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A novel controlled release formulation of the Pin1 inhibitor ATRA to improve liver cancer therapy by simultaneously blocking multiple cancer pathways

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ABSTRACT

Hepatocellular carcinoma (HCC) is the second leading cause of cancer deaths worldwide largely due to lack of effective targeted drugs to simultaneously block multiple cancer-driving pathways. The identification of all-*trans* retinoic acid (ATRA) as a potent Pin1 inhibitor provides a promising candidate for HCC targeted therapy because Pin1 is overexpressed in most HCC and activates numerous cancer-driving pathways. However, the efficacy of ATRA against solid tumors is limited due to its short half-life of 45 min in humans. A slow-releasing ATRA formulation inhibits solid tumors such as HCC, but can be used only in animals. Here, we developed a one-step, cost-effective route to produce a novel biocompatible, biodegradable, and non-toxic controlled release formulation of ATRA for effective HCC therapy. We used supercritical carbon dioxide process to encapsulate ATRA in largely uniform poly L-lactic acid (PLLA) microparticles, with the efficiency of 91.4% and yield of 68.3%, and ~4-fold higher C_{max} and AUC over the slow-releasing ATRA formulation. ATRA-PLLA microparticles had good biocompatibility, and significantly enhanced the inhibitory potency of ATRA on HCC cell growth, improving IC_{50} by over 3-fold. ATRA-PLLA microparticles exerted its efficacy likely through degrading Pin1 and inhibiting multiple Pin1-regulated cancer pathways and cell cycle progression. Indeed, Pin1 knock-down abolished ATRA inhibitory effects on HCC cells and ATRA-PLLA did not inhibit normal liver cells, as expected because ATRA selectively inhibits active Pin1 in cancer cells. Moreover ATRA-PLLA microparticles significantly enhanced the efficacy of ATRA against HCC tumor growth in mice through reducing Pin1, with a better potency than the slow-releasing ATRA formulation, consistent with its improved pharmacokinetic profiles. This study illustrates an effective platform to produce controlled release formulation of anti-cancer drugs, and ATRA-PLLA microparticles might be a promising targeted drug for HCC therapy as PLLA is biocompatible, biodegradable and nontoxic to humans.

1. Introduction

Although molecularly targeted drugs have changed cancer treatment, it has become evident that blocking a single pathway may not be as effective in solid tumors as in leukemias, especially aggressive or drug-resistant tumors, due to the feedback and simultaneous

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activation of a wide range of interactive and/or redundant pathways [1–4]. A notable example is hepatocellular carcinoma (HCC), which has extraordinarily high inter- and intra-tumor heterogeneity and complexity of etiology, with multiple cancer-driving pathways being often activated at the same time [5]. As a result, HCC is the second leading cause of cancer-related deaths in the world, although it is the sixth most common cancer [6]. However, so far there is no effective targeted therapy to block multiple activated signaling pathways at the same time [7]. Thus the development of novel molecularly targeted drugs to block multiple cancer-driving pathways at the same time is urgently needed for treating HCC and other aggressive cancers.

A common signaling mechanism in cell proliferation and transformation is Pro-directed Ser/Thr phosphorylation (pSer/Thr-Pro), which is regulated by a huge and diverse family of Pro-directed kinases and phosphatases and their upstream regulators [8,9]. The structure and function of these phosphorylated proteins are further controlled by a unique peptidyl-prolyl cis/trans isomerase (PPIase), Pin1 [10]. Pin1 is widely overexpressed and/or overactivated in most of human cancers, with its high levels being correlated with poor clinical prognosis [11,12]. For example, Pin1 is overexpressed in about 70% HCC [13–15] and high Pin1 expression is an independent factor for poor prognosis [16]. Pin1 overexpression promotes tumorigenesis by activating over 40 oncogenes or growth-promoting regulators, and inactivating over 20 tumor suppressors or growth-inhibitory regulators [10]. In contrast, PIN1 single nucleotide polymorphisms (SNPs) that lower Pin1 expression are associated with reduced cancer risk in humans [17–21]. Pin1-null mice, which develop normally [22,23], are highly resistant to tumorigenesis even after overexpression of oncogenes such as HER2 [24], RAS [24], Myc [25], Notch3 [26], or mutation [27] or ablation [28] of tumor suppressors such as p53. Thus, targeting Pin1 represents a novel anticancer strategy to block multiple cancer pathways simultaneously without general toxic effects on normal tissues [10,12,29]. However, because the available Pin1 inhibitors lacked the required specificity and/or potency, or cannot enter cells [30–32], it was challenging to evaluate Pin1 targeted therapy until our recent discovery of all-*trans* retinoic acid (ATRA) as a potent inhibitor of Pin1 *via* high throughput screening [33]. ATRA inhibits and ultimately degrades active Pin1 selectively in cancer cells, thereby blocking multiple Pin1-regulated cancer-driving pathways at the same time, an attractive property for treating aggressive and drug-resistant solid tumors [33].

