannan [a,c,*]

[a] Proteomics and Molecular Cell Physiology Laboratory, Department of Zoology, Bharathiar University, Coimbatore 641 046, TN, India
[b] Department of Virology, King Institute of Preventive Medicine and Research, Guindy, Chennai 600 032, TN, India
[c] Department of Zoology, Periyar University, Salem 636 011, TN, India

ARTICLE INFO

*Article history:*
Received 17 August 2013
Revised 3 January 2014
Accepted 14 January 2014
Available online 25 January 2014

*Keywords:*
Silica nanorattles
Epidermal growth factor receptor (EGFR)
Pyrrolidine-2
cPLA$_2\alpha$ activity
Drug delivery system (DDS)
Lung cancer therapy

ABSTRACT

Epidermal growth factor receptor antibody (EGFRAb) conjugated silica nanorattles (SNs) were synthesized and used to develop receptor mediated endocytosis for targeted drug delivery strategies for cancer therapy. The present study determined that the rate of internalization of silica nanorattles was found to be high in lung cancer cells when compared with the normal lung cells. EGFRAb can specifically bind to EGFR, a receptor that is highly expressed in lung cancer cells, but is expressed at low levels in other normal cells. Furthermore, in vitro studies clearly substantiated that the cPLA$_2\alpha$ activity, arachidonic acid release and cell proliferation were considerably reduced by pyrrolidine-2 loaded EGFRAb-SN in H460 cells. The cytotoxicity, cell cycle arrest and apoptosis were significantly induced by the treatment of pyrrolidine-2 loaded EGFRAb-SN when compared with free pyrrolidine-2 and pyrrolidine-2 loaded SNs in human non-small cell lung cancer cells. An in vivo toxicity assessment showed that silica nanorattles and EGFRAb-SN-pyrrolidine-2 exhibited low systemic toxicity in healthy Balb/c mice. The EGFRAb-SN-pyrrolidine-2 showed a much better antitumor activity (38%) with enhanced tumor inhibition rate than the pyrrolidine-2 on the non-small cell lung carcinoma subcutaneous model. Thus, the present findings validated the low toxicity and high therapeutic potentials of EGFRAb-SN-pyrrolidine-2, which may provide a convincing evidence of the silica nanorattles as new potential carriers for targeted drug delivery systems.



## Introduction

Cancer is a leading cause of death in economically developed countries and the second leading cause of death in developing countries (WHO, 2004). Advances in our knowledge of molecular biology of cancer and pathways involved in malignant transformation are revolutionizing the approach to cancer treatment with a focus on targeted cancer therapy. In the present study we have mainly focused on cytosolic phospholipase A$_2\alpha$ (cPLA$_2\alpha$) because it has fascinated attention to target the controlling arachidonic acid and eicosanoid related expressions in inflammation and cancer. Cytosolic phospholipase A$_2\alpha$ has been proposed to play an important role in cell cycle regulation. The unique functions of cPLA$_2\alpha$ are emphasized by the earlier findings endorsed that most of the tumor cells are found to produce elevated levels of eicosanoids, resulting in increased tumor growth, invasiveness and upgradation of metastatic activity of the tumor cells (Lagorce-Pages et al., 2004; Laye and Gill, 2003; Reich and Martin, 1996). Accordingly, cPLA$_2\alpha$ is remarkably found to be over-expressed in a range of human tumors, including non-small cell lung cancer (Heasley et al., 1997; Sundarraj and Kannan, 2010). Furthermore, PLA$_2$ inhibitors are able to suppress proliferation of tumor cells by inducing apoptosis (Korystov et al., 1998). In addition to the above factors, the 15S-hydroxyeicosatetraenoic acid has been shown to decrease the percentage of cells in S phase. This observation is concomitant with an increase in the numbers of cells in G0/G1 phase in prostate carcinoma cells (Shappell et al., 2001). These data may be acquainted with the pivotal role of cPLA$_2\alpha$ inhibitors as therapeutics for cancer treatment.

