Journal of Drug Delivery Science and Technology 31 (2016) 167–175

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

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

Research paper

Chemo-directed specific targeting of nanoparticle-doxorubicin complexes to tumor in animal model

S. Sreeja [a, b], Cherupally Krishnan Krishnan Nair [a, b, *]

[a] MACFAST, Thiruvalla, Kerala 689101, India
[b] Pushpagiri Institute of Medical Sciences and Research Centre, Thiruvalla, Kerala 689101, India

ARTICLE INFO

Article history:
Received 19 October 2015
Received in revised form
19 December 2015
Accepted 6 January 2016
Available online 11 January 2016

Keywords:
Doxorubicin
Iron oxide nanoparticles
Sanazole
Targeted delivery
Tumor hypoxia

ABSTRACT

The therapeutic potential of antineoplastic drugs is often limited by systemic toxicities which can be eliminated by targeted drug delivery to tumor sites. In the present study the chemotherapeutic doxorubicin (DOX), which causes cardiotoxicity, was complexed with iron oxide nanoparticles (NP) together with a hypoxic cell radiosensitizer Sanazole (SAN) for specific targeting to tumor sites. NP-DOX-SAN complexes increased apoptosis in tumor under *in vitro* conditions as characterized by apoptotic morphology, typical comet tail formation and the expression of specific genes. The tumor volume was reduced significantly in tumor- bearing mice administered with NP-DOX-SAN complexes compared to untreated control. Morphological studies of tumor and normal tissues including heart, liver as well as kidney, and also the data on serum biochemical parameters revealed the specificity of the action of the complexes on tumor. In tumor tissues, studies by quantitative Real Time- Polymerase Chain Reaction revealed significant down regulation in the transcription of the gene hypoxia- inducible fator-1α (*hif- 1α*), an adaptive response initiated by hypoxic solid tumor and up regulation of *bax* and *caspases*. Thus the present study reveals the potential application of SAN to direct and specifically target nano- complexes of antineoplastic drug to the cells of solid tumors, to cause tumor regression and reduce unwanted toxicities.

1. Introduction

Doxorubicin (DOX), an anthracycline antibiotic, is widely used as a chemotherapeutic drug to treat various types of cancers including multiple myeloma, some types of leukaemia as well as breast cancer, stomach cancer, ovarian cancer etc [1]. DOX is effective against a large number of malignancies by intercalating DNA [2]. However, its therapeutic efficacy is limited by systemic toxicity particularly to the heart. DOX induced cardiotoxicity might be due to the generation of oxygen free radicals by several enzymes of mitochondria such as xanthine oxidase, cytochrome P- 450 and NADH dehydrogenase [3,4]. Also DOX causes the depletion of GATA-4, one of the anti-apoptotic factors responsible for the survival of adult heart which in turn results in programmed cell death of cardiomyocytes [5].

The lack of specificity, the major limitation of conventional chemotherapy, can be overcome by the targeted delivery of drugs to tumor site. The nanoparticle- based drug delivery has been gained a lot of significance in this contest. Iron oxide nanoparticles (NP) have been widely used in diagnosis and therapy because of its biocompatibility and magnetic property. The externally applied magnetic field can be used to target these nanoparticles to the tumor site [6]. An isoquinoline alkaloid, Berberine is targeted to tumor site with the help of an applied external magnetic field and showed significant regression in tumor [7] [8]. targeted methotrexate, a chemotherapeutic agent, specifically to tumor with the help of iron oxide nanoparticles surface- modified with citrate, thereby protected normal tissues [8]. D-aminoacid oxidase- bound iron oxide nanoparticles were orally administered to tumor-bearing animals and specifically targeted to tumor by an externally applied magnetic field. When D-aminoacid was administered, it underwent oxidative deamination and producing alpha-keto acid, ammonia and H_2O_2 and thereby generating free radicals to damage tumor cells [9]. The efficacy of the drug, DOX has been increased by complexing with magnetic iron oxide nanoparticles and targeting

* Corresponding author. Present address: St. Gregorios Dental College and Research Centre, Kothamangalam, 686681, Kerala, India.
E-mail addresses: sreebio14@gmail.com (S. Sreeja), ckknair@yahoo.com (C.K. Krishnan Nair).

http://dx.doi.org/10.1016/j.jddst.2016.01.003
1773-2247/

them to tumor with the aid of an external applied magnetic field [6]. The application of an electro-magnetic field around tumor tissues, after the administration of magnetic nanoparticles- DOX complexes, enhances cytotoxicity in the tumor by the generation of hyperthermia [10].

