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# Hepatic stellate cell-targeted imatinib nanomedicine *versus* conventional imatinib: A novel strategy with potent efficacy in experimental liver fibrosis

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ABSTRACT

Liver fibrosis is a global health problem without approved treatment. Imatinib inhibits two key profibrotic pathways; platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) and thus can be used to treat liver fibrosis. However, conventional imatinib therapy is hampered by low concentration at target tissue and increased toxicity to other tissues especially heart, lung and liver. Since hepatic stellate cells (HSCs) are the main contributors to liver fibrosis pathogenesis and sole hepatic vitamin A ($V_A$) storage cells, they can be actively targeted by coupling liposomes to $V_A$. In this study, novel $V_A$-coupled imatinib-loaded liposomes (ILC) were prepared and optimized regarding $V_A$-coupling efficiency, imatinib entrapment efficiency, and particle size. Preferential accumulation of the selected formula in liver was proved by tracing intraperitoneally (i.p.)-injected $V_A$-coupled liposomes loaded with Nile Red (LCNR) to rats with $CCl_4$-induced liver fibrosis using live animal imaging. Co-localization of LCNR with immunofluorescently-labeled PDGFR-β in frozen liver tissue sections confirmed HSCs targeting. ILC bio-distribution, following single i.p. injection, revealed 13.5 folds higher hepatic accumulation than conventional imatinib in addition to limited bio-distribution to other organs including heart and lung reflecting diminished adverse effects. ILC therapy resulted in a potent inhibition of phosphorylated PDGFR-β expression when compared to conventional imatinib. Subsequently, there was a statistically significant improvement in liver function tests and reversal of hepatotoxicity along with liver fibrosis. Anti-fibrotic effect was evident from histopathologic Ishak score reduction as well as normalization of the level of profibrotic mediators (hydroxyproline, TGF-B and matrix metalloproteinase-2). Thus, HSC-targeted imatinib therapy shows outstanding anti-fibrotic effects with reduced cytotoxicity compared to conventional imatinib. It can represent a promising novel approach for liver fibrosis treatment.

## 1. Introduction

Liver fibrosis is a serious health problem with high worldwide dominance and poor prognosis [1]. Clinical consequences of unopposed fibrosis are: cirrhosis, and hepatocellular carcinoma development, with a further increase in the relative mortality rate [2]. So far, tested anti-fibrotic drugs were not approved for liver fibrosis treatment due to lack of tissue specificity and low drug concentration at the target organ [3]. Liver transplantation, an extremely costly procedure, is the only efficient solution for end-stage liver disease patients [4]. Intensive research about cells and fibrogenic mediators governing this hepatic scar formation over the past decades shed light on two universally accepted facts. The first is the potential liver regeneration capacity and the reversibility of liver fibrosis, while the second is that hepatic stellate cells (HSCs) are the main regulators for liver fibrosis pathogenesis [5]. These facts paved the way for the idea that HSCs targeted-therapy may provide a cure for this reversible morbid condition.

In quiescent state, HSCs are the main reservoir of body's retinol (vitamin A ($V_A$)) as they store 80% of total body retinoids in lipid droplets in their cytoplasm [6]. During hepatic injury, these $V_A$-storing cells become activated; shed their retinoid and lipid droplets and transdifferentiate into highly proliferative, contractile and matrix-producing myofibroblasts [7]. HSCs activation and fibrogenesis are orchestrated by a complex network of cytokine-mediated signaling

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pathways including transforming growth factor-beta (TGF-β) and platelet-derived growth factor-beta (PDGF-β) [8]. TGF-β is the strongest known inducer of hepatic fibrogenesis [9]. PDGF-β and its receptor (PDGFR-β), a member of the type III class of tyrosine kinase receptors, are markedly over-expressed in fibrous tissues. They are key regulators of connective tissue formation, and their activity increase with the extent of liver fibrosis [10]. Moreover, PDGF-β is the strongest mitogen to HSCs among all polypeptide growth factors [11]. Accordingly, neutralization of TGF-β and PDGF-β activity would be a potentially useful approach for liver fibrosis treatment.

Imatinib, a receptor tyrosine kinase inhibitor, is extensively used for the treatment of chronic myelogenous leukemia and gastrointestinal stromal tumors [12]. Since imatinib inhibits simultaneously and also rather selectively TGF-β and PDGF signaling, two major pro-fibrotic pathways, it can be an effective anti-fibrotic therapy. However, the half maximal inhibitory concentration (IC50s) of imatinib on PDGFR-β was 100 nM which is far beyond the plasma concentration following the usual imatinib dose of 400 mg/day [13,14]. Moreover, most patients receiving imatinib therapy experience dose-dependent adverse drug effects including cardiac, pulmonary and even hepatic toxicity throughout imatinib treatment. The extensive imatinib bio-distribution to almost all organs is the basis for the development of these diverse side effects [15,16]. These factors either precipitate imatinib treatment failure or discontinuation. Therefore, loading imatinib in an appropriate drug delivery system may hold exceptional potential for novel therapeutic approach to target HSCs that increases the concentration at the critical target cell or tissue, while reduces the putative toxicity for other cell types.

