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PLGA nanoparticle formulation of RK-33: an RNA helicase inhibitor against DDX3

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Abstract

Background—The DDX3 helicase inhibitor RK-33 is a newly developed anticancer agent that showed promising results in preclinical research (Bol et al. *EMBO Mol Med*, 7(5):648–649, 2015). However, due to the physicochemical and pharmacological characteristics of RK-33, we initiated development of alternative formulations of RK-33 by preparing sustained release nanoparticles that can be administered intravenously.

Methods—In this study, RK-33 was encapsulated in poly(lactic-*co*-glycolic acid) (PLGA), one of the most well-developed biodegradable polymers, using the emulsion solvent evaporation method.

Results—Hydrodynamic diameter of RK-33-PLGA nanoparticles was about 245 nm with a negative charge, and RK-33-PLGA nanoparticles had a payload of 1.4 % RK-33. RK-33 was released from the PLGA nanoparticles over 7 days (90 ± 5.7 % released by day 7) and exhibited cytotoxicity to human breast carcinoma MCF-7 cells in a time-dependent manner. Moreover, RK-33-PLGA nanoparticles were well tolerated, and systemic retention of RK-33 was markedly improved in normal mice.

Conclusions—PLGA nanoparticles have a potential as a parenteral formulation of RK-33.

Keywords

DDX3; PLGA; RK-33; Nanoparticles; Drug release

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Compliance with ethical standards

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Introduction

Targeted cancer therapies give clinicians an alternative way to tailor cancer treatment with better patient outcomes and less side effects. DDX3, a member of the DEAD-box RNA helicase family, is involved in the cellular stress response by controlling apoptosis, cellular transformation, proliferation via the Wnt-pathway, and non-homologs end joining [1–11]. Therefore, DDX3 could be an interesting target in cancer therapy. Novel compounds that inhibit DDX3 activity have been recently developed [12–17], with 5:7:5-fused tricyclic diimidazo[4,5-d:40,50-f]—[2, 4] diazepine ring (designated as RK-33) for cancer treatment. RK-33 effectively inhibits DDX3 and was shown to induce G1 arrest and with little or no toxicity in mice [1]. However, due to the hydrophobicity of RK-33, we initiated development of alternative formulations of RK-33 using nanoparticles (NPs).

NPs enable encapsulation of a wide variety of drugs, such as hydrophilic drugs, hydrophobic drugs, macromolecules, and at the same time improve their physicochemical properties and pharmacokinetics [18]. The most widely used biodegradable polymer for NPs is poly(lactic-*co*-glycolic acid) (PLGA), which is composed of poly(lactic acid) (PLA) and poly(glycolic acid) (PGA). PLGA is hydrolyzed into monomers and easily metabolized, thus ensuring minimal systemic toxicity of PLGA [19]. PLGA NPs have been demonstrated to be suitable for systemic delivery and controlled release of hydrophobic therapeutic agents in a number of biomedical applications [20–22]. In this study, we prepared and characterized PLGA-based NPs of RK-33 to maximize future clinical utility of RK-33.

Materials and methods

Chemicals, cell lines, and animals

Poly(D,L-lactide-*co*-glycolide) (PLGA, M.W. = 40–75 kDa), poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) copolymer (PEG–PPG–PEG, average M.W = 8.4 kDa) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Female athymic NCr-nu/nu mice (4–5 weeks old) were purchased from NCI Frederick. Mice were maintained under pathogen-free conditions and given food and water ad libitum in accordance with guidelines from the Johns Hopkins University Animal Care and Use Committee.

Preparation of RK-33-loaded PLGA nanoparticles

PLGA NPs incorporating RK-33 were prepared using an emulsion solvent evaporation method. Briefly, 200 mg of PLGA and 10 mg of RK-33 were dissolved in 20 mL dichloromethane and slowly added to 30 mL PBS containing 1 % PVA, 0.2 % PEG–PPG–PEG, 1 % NaCl while sonicated on ice for 8 min. Subsequently, dichloromethane was evaporated, and NPs were washed with purified water five times, lyophilized, and stored at –20 °C until use. To optimize encapsulation of RK-33, we prepared NPs with two different PLGA–RK-33 ratios: 20:1 (5 %) and 10:1 (10 %).

