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Bone metastasis targeting: A novel approach to reach bone using Zoledronate anchored PLGA nanoparticle as carrier system loaded with Docetaxel

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ABSTRACT

In spite of good research in drug delivery, bone targeting remains largely unexplored. Even some of the bone diseases are seldom cured just because of poor distribution of drug at the bone site. Zoledronate (ZOL) having strong affinity towards bone and its utility in bone metastasis management makes it perfect ligand for bone targeting. Recent studies revealed that ZOL in combination with docetaxel showed significant synergism in the management of bone metastasis. From the results, it is clear that ZOL-conjugated PLGA nanoparticles (NPs) showed more cellular uptake than pegylated PLGA NPs with change in cellular uptake route. *In vitro* studies on MCF-7 and BO2 cell line revealed that ZOL anchored PLGA-PEG NPs showed enhanced cell cytotoxicity, increase in cell cycle arrest and more apoptotic activity. PLGA-PEG-ZOL NPs found to block mevalonate pathway and increase accumulation of apoptotic metabolites such as ApppI. *In vivo* animal studies using technetium-99 m radiolabeling showed prolong blood circulation half-life, reduced liver uptake and significantly higher retention of ZOL tagged NPs at the bone site with enhanced tumor retention. Here, we can conclude that the targeting ability of ZOL enhanced by strong affinity to bone, enhanced endocytosis of ZOL anchored PLGA-PEG NPs.

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1. Introduction

Metastasis is the fatal stage in all cancers which remains incurable in spite of in depth research. Bone is the most prone site for metastasis because of its physiological environment which supports tumor inoculation and progress. Some common human cancers such as prostate cancer, breast cancer, renal carcinoma, thyroid cancer and multiple myelomas have high vulnerability (> 50%) to get metastasized at bone site in advanced stage [1].

Bisphosphonates (BPs) are group of drugs which reduce bone erosion and restore bone density in osteoporosis and bone related diseases. Many clinical trials have already proved that BPs restore bone homeostasis and reduce risk of osteoporotic complication with good tolerance and safety [2]. BPs are found to induce apoptosis of osteoclasts which are responsible for bone erosion [3]. Additionally, BPs possess strong affinity towards bone mass as they distribute faster and accumulate as high as 100 times in bone, in comparison to C_{max} even after 6 months post injection [4]. Because of its strong selectivity and affinity, BPs are now widely used as a bone imaging agents in conjugation with radiopharmaceuticals. Because of the unique features of BPs, many attempts have been made to conjugate bone therapeutic agents in order to get osteotropicity. These include estradiol [5], prostaglandin E2 [6], Src (protein tyrosine kinase pp60c-Src) homology 2 inhibitors [7], diclofenac [8], fluroquinolone, cisplatin, melphalan, methotrexate [9], radiopharmaceuticals like technetium (^{99m}Tc) hydroxyethylidene disphosphonate, ^{99m}Tc methylene disphosphonate, ^{99m}Tc hydroxymethylene disphosphonate [10] and samarium (153Sm) lexidronam (QuadrametR) [11]. Peptides and proteins have also been proposed by Gittens et al. for conjugation with BPs to induce bone specificity [12]. Hengst et al. [13] has suggested use of CHOL-TOE-BP as targeting moiety for liposomal drug delivery to bone. BP conjugates were also used as delivery anchor for treatment of osteoporosis [6]. Liu et al. [14] demonstrated use of alendronate-β-cyclodextrin conjugate as a bone anabolic agent.

Along with osteotropicity and utility as bone homeostasis enhancer, Zoledronic acid (ZOL), a nitrogen containing BP recently found to have

Abbreviations: BPs, Bisphosphonates; ZOL, Zoledronic acid; DTX, Docetaxel; ApppI, 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester; IPP, Isopentenyl pyrophosphate; PLGA, Poly(lactide-Glycolide) acid; NPs, Nanoparticles; ^{99m}Tc, Technetium-99 m; FPP synthase, Farnesyl pyrophosphate synthase; PEG, Polyethylene glycol.