The silica nanoparticles are believed to be non-toxic and are currently used in several industrial and biomedical applications including food, cosmetics and drug delivery carrier systems. The drug delivery system will not only be an important therapeutic and pharmacological application, but also be of great interest in medical imaging and diagnosis. The EGFR and its ligands are important in normal and neoplastic epithelial cell growths. EGFR has been recognized as a potential cancer biomarker since the activation of EGFR is associated with the tumorigenic mechanisms such as autonomous cell growth, invasion, angiogenesis, and metastasis (Grünwald and Hidalgo, 2002; Ritter and Arteaga, 2003). The epidermal growth factor receptor is used as the target ligand (Rusch et al., 1996), and it can be employed for more specific recognition and interaction with cancer cells because EGFR is overexpressed in human

* Corresponding authors. Fax: +91 4272345124.
*E-mail addresses:* sundarrajbu09@gmail.com (S. Sundarraj), skperiyaruniv@gmail.com (S. Kannan).

0041-008X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2014.01.012

tumors, especially on non-small cell lung cancer (Fontanini et al., 1998; Fujino et al., 1996; Rusch et al., 1993, 1997; Salomon et al., 1995; Volm et al., 1998). Xu and Amiji (2012) recently reported the therapeutic gene delivery through EGFRAb conjugated gelatin nanoparticle to target human pancreatic cancer. Slowing et al. (2006) have confirmed that surface-functionalized 100 nm size silica particles are effectively internalized by HeLa cells regardless of the surface composition of the particles. Li et al. (2010, 2011) have proved that the SN-PEG–Dtxl has low systemic toxicity and high therapeutic efficacy, which provides convincing evidence for the silica nanorattles as a promising candidate for a drug delivery system. The recent study witnessed that the silica nanoparticles have internalized in Balb/3T3 mouse fibroblasts as it did not trigger any cytotoxic or genotoxic effect and did not induce a morphological transformation (Uboldi et al., 2012). To our knowledge, no reports are available with emphasis on the use of EGFRAb conjugated silica nanorattles to delivery drug of interest.

Recently a class of pyrrolidine containing compounds has been reported to act as potent inhibitors of $cPLA_2\alpha$ in vitro and to block arachidonate release in calcium ionophore-stimulated human acute monocytic leukemia THP-1 cells (Seno et al., 2000) and CHO cells (Ghomashchi et al., 2001). They block the production of prostaglandins E2 and leukotriene (Ghosh et al., 2007; Seno et al., 2000).

In this study, we aimed to develop as a novel targeted drug delivery system for the $cPLA_2\alpha$ inhibitor pyrrolidine-2 against non-small cell lung carcinoma cells. We further investigated the Silica nanorattles encapsulated pyrrolidine-2 reduced systemic toxicity and also enhanced therapeutic efficacy in tumor bearing Balb/c mice.

## Materials and methods

### *Synthesis and characterization of EGFRAb-SN-pyrrolidine-2*

Silica nanorattles were fabricated according to a procedure described by Chen et al. (2010) with a slight modification. In a typical synthesis, 0.5 g of tetraethyl orthosilicate (TEOS) (Sigma-Aldrich, India) was mixed under inert atmosphere and added to an ethanol solution containing the structure directing agent sodium do-decyl sulfate (SDS, 99%). The resulting synthesis mixture had a molar ratio of 0.4 TEOS:0.5 SDS:1439 EtOH:2560 $H_2O$. The solution was stirred 24 h at room temperature, and thereafter aged for 6 h at static conditions. The precipitate was filtered off, washed with ethanol, and dried at 60 °C in vacuo for 48 h. The SDS was subsequently removed by ultrasonication in ethanol three times (Moller et al., 2007). Morphology and structure of the resulting SNs were observed with a Technai $G^2$ Transmission Electron Microscope (TEM); the size of nanorattles was determined by Dynamic Light Scattering (DLS) (Malvern ZetaSizer Nano); Fourier transform infrared (FTIR) analysis was carried out using KBr disks in the region of 4000–400 $cm^{-1}$ (Shimadzu, IR Affinity-1, Japan).

Polyethylenimine (PEI) was grown onto the silica nanorattles by hyperactive surface polymerization according to a modified reaction described by Rosenholm et al. (2009). Thus, before polyethylenimine alteration, the surfactant extracted nanorattles were carefully vacuum-dried and subsequently subject to argon atmosphere. The nanorattles (0.125 g) were immersed in toluene under inert atmosphere. Catalytic amounts of acetic acid and 45 μL of aziridine were added, and the reaction mixture was stirred overnight at 75 °C. After the reaction, the nanorattles were filtered off, washed with copious amounts of toluene, and vacuum-dried for at least 24 h.