Characterization of PLGA nanoparticles

To determine particle size, size distribution, and ζ -potential of the PLGA NPs, dynamic laser-light-scattering (DLS) measurements were performed using a Zetasizer Nano-ZS90 (Malvern Instruments Ltd., Worcestershire, UK) with a He–Ne laser system, which was operated at 633 nm and 25 ± 1 °C, and the scattered light was measured at an angle of 90°. To determine ζ -potential, NPs were dispersed in 10 mM NaCl. The morphology of PLGA NPs was characterized with a scanning electron microscope (LEO 1550 FESEM, Carl Zeiss, Inc., Thornwood, NY, USA). For sample preparation, PLGA NPs were dispersed in purified water (0.05 mg/mL), and the droplets were dried on the surface of a cover glass at room temperature, and then coated with gold sputter from the vapor on an auto fine coater.

Loading amounts of RK-33 were determined by reverse-phase high-performance liquid chromatography (HPLC) with a UV detector. The quantification analysis with HPLC was performed as described earlier [23], with minor modifications. For burst release, 0.5 mg PLGA was dissolved in 1 mL acetonitrile. For the release characteristics, 1.6 mg NPs were dispersed in 2 mL of PBS/1 % Tween 20 and divided into five separate tubes. These samples were incubated in a shaker at 37 °C, and an aliquot from each sample was analyzed at a predetermined time point. The NPs were first spun down, after which 30 μ L of the supernatant was added to 70 μ L acetonitrile. After vortexing, 20 μ L of the sample solution was directly injected into a Waters 1525 binary HPLC pump equipped with a Waters Symmetry® C18 reversed phase column (4.6 mm $\times$ 150 mm) and a Waters Ultrahydrogel Guard Column (6.0 mm $\times$ 40 mm). A Waters 2487 dual λ absorbance detector was set at 390 nm. Acetonitrile and H₂O were used as the mobile phase at 75 and 25 %, respectively. The flow rate was 1 mL/min, and HPLC analysis was performed at room temperature. The Breeze 3.30 program (Waters Corp., Milford, MA, USA) was used as acquisition and analysis software. The experiment concerning the release characteristics was repeated with two different batches.

In vivo retention

Ten milligram RK-33-PLGA NPs (equivalent to 0.14 mg RK-33) was intravenously administered into athymic female mice. As a control group, 0.8 mg free RK-33 was intraperitoneally administered into athymic mice, which is the standard dose and route for RK-33 therapy. Animals were killed 48 h after the administration, and blood was withdrawn intracardially, and organs were excised. Blood was collected into a heparin-treated Eppendorf tube and spun at 8000 rpm for 5 min. The liver and the lungs were weighed and subsequently homogenized in three times volume of cold PBS using a glass homogenizer with a Teflon Pestle.

RK-33 contained in the sample solution was extracted as previously described [24]. Briefly, 1 mL of chloroform/isopropanol (1:1, v/v) mixture was added to 0.1 mL of plasma sample or homogenate. The obtained mixture was shaken vigorously for 3 min and centrifuged to obtain the supernatant. The supernatant was transferred to a glass tube and evaporated to dryness at 37 °C under nitrogen gas. The residue was dissolved in 100 μ L of acetonitrile, and the resultant solution was analyzed by HPLC to determine RK-33 in the sample.

Cytotoxicity study

Viability of cancer cells was determined by MTS assay (CellTiter 96[®] AQueous One Solution, Promega, Madison, WI, USA) as described by manufacturer. Briefly, 8×10^2 MCF-7 cells were seeded in a 96-well plate, and treated with different concentrations of RK-33 loaded nanoparticles and unloaded nanoparticles next day. After 72-h incubation, MTS reagent was added to the cells, and the absorbance was measured at 490 nm after 2-h incubation with MTS reagent. The experiment was repeated three independent times.

Results

Characteristics of RK-33-loaded nanoparticles

PLGA nanoparticles were prepared with 5 % w/w, 10 % w/w RK-33 and without drug by means of an oil-in-water emulsion/solvent evaporation method. Furthermore, these nanoparticles were coated with PVA and PEG-PPG-PEG to improve solubility and decrease flocculation in vivo. This resulted in RK-33 loaded nanoparticles of 245 nm (5 % RK-33) and 266 nm (10 % RK-33) with a ζ -potential of -2.17 mV in 10 mM NaCl (Table 1).

Scanning electron micrographs (SEM) were used to confirm size and shape. The nanoparticles were spherical in shape and had a consistent size of about 200 nm (Fig. 1).