ATRA, one of the active derivatives of vitamin A, is becoming as a promising compound for cancer therapy and prevention [34–36]. Nowadays ATRA has become the standard frontline drug for acute promyelocytic leukemia (APL) therapy with almost complete remission, however, its therapeutic efficacy on solid tumors remains poor [37]. Conventional systemic delivery such as oral administration of ATRA to these tumors is inefficient which always lead to side effects like drug resistance, plasma drug concentration reduction, and cancer relapse after a brief remission [37–39]. The short half-life of 45 min in humans [40] and poor aqueous solubility of 0.21 μM under physiological conditions [41] are two main obstacles for delivery ATRA to tumors. In addition, ATRA is chemically unstable and susceptible to light, heat and oxidants, which further limit its clinical application.

To overcome these problems, it is needed to develop new formulations to deliver ATRA at a sustained rate to tumors while maintaining its activity and stability. Micro/nano-particles provide powerful tools to deliver anti-cancer molecules into cancer tissues [42–44]. Some formulations for ATRA delivery including liposomes, solid lipid nanoparticles, and polymeric material based particles have been developed by a number of techniques such as hot melting homogenization method and emulsification–solvent evaporation [45–50]. Although most of them demonstrated improved anti-cancer activities, almost none of them had been performed in clinical application especially in solid tumor therapy. A possible exception is liposomal ATRA, which has been shown to have some promising antitumor activity against renal cancer in phase I/II clinical trials, but further evaluation was stopped due to halt of liposomal ATRA production [51–53]. In our previous study, we showed that ATRA slow-releasing pellets exerted potent anticancer activity against both APL and aggressive triple negative breast cancer by inhibiting and ablating Pin1 and thereby turning off and on numerous oncogenes and tumor suppressors, respectively, at the same time [33]. However, this formulation of slow-releasing ATRA pellets can be used only in animals but not humans. In addition, some issues such as low ATRA encapsulation efficiency and stability and fast release rate are still needed to be addressed. What's more, these preparation processes are lengthy and additional procedures are needed for organic solvent removal and product drying which may result in damage to the physical structure of carriers. Thus, it is highly desirable to develop a convenient and cost-effective route to prepare a biocompatible and biodegradable formulation for efficient sustained release of ATRA that can be used in humans.

Supercritical fluid technology, in particular of supercritical carbon dioxide (sc-CO_2) process is growing into an attractive method for production of drug delivery carriers [54–56]. Comparing to conventional methods for particle preparation, the sc-CO_2 process has many inherent advantages: operation at moderate temperature (above 31.2 °C) and in an inert medium that avoid degradation and oxidation of the products, efficient phase separation, direct obtaining solvent-free dry products, non-toxicity and environmental acceptability. However, to date, there is no report on preparation of ATRA controlled release formulation by sc-CO_2 process. Poly lactic acid is a biocompatible, biodegradable and non-toxic material, and has been used in drug carrier preparation [48,57].

The present study encapsulated ATRA into PLLA microparticles by sc-CO_2 process for the first time and further evaluated its efficacy in treating HCC cell growth *in vitro* and tumor growth *in vivo*. Our results show that our novel ATRA-loaded PLLA microparticles have significantly enhanced the efficacy of ATRA against HCC through reducing Pin1 *in vitro* and *in vivo*, with a better potency than the commercial available ATRA slow-releasing pellets, which can be used only in animals. Given that PLLA is biocompatible, biodegradable and nontoxic to humans, these results suggest that the ATRA-PLLA microparticles might be a promising targeted drug for HCC therapy.

2. Materials and methods

2.1. Materials

ATRA (CAS 302-79-4, purity ≥ 98%), acitretin (CAS 55079-83-9, purity ≥ 98%), fluorescein isothiocyanate isomer I (FITC) (CAS 3326-32-7, purity ≥ 90%), and corn oil (CAS 8001-30-7, d: 0.9 g/mL) were purchased from Sigma-Aldrich (MO, USA). Ester-terminated PLLA (Mw 50,000 g/mol) was obtained from Jinan Daigang Biomaterial Co., Ltd. (Jinan, China). CO_2 (purity > 99.9%, v/v) was supplied by Xiamen Rihong Co., Ltd. (Xiamen, China). ATRA slow-releasing pellets were purchased from Innovative Research of America (Florida, USA). All other chemical reagents were of analytical purity.

2.2. Cell culture and animal

293T cells, human HCC cell lines HuH7 and PLC/PRF/5 (hereinafter referred to as PLC), and normal human liver cell L-02 were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). Cell lines were checked for contamination by *Mycoplasma* detection, and were authenticated by Short Tandem Repeat profiling. 293T, HuH7 and L-02 cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) (HyClone), and PLC cells were cultured in Minimum Essential Medium (MEM) (HyClone). These mediums were supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) and 1% (v/v) penicillin–streptomycin (HyClone). All cells were routinely maintained at 37 °C under 5% CO_2 in humidified incubator. The medium was refreshed every 3 days. When confluent, the cells were harvested with