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anticancer activity. ZOL is known to have multiple targets and activities such as induction of apoptosis, anti-angiogenesis, reduction of vascular endothelial growth factor level and circular occult tumor cells in blood, anti-adhesion activity to tumor and osteoclasts which increase its potential as an anticancer drug [15]. ZOL inhibits farnesyl pyrophosphate synthase (FPP synthase), an enzyme in the mevalonate pathway. Inhibition of this enzyme prevents the formation of some important signaling molecules and modification of proteins, which leads to the loss of osteoclast/cancer cell function and induce apoptotic cell death [16]. A mechanism has been earlier established that ZOL induces the formation of a novel ATP analog, triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester (Apppl) by inhibiting mevalonate pathway, which causes apoptotic cell death [17]. There is a clear correlation between the ability of ZOL to inhibit FPP synthase and protein prenylation *in vitro* [18], and its ability as anti-osteoclast and anticancer *in vivo*, by induction of IPP and Apppl formation [19,20].

Combination therapy of ZOL and Docetaxel (DTX) are already proven to have synergistic effects. Karabulut et al. [21] have explored the possible synergistic cytotoxic effects of combination therapy of DTX and ZOL in hormone-refractory PC-3 and DU-145 prostate cancer cell lines. Phase I clinical studies on prostate cancer cells reveals that the ZOL in combination with DTX shows synergistic cytotoxic effect when given sequentially [22].

Bone accounts for more than 50% of body mass, but still shares only 7% of cardiac output. Moreover, major part of the bone remains less perfused and isolated. Thus, chemotherapeutic drug never achieve desired concentration for tumor suppression. Brubaker et al. [23] examined the effects of DTX and ZOL on LuCaP 23.1 prostate cancer xenograft model and showed that ZOL decreased the proliferation of LuCaP 23.1 in the bone environment *in vivo*, while DTX alone fails to inhibit growth of tumor with the concentration which was effective for subcutaneous tumor. The contradictory *in vitro* and *in vivo* results, explain the inability of DTX to gain desired concentration at the tumor site. The proposed nano-scale targeting system can potentially be applied to localize DTX and ZOL to obtain high concentration at bone metastasis site to enhance therapeutic outcomes. Nanocarriers are known for alteration of body distribution a selective retention at tumor site due to Enhanced Permeation and Retention effect (EPR). The higher localized concentration explains higher efficacy and low systemic toxicity of nanocarriers. Poly(lactide-Glycolide) acid (PLGA) has been approved for human use by US FDA because of its biocompatibility and biodegradability [24,25]. Here, PLGA nanoparticles (NPs) were used as a nanocarrier based drug delivery system.

2. Materials and methods

2.1. Materials

PLGA 502H, (lactide/glycolide ratio 50:50, inherent viscosity 0.22 dl/g) was obtained as a gift sample from Boehringer Ingelheim, Germany. DTX and ZOL were obtained as a gift sample from Sun Pharma Advanced Research Centre (SPARC), Vadodara, India. PEG bisamine (Mol wt. 3350 Da), CDI, NHS, DCC and 6-coumarin were purchased from Sigma Aldrich, India. Poloxamer 188 was kindly gifted by BASF, India. HCl, NaOH, SDS, DMF and DMSO were obtained from S. D. Fine Chemicals, India. MCF-7 cell line was obtained from ECACC (Salisbury, UK), RAW264 cell line was obtained from ATCC (USA) and BO2 cell line was gifted from INSERM, France. DMEM 21885, DMEM 31885, FBS, PBS, Penicillin, streptomycin, tripsin, EDTA, HBSS were purchased from GIBCO, Finland. RPMI 1640 was purchased from Lonza, Finland. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Propidium Iodide (PI) were purchased from Sigma Chemicals, Finland. All well plates and tissue culture flask were purchased from NUNC, Finland. Hoechst 33342 and RNAse A was purchased from Invitrogen, Finland. Annexin V-FITC and binding buffer were purchased from Biolegend. Stannous chloride dihydrate ($SnCl_2.2H_2O$) was purchased from Sigma Chemical Co. (St. Louis, MO), sodium pertechnetate, separated from molybdenom-99 (99 m) by solvent extraction method, was provided by Regional Center for Radiopharmaceutical Division, (Northern Region) Board of Radiation and Isotope Technology (BRIT, Delhi, India).