Tumor hypoxia is the major factor contributing to resistance for radiotherapy and chemotherapy [11,12]. Although the cells in hypoxic conditions are more prone to cell death due to the deficiency of oxygen and nutrients, tumor itself make an effort to acclimatize O_2 and nutrient deprived state to survive in their hostile environment. This is carried out by the activation of several transcription factors especially hypoxia- inducible factor- 1α (*hif-1α*) to induce angiogenesis, cell proliferation and glycolysis [13]. The treatment of tumor with chemotherapeutic drug including DOX would kill tumor cells close to blood vessels, resulting in improvement of nutrient supply to the previously hypoxic cells, leading to survival and the recurrence of tumor. Several compounds are shown to sensitize cells in hypoxic environment to chemotherapy and radiation [14,15]. Nitric oxide (NO) donors are found to be effective in regulating hypoxia by inhibiting the expression of *hif-1α* and its target genes, thereby enhancing tumor therapy [16] The nitrotriazole compound, Sanazole (SAN), is a potent hypoxic cell radiosensitizer which has completed phase III clinical trials [15]. This compound has a peculiar property of getting accumulated in hypoxic tumor tissues [17]. Silver nanoparticles when complexed with DOX and SAN were more effective in causing tumor regression in murine system [18]. Toxicity of silver nanoparticles could be a major limitation in translating this for therapeutic application. In the present study biocompatible and nontoxic iron oxide nanoparticles were complexed with the hypoxia seeking SAN and antineoplastic DOX, and these complexes were examined for the therapeutic efficiency under *in vitro* and *in vivo* conditions.

2. Materials and methods

2.1. Chemicals and reagents

Ferric and ferrous chlorides (PubChem CID: 24380 and 24458) were purchased from Merk, India. Chemicals for RNA isolation such as Guanidium thiocyanate, Isoamyl alcohol (PubChem CID: 31260), Isopropanol, Sodium acetate (PubChem CID: 517045) and Ethyl alcohol (PubChem CID: 702) were purchased from Sigma Aldrich, India. Doxorubicin is purchased from Fresenius Kabi Oncology Limited, India and Sanazole was obtained as a gift from Dr. V.T.Kagiya, Health Research Foundation, Kyoto, Japan. All other chemicals were obtained from reputed national manufactures-Otto Chemie Pvt. Ltd. and Himedia Pvt. Laboratories Ltd.

2.2. Animals

Female Swiss albino mice weighing 23–25 g were purchased from the Small Animal Breeding Section (SABS), Government Veterinary College, Mannuthy, Thrissur, Kerala [7]. They were kept under standard conditions of temperature and humidity in the Centre's Animal House Facility. The animals were provided with standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad-libitum*. All animal experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly adhering to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India.

2.3. Preparation and characterization of nanoparticles

Fe_3O_4 nanoparticles were prepared by co-precipitation method. In the solution of metal chlorides- ferric chloride and ferrous chloride- in 2:1 ratio, ammonia solution (28%) was added under vigorous stirring until the pH reaches 11–12. The temperature was maintained at 80 °C. Polyvinyl pyrrolidone was added to the slurry and cooled to room temperature. The surfaces of the nanoparticles were modified in order to make them hydrophilic. The prepared NPs were characterized by FTIR and X-ray diffraction as described earlier [7]. NPs were complexed with DOX and SAN in the ratio of 10:1:1 by ultra-sonication. FT-IR (Nicolet iS10, thermoscientific) and TEM analyses of these samples were also performed.

2.4. In vitro studies

2.4.1. Cells

All experiments under *in vitro* condition were performed in mouse cancer cell line- Dalton's Lymphoma Ascites (DLA) cells. 1×10^6 cells/ml were suspended in DMEM with 12% foetal bovine serum and used for all *in vitro* analysis.

2.4.2. Experimental design

1: Control: untreated DLA cells; 2: NP: Cells treated with NPs alone (500 μg/ml); 3: DOX: cells treated with DOX (50 μg/ml, 91.99 μM); 4: SAN: cells treated with SAN (50 μg/ml, 218.15 μM); 5: NP-DOX: cells treated with NP-DOX complexes (500 μg/ml NP and 91.99 μM DOX); 6. NP- SAN: cells treated with NP- SAN complexes (500 μg/ml NP and 218.15 μM SAN) and 7: NP-DOX- SAN: cells treated with NP-DOX- SAN complexes (500 μg/ml NP, 91.99 μM DOX and 218.15 μM SAN in 10:1:1 ratio).

2.4.3. Apoptosis by dual staining method

1×10^6 cells/ml were incubated with DOX and its complexes with NPs for 4 h and stained with propidium iodide and acridine orange (20 μg/ml). Membrane blebbing as one of the characteristics of apoptosis were viewed under fluorescent microscope and the percentage of apoptotic cells were calculated by formula [19] [20],

Percentage of apoptosis cells = Number of apoptotic cells × 100 / Total number of cells

2.4.4. Alkaline single cell gel electrophoresis

The cellular DNA damages were assessed by alkaline single cell gel electrophoresis or Comet assay. The cells following various treatments were immobilized with 1% low melting agarose on 1% normal agarose coated slides and kept in lysis buffer for 90min. Finally, electrophoresis was carried out in alkaline buffer for 20–30min (25 V, 300 mA). The cells in the slides were stained with propidium iodide (25 μg/ml) and photographed under fluorescent microscope (40× magnification) [19,21–23]. CASP was used to calculate comet parameters such as tail length, % DNA in tail, tail moment and olive tail moment [24].

2.4.5. Gene expression studies

RNA isolation was performed in cells after various treatments by acid guanidium thiocyanate method [25]. 1 *μg* RNA was used to synthesize cDNA in 20 μl reaction. Polymerase chain reaction (reverse transcriptase) was used to amplify the genes-*gapdh* [GenBank: XR405643.1], *bax* [GenBank: NM007527.3], *bcl2* [GenBank: BC068988.1] and *p53* [GenBank: AB020317.1] (please see supplementary data for primer sequences).