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Research Paper

siRNA-loaded biodegradable nanocarriers for therapeutic MAPK1 silencing against cisplatin-induced ototoxicity

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ABSTRACT

Ototoxicity represents a major adverse side-effect of *cis*-diamminedichloroplatinum-II (cisplatin, CDDP). The mitogen-activated protein kinase (MAPK) pathway is thought to play a central role in potentiating the apoptotic effect of CDDP within the cochlea. We hypothesized that prophylactic inhibition of MAPK signaling, using small interfering RNA (siRNA), might confer a protective effect against CDDP-induced apoptosis within the auditory sensory epithelia. To enhance the therapeutic utility of this approach, we synthesized biocompatible *siMAPK1*-loaded nanoparticles (NPs) and performed physicochemical characterizations for size, morphology, drug loading and release kinetics, using dynamic light scattering, electron microscopy and spectrophotometric analyses, respectively. Our findings show 183.88 ± 6.26 nm-sized spherical *siMAPK1*-loaded NPs with -27.12 ± 6.65 mV zeta potential and 112.78 ± 0.24 pmol/mg of *siMAPK1* loading that exhibit a sustained release profile for prolonged therapeutic efficacy. Synthesized NPs were validated for biocompatibility and prophylactically protected against CDDP-induced cytotoxicity in HEI-OC1 cells and hair cell loss in murine organotypic cochlear explants. Our study confirms a pivotal role for MAPK1 signaling as a potentiating factor for CDDP-induced apoptosis and cochlear hair cell loss, and highlights *siMAPK1* NP treatment as a therapeutic strategy for limiting the ototoxic side-effects associated with systemic CDDP administration.

1. Introduction

Cisplatin (*cis*-dichlorodiamminoplatinum (II); CDDP) is one of the most potent and efficacious anti-neoplastic agents employed for the treatment of a variety of cancers, including testicular, germ cell, head and neck, bladder, cervical, non-small cell lung, breast, esophageal, cervical, stomach and prostate cancers (Florea and Büsselberg, 2011). However, CDDP treatment and therapeutic dosing are limited by detrimental side effects, such as neurotoxicity, ototoxicity, gastrointestinal disturbance, and acute nephrotoxicity (Arany and Safirstein, 2003; Travis et al., 2014; Karasawa and Steyger, 2015). The major dose-related adverse side effect of cisplatin treatment is irreversible sensorineural hearing loss (Sun et al., 2015). Ototoxicity is one of the frequent side effects of CDDP therapy in patients treated with higher dose regimens, with 75–100% of patients receiving 150–225 mg/m^2 CDDP showing some degree of hearing loss (Kopelman et al., 1988; McKeage, 1995). Ototoxicity caused by CDDP can occur within hours to days after drug administration (Rybak et al., 2009). Outer hair cells (OHCs) have been found to be the most vulnerable to CDDP damage (Usami et al., 1996). Experimental and clinical studies have revealed that, OHCs in the basal turn are particularly susceptible to CDDP-induced cytotoxicity (Rybak et al., 2007; Ding et al., 2011). Indeed, histological evaluations of cochlear tissues in animal models of clinical CDDP administration have revealed that ototoxic destruction is primarily focused among the OHCs, particularly within the basal turn (high frequency region) of the cochlea (García-Berrocal et al., 2007; Hellberg et al., 2013; Kuduban et al., 2013).

Over the last 30 years, considerable effort has been devoted to determine the underlying pathophysiology that governs CDDP-induced ototoxicity (Callejo et al., 2015; Karasawa and Steyger, 2015). At cellular level, acute CDDP exposure results in a progressive wave of programmed cell death (apoptosis) within the OHC population that is, at least in part, initiated and perpetuated by DNA damage and oxidative stress (Kopke et al.,

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1999; Dehne et al., 2001; Karasawa and Steyger, 2015). These intrinsic stressors induce extrinsic signaling through activation of tumor necrosis factor α (TNFα)-like death receptors that further drive the apoptotic response (So et al., 2007). Intrinsic and extrinsic pathways of CDDP-induced OHC pro-apoptotic signaling mediate their destructive influence through a family of cysteine proteases, called caspases, whose cumulative proteolytic activities culminate in OHC destruction.

While many signaling pathways have been implicated in governing and perpetuating this CDDP-induced pathologic response, the mitogen-activated protein kinase (MAPK) family of signaling factors has been shown to play a central role in tipping the balance towards an apoptotic fate. At the nexus of these pro-apoptotic signaling cascades for CDDP ototoxicity, are two isoforms of an extracellular signal-regulated kinase named MAPK1 and MAPK3 (also known as ERK 2 and 1, respectively). Upon activation, MAPK1/MAPK3 phosphorylate a number of key cytoplasmic and nuclear factors that, in this cytotoxic context, promote both intrinsic and extrinsic caspase-mediated cell death (Cagnol and Chambard, 2010). While other members of the MAPK family, such as p38 MAPK and c-Jun-NH2-terminal kinase (JNK), have been shown to play roles in CDDP-induced apoptosis, *in vitro* studies have revealed that MAPK1/MAPK3 activation (i.e. phosphorylation) and subsequent signaling represent key, rate-limiting events for driving OHC death in this context (Wang et al., 2004; So et al., 2007; Lee et al., 2010). Moreover, the specificity and preeminent status of this signaling pathway is conserved in *in vitro* models of CDDP-induced nephrotoxicity (Arany et al., 2004; Kim et al., 2005), suggesting that MAPK1/3 signaling may represent a highly attractive and specific target for controlled therapeutic inhibition to mitigate the dose-limiting side-effects of CDDP chemotherapy.