2.2. Preparation of Conjugates and Nanoparticles

2.2.1. Synthesis and characterization of PLGA-PEG and PLGA-PEG-ZOL conjugate

PLGA-PEG conjugate was synthesized using N-Hydroxysuccinimide (NHS) and Dicyclohexylcarbodiimide (DCC) as an activator [26]. Conjugation of ZOL with PLGA-PEG-NH_2 was performed using N,N'-Carbonyldiimidazole (CDI) as a conjugation linker [27]. In brief, ZOL (100 mg) was dissolved in distilled DMF with triethylamine (TEA). CDI (90 mg, moisture free) was added to that solution in tightly closed vessel under nitrogen blanket for 24 h at 60 °C. TEA was then evaporated and precipitates were washed twice with acetonitrile. PLGA-PEG (1 g) and activated ZOL (22.6 mg) were dissolved in DMSO with TEA in tightly closed vessel under nitrogen blanket and allowed to react for 12 h. The reaction mixture was dialyzed against distilled water to remove excess activated ZOL.

2.2.2. Preparation of PLGA NPs

DTX loaded PLGA NPs were prepared by solvent diffusion (nanoprecipitation) technique as described by Fessi et al. [28]. The organic phase (acetone) containing DTX (7.5 mg) and PLGA (100 mg) were slowly injected (0.5 ml/min) into 20 ml of aqueous phase containing Poloxamer 188 (0.5% w/v) as stabilizer on a magnetic stirrer (Remi Equipments, Mumbai). NPs were recovered by centrifugation at 25,000 rpm for 30 min (Sigma 3 K30, Germany) and lyophilized for 48 h (Heto Drywinner, Denmark) using trehalose as a cryoprotectant. PLGA-PEG and PLGA-PEG-ZOL NPs were prepared in same way by replacing PLGA with PLGA-PEG and PLGA-PEG-ZOL in a fixed molar ratio ranging from 10-30%. 6-coumarin loaded NPs were prepared in the similar way with addition of 6-coumarin instead of DTX.

2.2.3. Salt and Serum Induced Aggregation and In vitro bone binding affinity Studies

Colloidal stability of NPs was tested using salts such as sodium sulphate (1 M), calcium chloride (30 mM) and 1% bovine serum (FBS) which are well known as aggregation inducer using our earlier reported method by Chaudhari et al. [29]. ZOL solution and PLGA-PEG-ZOL NPs were evaluated for *in vitro* bone binding affinity. Both samples having same amount of ZOL were diluted with 100 ml PBS and kept on slow stirring along with human bone powder and binding was estimated at different time interval.

2.2.4. Phagocytosis uptake, Quantitative cell uptake, NPs uptake route characterization and Confocal microscopy

In vitro phagocytic uptake of PEGylated PLGA NPs using RAW264 cells were carried out using our earlier reported method by Chaudhari et al. [29]. Cell uptake was carried out using MCF-7 and BO2 cells (a subclone of MDA-MB-231, derived after six *in vivo* passages in nude mice and characterized by its unique morphology and affinity resembling to bone metastasis [30]). 6-coumarin loaded PLGA-PEG20 NPs and PLGA-PEG-ZOL NPs were incubated and the uptakes were tested using FACS (Canto-II, BD) analysis and confocal analysis (Carl Zeiss, Axiovert 135 M) was performed. The cell uptake route characterization was performed on BO2 cells for 6-coumarin loaded PLGA-PEG20 NPs and PLGA-PEG-ZOL NPs using various cell uptake route inhibitors as listed in Electronic Supplimentary Material (ESM) Table T1. After treatment with specific inhibitor, each well was tested for cell uptake of 6-coumarin loaded PLGA-PEG20 NPs and PLGA-PEG-ZOL NPs using FACS (Canto-II, BD).

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