We were able to make about 175 mg of RK-33 loaded PLGA nanoparticles in one experiment while ensuring consistency in size (RK-33 PLGA (5 %) = 245 ± 37 nm, RK-33 PLGA (10 %) = 266 ± 28 nm), keeping the polydispersity index (PDI) to a minimum (<0.250) and maintaining efficient production (>80 %). Encapsulation of RK-33-PLGA (10 %) was highly variable and was therefore not considered. The encapsulation efficiency of RK-33 PLGA (5 %) was 27 ± 7.4 %, which means every 100 mg of nanoparticles contained 1.4 mg of RK-33. Effectively, we were able to consistently and efficiently load PLGA nanoparticles with the hydrophobic DDX3 inhibitor RK-33.

Release kinetics of RK-33-loaded nanoparticles

To quantitate RK-33 released from PLGA NPs, HPLC was carried out following extraction of free RK-33 from the release media. HPLC chromatogram of RK-33 showed a distinct peak at 4.5-min retention time, indicating that RK-33 molecules remain intact after encapsulation and subsequent burst release from these NPs (Fig. 2a, b). Furthermore, breakdown products of the NP, lactic acid and glycolic acid, did not interfere with HPLC measurements (Fig. 2c).

The release of two different batches of RK-33-loaded NPs was assessed in siliconized tubes, as polypropylene tubes may facilitate the adherence of RK-33 to the tubes sidewalls. An initial release in the first 24 h (25 ± 4.2 %) was followed by a linear release of RK-33 from the NPs prepared from PLGA:RK-33 ratios of 20:1. In 7 days, 90 ± 5.7 % of RK-33 was released from the NPs (Fig. 3). In vitro release of RK-33 from the NP formulation prepared from PLGA:RK-33 ratios of 10:1 was not consistent (data not shown).

Cytotoxicity of RK-33-loaded nanoparticles

To determine whether RK-33-loaded NPs could efficiently kill cancer cells, we carried out an MTS assay following incubation of a human breast carcinoma cell line, MCF-7, with the RK-33 NPs. As shown in Fig. 4a, RK-33-loaded NPs demonstrated cytotoxicity to MCF-7 cells in a dose-dependent manner, while equivalent doses of empty NPs had no killing effect. The IC₅₀ value of 5 % RK-33 loaded NPs was 49 µg/mL, and the IC₅₀ value of 10 % RK-33 loaded NPs was 25 µg/mL (Fig. 4a). Next, we assessed whether RK-33 NPs (5 %) were cytotoxic to MCF-7 breast cancer cells in a time-dependent manner. The IC₅₀ value of the RK-33-loaded NPs was 57 µg/mL after 2-day incubation, further decreasing to 40 µg/mL for 4-day incubation (Fig. 4b), indicating that the slower release of RK-33 from the NPs promoted cytotoxicity in a time-dependent manner.

In vivo retention of RK-33

To assess the release of RK-33 in the context of circulation and metabolism in human patients, we tested two mice with RK-33 loaded nanoparticles injected intravenously. We injected 10 mg of nanoparticles intravenously into mice, which was equivalent to a treatment dose of 0.14 mg RK-33 per mouse. As a control, three mice were treated following the standard protocol (intraperitoneal injection of 0.80 mg RK-33). In the mice treated with free RK-33 (control), we could not detect any RK-33 (<1 µg/mL) in the plasma, lungs, or liver 48 h after treatment. This was expected, considering the systemic half-life of free RK-33 ($t_{1/2}$ = 7.3 h). Conversely, in the mice treated with RK-33-PLGA, we could still detect RK-33 in the plasma (34 µg/mL) and liver (28 µg/g), but not lungs (Fig. 4). The mice treated with nanoparticles received six times less RK-33 compared with the control mice (due to technical limitations), yet RK-33 was still detected 48 h after treatment with RK-33-PLGA.

Discussion

Hydrophobic compounds are not easy to handle in order to maximize clinical utility. Although paclitaxel, one of the hydrophobic anticancer agents used clinically is dissolved in the mixture of Cremophor EL and dehydrated ethanol, and administered intravenously, excipients including Cremophor EL are relatively toxic and induce severe hypersensitive reactions [25]. One strategy to increase water solubility of hydrophobic compounds is to make salt formation. An example of such drugs includes doxorubicin hydrochloride. Another strategy is to encapsulate hydrophobic compounds into biodegradable and biocompatible NPs. The use of NPs in medicine provides a new platform to increase the efficiency of drug delivery, and to maintain a steady-state level of the therapeutic dose of the drug in the circulatory system (Fig. 5).