Small interfering RNA-mediated silencing of mRNA transcripts is a canonical approach for gene-specific inhibition (Carthew and Sontheimer, 2009; Ghildiyal and Zamore, 2009). However, several factors limit the clinical application of siRNA. Those factors include poor cellular internalization, physical and chemical instability within bodily fluids (half-life ~10 min in plasma), and rapid degradation in the lysosome (Grimm, 2009; Takahashi et al., 2009; Whitehead et al., 2009). Encapsulation of siRNA within biocompatible poly (lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) represents a reliable approach to overcome the above disadvantages, including physical protection of siRNA against RNase activity, enhanced cellular uptake, and prolonged *in vivo* half-life (Woodrow et al., 2009; Wang et al., 2012). Moreover, encapsulation within PLGA NPs allows increased surface exchange, nuclease resistance, controlled drug release and extending the therapeutic window of siRNA-mediated knockdown (Cun et al., 2011). In the present study, we evaluated the efficacy of siRNA-loaded PLGA NPs as delivery vehicles for mediating transcriptional silencing of MAPK1 (*ERK2*) as a means of mitigating CDDP-induced ototoxicity and provide a proof-of-concept support for their potential therapeutic utility against this chemotherapeutic adverse effect.

2. Materials and methods

2.1. Materials

Poly D,L-lactic-co-glycolic acid (PLGA, L/G ratio of 50:50 and molecular weight ranging between: 7 to 17 kDa) was purchased from Boehringer Ingelheim (Ridgefield, CT). Dichloromethane (DCM) was obtained from Burdick and Jackson (Muskegon, MI). Polyvinyl alcohol (PVA, MW: 30–70 kDa) was provided by Sigma Aldrich (Saint-Louis, MO). TE Buffer Solution OmniPur® Grade, pH 8.0 was purchased from EMD Millipore (Billerica, MA). Fluorescein-conjugated non-targeting (scrambled) siRNA (Ambion *Silencer* FAM-labeled Negative Control #1 siRNA) was purchased from ThermoFisher Scientific Inc. (Rockford, IL). For this project, unlabeled non-targeting (scrambled) siRNA and *siMAPK1* were designed through custom synthesis by Dharmacon, Inc. (Lafayette, CO). All other chemicals were of analytical grade without further purification.

2.2. Preparation of siMAPK1-loaded nanoparticles

siMAPK1-loaded PLGA nanoparticles (NPs) were prepared by the water-in-oil-in-water (w_1/o/w2) double emulsion solvent evaporation method as previously reported (Cun et al., 2010). Briefly, *siMAPK1* was dissolved in 50 μL TE buffer (10 mM Tris–HCl and 1 mM EDTA in MilliQ water, pH 8.0) and added to 1,000 μL of DCM containing 100 mg of PLGA. The mixture was emulsified by sonication (Microson ultrasonic cell disruptor XL Misonix Inc., Farmingdale, NY) into a primary W_1/O emulsion. Four milliliters of 5% PVA (w/v) in MilliQ water (Millipore Co., Billerica, MA) were directly poured into the primary emulsion prior to further emulsification and sonication (3×30 s) to form a W_1/O/W_2 double emulsion. The resulting emulsion was diluted in 50 mL of 0.3% (w/v) PVA in MilliQ water and magnetically stirred for two hours at room temperature (RO 10, IKA-Werke Gmbh & Co, Staufen, Germany) to evaporate the DCM. PLGA NPs were collected by ultracentrifugation at 13,000*g* for 20 min at 10 °C (TOMY MX-201 Highspeed Refrigerated Microcentrifuge), washed thrice with MilliQ water, resuspended in 5 mL of MilliQ water, and freeze-dried at −100 °C under 40 mTorr (Virtis Benchtop freeze-dryer, Gardiner, NY) for three consecutive days. The obtained powdered NPs were sterilized under UV light for 45–60 min at room temperature and stored at −80 °C until further use. Fluorescent FAM-scRNA-loaded NPs were prepared, characterized, sterilized and stored using the previously described methods.

2.3. Physicochemical characterization of siMAPK1 nanoparticles

2.3.1. Particle mean diameter, polydispersity index and zeta potential analyses

The particle mean diameter (PMD), polydispersity index (PDI) and zeta potential (ZP) of *siMAPK1*-loaded NPs were measured by dynamic and phase analysis light scattering (DLS and PALS, respectively) (Zetasizer Nano ZS series, Malvern Instruments Ltd., Worcestershire, UK). Colloidal suspensions were diluted ten times with Milli-Q water and subsequently sonicated for 2 min. Sample measurements were completed at a scattering angle of 173°. The average of PMD, PDI and ZP were recorded in sextuplicate at 25 °C. According to the National Institute of Standards and Technology (NIST), a sample with a (PDI) < 0.15 is considered monodisperse and otherwise polydisperse (Hackley, 2001). Furthermore, a complementary analysis of particle size and distribution was performed by nanoparticle tracking analysis (NTA) using a NanoSight LM20 (Malvern Instruments Inc, Houston, TX) equipped with a sample chamber, a 640-nm laser, and aViton fluoroelastomer O-ring. NTA version 2.3 Build 0017 software was used for image capture and data processing. Samples were injected in the sample chamber with sterile pipettes and analyzed at room temperature.

2.3.2. Determination of drug loading and percent drug encapsulation efficiency

Powdered *siMAPK1*-loaded NPs (2 mg) were dissolved in 200 μL of DCM, vortexed (Vortex-Genie, Fisher Scientific Inc., Bohemia, NY) and sonicated at room temperature for 3 min. A volume of 500 μL of TE buffer pH 8 was added, and samples were vortexed for 2 min. The obtained suspension was centrifuged at 18,000 rpm at 4 °C for 20 min. The aqueous supernatant was collected and incubated in a horizontal SHEL LAB water bath (Sheldon