The nanorattles plain and PEI coated, were labeled with FITC (fluorescein isothiocyanate, 98%) by suspending 25 mg of nanorattles in carbonate buffer (pH 9.0), to which 250 μL of an ethanolic FITC solution (1 mg/mL) was added and stirred for 30 min. After this, the PEI-functionalized nanorattles were collected by centrifugation, washed with de-ionized water repeatedly, and subsequently suspended in MES buffer (pH 5.0). Anti EGFR was purchased from Santa Cruz Biotechnology (sc-1724, Santa Cruz, CA). 50 μg of EGFRAb was sonicated in MES, to which 20 μL of a 1 μL/mL EDC solution (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) was added to activate the carboxylic acid groups of EGFRAb. This solution was rapidly added to the nanorattle suspension, after which 25 μL of (1 mg/mL in MES) NHS (*N*-hydroxysuccinimide) solutions was mixed with the suspension. The suspension was disturbed for over-night and washed with copious amounts of de-ionized water and ethanol, dried in vacuo and stored at 4 °C. The ζ-potential was measured as a function of pH by titrating with 0.1 or 0.5 M HCl and NaOH at 25 °C. The detailed schematic synthesis of mesoporous silica nanorattles with a functional core, hollow, and mesoporous structure was explained in Scheme 1.

Pyrrolidine-2 was synthesized as described previously by Seno et al. (1998). To load pyrrolidine-2 into the pores of the nanorattles, SN and EGFRAb-SN were dispersed in a solution of pyrrolidine-2 (10 mg/mL in ethanol) and stirred for 24 h, followed by centrifugation with extensive washing of PBS to obtain the pyrrolidine-2 loaded SN and EGFRAb-SN spheres, which were used for subsequent in vitro and in vivo studies.

### *Cell culture and culture conditions*

The human lung epithelial cell line L-132 and human large cell lung cancer H460 cells were purchased from the National Centre for Cell Sciences (Pune, India). L-132 cells were maintained in DMEM with 10% fetal bovine serum and H460 cells were cultured in RPMI 1640 (Hi-Media, Inc.) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Both the cells were maintained at 37 °C with 5% $CO_2$ in a humidified incubator.

### *Quantification of pyrrolidine-2-loaded EGFRAb-SN uptake by L-132 and H460 cells*

To compare the uptake rate of pyrrolidine-2-loaded EGFRAb-SN, L-132 and H460 cells were plated 24 h prior to starting the experiment in chamber slides at a density of $5 \times 10^3$ cells/$cm^2$. After incubation with 10 μg/mL FITC or RITC labeled sphere shaped EGFRAb-SN-pyrrolidine-2 for 4 h, the L-132 and H460 cells were washed twice with PBS and incubated with 0.1% Triton X-100 plus 1% BSA in PBS at room temperature for 15 min. The slides were washed twice with PBS and then examined with a LEICA-Sp5 confocal microscope.

L-132 and H460 cells were seeded $5 \times 10^5$ cells/well in six-well plates and allowed to adhere for 24 h. To determine the quantity of fluorescence silica nanorattles (FSNs) taken up by L-132 and H460 cells, the cells were incubated with FSN in the specific medium for 4 h and the cells were washed thrice with PBS, and finally harvested through trypsinization. After the cell pellet was centrifuged, it was re-washed once again and re-suspended with PBS containing 0.1% FBS. The cellular uptake of FSN was quantitatively determined by Fluorescence Activated Cell Sorting (FACS) (Beckman Coulter Inc., CA).

### *Cell viability assay*

The cytotoxicity of SN-PEI, pyrrolidine-2, pyrrolidine-2-loaded SN, and pyrrolidine-2-loaded EGFRAb-SN was evaluated by MTT cell viability assay (Mosman, 1983; Sundarraj et al., 2012a). Pyrrolidine-2 was dissolved using DMSO, and the final concentration of DMSO in culture media was less than 0.5%. For 24 h detection, the cells were seeded at a density of 8000 cells/well on 96-well plates (Nunc, USA), and for 72 h detection, the cell density was 2000 cells/well. After incubating the cells with SN, pyrrolidine-2, SN-pyrrolidine-2, and EGFRAb-SN-pyrrolidine-2 for 24 h, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Hi-Media, India) (final concentration of 5 mg/mL) was added to each well. After 4 h of incubation at 37 °C, colorimetric measurements were performed at 570 nm on a microtiter plate reader (Thermo Electron Corporation, USA). Data were