In this study, we evaluated the use of PLGA NPs as a carrier for a new DDX3 helicase inhibitor, RK-33, which is highly hydrophobic and has the potential to be an effective anticancer agent. We constantly generated PLGA NPs with 80 % production efficiency and keeping PDI to low (<0.250), indicating that the properties of RK-33 were not a hindrance as a payload for the PLGA NPs.

The NPs prepared from PLGA:RK-33 ratios of 20:1 had a consistent encapsulation efficiency ($27 \pm 7.4\%$), which is within the normal range [19]. The initial burst release is thought to be caused by poor drug entrapment or drug adsorption onto the outside of the particles [19]. Burst release of RK-33 NPs was reasonable, 25 % release in the first 24 h versus 11 % release per 24 h afterward, likely due to the vigorous washing step before lyophilization.

Subsequently, we evaluated the systemic retention of RK-33 in PLGA NP formulation in normal mice. The results revealed that 10 mg of RK-33-PLGA NPs (0.14 mg eq. RK-33) was safe for intravenous administration and that RK-33 was retained in the systemic circulation even 48 h after intravenous administration of the NPs. Although total drug encapsulation was not sufficient, an active targeting strategy by surface modification [26] could maximize the therapeutic efficacy of RK-33-PLGA for the future study.

Based on the data obtained, RK-33 was slowly released from the NPs in vitro in a linear manner over 7 days and retains its cytotoxic effect in vitro. Furthermore, an in vivo pilot study revealed longer systemic retention of RK-33 compared to free RK-33. Taken together, PLGA NPs can be used as a carrier of RK-33 by achieving sustained release as well as the formulation that can be administered intravenously and may facilitate efficient delivery for effective cancer treatment in the future.

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Fig. 1. Scanning electron microscopic (SEM) images of RK-33-PLGA nanoparticles. *Scale bar* 200 nm

Fig. 2.

Reverse-phase high-performance liquid chromatography (HPLC) of nanoparticles. **a** HPLC chromatogram of RK-33 dissolved in acetonitrile; **b** HPLC chromatogram of RK-33-PLGA NPs dissolved in acetonitrile (burst release); **c** HPLC of RK-33-free PLGA NPs dissolved in acetonitrile

Fig. 3.

Release characteristics of RK-33-PLGA nanoparticles prepared from PLGA:RK-33 ratios of 20:1. RK-33 release from the NPs is expressed as a percentage of the total RK-33 content in the sample at each time point. *Error bars* represent $\pm$ SD

Fig. 4.

In vitro cytotoxicity of RK-33-PLGA nanoparticles. **a** Dose response curve of the cytotoxicity of RK-33-PLGA NPs to human breast carcinoma MCF-7 cells. *Squares* PLGA NPs (no RK-33); *circles* RK-33-PLGA NPs prepared from PLGA:RK-33 ratios of 20:1; *diamonds* RK-33-PLGA NPs prepared from PLGA:RK-33 ratios of 10:1. *Error bars* represent $\pm$ SD. **b** IC₅₀ values of RK-33-PLGA NPs prepared from PLGA:RK-33 ratios of 20:1 in relation to incubation time. *Error bars* represent 95 % confidence interval

Fig. 5.

In vivo retention of RK-33 in normal mice 48 h after intravenous administration of RK-33-PLGA nanoparticles (equivalent to 0.14 mg RK-33) and after intraperitoneal administration of free RK-33 (0.8 mg). Error bars represent $\pm\text{SD}$. Open columns RK-33-PLGA NPs; closed columns Free RK-33

Table 1

Physicochemical characteristics of PLGA nanoparticles

Samples	Hydrodynamic diameter $\pm$ SD (nm)	PdI $\pm$ SD	ζ -Potential $\pm$ SD (mV)
PLGA	220 $\pm$ 25	0.140 $\pm$ 0.08	-2.37 $\pm$ 0.7
PLGA RK-33 (20:1)	245 $\pm$ 37	0.167 $\pm$ 0.08	-2.17 $\pm$ 0.3
PLGA RK-33 (10:1)	266 $\pm$ 28	0.203 $\pm$ 0.04	-2.17 $\pm$ 0.9

Hydrodynamic diameter, polydispersity index (PdI), and ζ -potential were measured for unloaded NPs (PLGA), RK-33 loaded NPs prepared from PLGA–RK-33 ratios of 20:1 and 10:1