In the current study, the retinol-storing function of HSCs was used as a mechanism for their targeting using $V_A$-coupled liposomes. As far as we know, this is the first time to use imatinib as a targeted nanomedicine for liver fibrosis treatment. Using animal model of liver fibrosis, we aimed at proving hepatic accumulation and efficient HSCs targeting of the prepared formula. This was done by tracing fluorescently-labeled $V_A$-coupled liposomes following administration to rats using *in vivo* imaging and locating them inside the isolated livers. Another goal was to compare the bio-distribution of HSC-active targeted imatinib therapy *versus* conventional imatinib. Efficacy of therapy was evaluated by testing efficient blockade of PDGFR-β and resultant improvement in liver histology, hepatic functions and level of profibrotic mediators (TGF-β, matrix metalloproteinase-2 (MMP-2) and hydroxyproline).

## 2. Materials and methods

### 2.1. Materials and assay kits

Egg Phosphatidyl Choline (PC), cholesterol (Chol.), vitamin A (Retinol), acetonitrile (HPLC grade) and Nile Red were purchased from Sigma Aldrich (St. Louis, United States). *N*-[1-(2, 3-Dioleoyloxy) propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate (DOTAP) was a gift from LIPOID Co. (Nattermannallee, Germany). Imatinib (Gleevec®) was obtained from Novartis (Basel, Switzerland). Carbon tetrachloride ($CCl_4$) 98% was from Iso-Chem fine chemicals (Vert-le-Petit, France). Phosphate buffered saline tablets (PBS), iodine, methanol, ethanol, dimethyl sulfoxide (DMSO), potassium dihydrogen phosphate (and chloroform were of analytical grade. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) colorimetric assay kits and prothrombin time (PT) assay vials were purchased from N.S. Bio-Tec (Cairo, Egypt). Albumin assay kit was from BioMed (Cairo, Egypt). Rat MMP-2 enzyme-linked immunosorbent assay (ELISA) was from Kono Biotechnology Co., Ltd. (Zhejiang, China). Rat transforming growth factor-β (TGF-β) ELISA kit was from Glory Science Co., Ltd. (Del Rio, USA). Polyclonal anti-PDGFR-β (phosphorylated Y751) antibody labeled with maxlight™ 550 was purchased from US biological (Massachusetts, USA) and hydroxyproline (HP) colorimetric assay kit was from Elabscience Biotechnology Co., Ltd. (Bethesda, USA).

### 2.2. Animals

The present study was performed on healthy male Sprague Dawley albino rats of a locally bred strain, weighing 200 ± 10 g each. The rats were purchased from and housed in animal house of Medical Research Institute, Alexandria University (Alexandria, Egypt). Use of animals and experimental procedures were approved by the Ethics Committee, Medical Research Institute, Alexandria University (Alexandria, Egypt) in fulfillment of the Guide for the Care and Use of Laboratory Animals [17].

### 2.3. Preparation of liposomes

#### 2.3.1. Free liposomes

Free liposomes were developed by applying thin film hydration method, [18] utilizing different molar ratios (4:8:1, 4:6:1, 4:4:1, and 4:2:1) of PC: DOTAP: Cholesterol, respectively. The optimum ratio was chosen with regard to the optimum physicochemical properties. Briefly, the specified amounts of lipids (PC, DOTAP and cholesterol) were dissolved in a mixture of methanol and chloroform in a volume ratio 1:9. They were then dried to a thin film using rotary evaporator (Eyela N-1200, Tokyo, Japan) at 45 °C and further dried under vacuum to remove traces of organic solvents. The lipid film was hydrated with PBS, pH 7.4.

#### 2.3.2. Imatinib-loaded liposomes

Imatinib-loaded liposomes (IL) were prepared by the above mentioned methodology [18]. Imatinib encapsulation was driven by transmembrane pH-gradient method [19]. Ammonium sulfate (300 mM) was applied to hydrate the formed lipid film of the selected lipid ratio using rotary evaporator for 30 min at 60 °C with occasional vortex mixing. The extraliposomal medium was replaced with PBS, pH 7.4. This was done by placing samples in microfiltration system (Centrisart 2.5 mL concentrator 20,000 MWCO, Sartorius, Goettingen, Germany) followed by centrifugation at 15000 rpm for 10 min three times using cooling centrifuge and each time the supernatant was discarded and replaced with equivalent volume of buffer so as to establish a transmembrane pH gradient.

For active imatinib loading, the last wash was replaced by half the discarded volume PBS and the other half with deionized water containing imatinib at different imatinib-to-total lipid (I/L) ratios (1:1, 1:2, 1:4, 1:6, and 1:8). Preparations were allowed to develop overnight then sonicated for 15 min using ultrasonic cleaner (Sonica, Milano, Italy).

#### 2.3.3. Coupling liposomes with vitamin A ($V_A$)

In order to prepare $V_A$-coupled free liposomes (LC) or imatinib loaded (ILC), 200 nmol of $V_A$ dissolved in DMSO were mixed with the selected liposome suspensions (100 nmol as DOTAP) by vortexing at 25 °C. Any uncoupled $V_A$ was separated from the liposomal suspensions using centrisart 2.5 mL concentrator 20,000 MWCO. The prepared liposomal suspension was added to the microfilters and centrifuged at 15000 rpm for 10 min at 10 °C using cooling centrifuge. The material trapped in the filter was reconstituted with deionized water to get the desired dose for *in vitro* or *in vivo* use [20]. Due to $V_A$ photosensitivity, formulations were kept shielded from light.

#### 2.3.4. Nile Red-loaded liposomes

Fluorescently labeled liposomes were synthesized for *in vivo* formula tracing to prove targeting. Nile Red, as a hydrophobic fluorescent dye, was trapped in the lipid bilayer of liposomes. Nile Red was added to the lipid mixture at dye-to-total lipid ratio (1:300) and liposomes were prepared as described above [21].