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Nitroxide radical-containing nanoparticles as potential candidates for overcoming drug resistance in epidermoid cancers

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ABSTRACT

Multidrug resistance in cancer cells contributes to the failure of conventional chemotherapy in more than 90% of cancer patients (metastatic). This is attributed to reactive oxygen species (ROS)-regulated drug efflux proteins, P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1). In this study, we focused on overcoming multidrug resistance with a therapeutic application of ROS-scavenging nitroxide radical-containing nanoparticles, RNP^N (pH-sensitive) and RNP^O (pH-insensitive), in combination with the conventional chemotherapeutic drug, doxorubicin (Dox), in drug-resistant epidermoid cancer cell lines, KB-C2 (P-gp expressing) and KB/MRP (MRP1 expressing). We confirmed that the combination treatment with RNPs increased Dox uptake in multidrug-resistant cancer cells, which further enhanced cell cytotoxicity. The abrogation of the crucial ROS signaling was confirmed with RNP treatment, which deterred ROS-regulated drug efflux protein (P-gp and MRP1) expression, resulting in the sensitization of resistant cells to Dox. These results establish ROS-scavenging RNPs as potential therapeutic candidates to overcome drug resistance in multidrug-resistant cancers.

1. Introduction

The effectiveness of anticancer drugs is greatly limited owing to the drug-resistant characteristics of tumor cells, which are either innate (primary) or acquired during chemotherapy. Acquired resistance is more challenging to deal with as it is not only limited to the original initiating drug, but also, over time, the tumors acquire cross-resistance to other anticancer drugs from a diverse resistance mechanism, hence further complicating cancer therapeutics [1]. Resistance to chemotherapy has been reported to be the cause of treatment failure in more than 90% of cancer patients (metastatic) [2].

Chemoresistance is attributed to various resistant mechanisms, for instance, drug inactivation [3,4], alteration of molecular drug targets by mutation [5,6], repair of drug-damaged DNA [7,8], evasion of cell death (apoptosis and autophagy pathway) [9–12], epithelial–mesenchymal transition [13,14], cancer heterogeneity [15], and drug efflux [16]. Of these, drug efflux is one of the most established drug-resistant mechanisms, which limits the accumulation of drugs by enhancing its efflux. Other than attaining drug resistance in cancer cells, a drug efflux system prevents toxin accumulation in healthy cells, the bile duct, intestines, and the blood–brain barrier [17,18].

The most extensively studied drug efflux proteins implicated in cancer are members of the ATP-binding cassette (ABC) transporter superfamily, which includes P-glycoprotein (P-gp), a multidrug resistance protein (MDR) also called ABCB1, and multidrug

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resistance-associated protein-1 (MRP1) encoded by ABCC1 [19]. P-gp is a 170 kDa transmembrane glycoprotein that functions as an ATP-dependent efflux pump [20,21] and is overexpressed in various cancers owing to drug resistance [22,23]. P-gp transports hydrophobic compounds and has been implicated in resistance against paclitaxel and doxorubicin (Dox) [24]. On the contrary, MRP1, a 190 kDa transmembrane glycoprotein, not only transports organic anions and hydrophobic compounds but also confers resistance to Dox [25]. P-gp and MRP1 have been reported to be associated with poor clinical outcomes in acute myeloid leukemia and neuroblastoma, respectively [26,27]. Their co-expression is associated with poor outcomes in acute myeloid leukemia [28,29].

Since the mechanism of chemoresistance contributed by P-gp and MRP1 have different target substrates and distinct transport pathways [30], a combination therapy may be quite effective to overcome the multidrug resistance. For instance, treatment with one drug to sensitize cancer cells by altering the gene expression of crucial survival proteins followed by a cytotoxic drug treatment for already vulnerable cancer cells. Furthermore, the regulation of P-gp and MRP1 by reactive oxygen species (ROS) in cancers has been reported [31,32], which may be a crucial target for antioxidant functioning as chemosensitizers in resistant cancers for anticancer drugs.

Recently, the therapeutic application of nanoparticles in resistant tumors has become the most sought-after strategy to overcome multidrug resistance in cancers [34]. Nanoparticles, in combination therapy (nanoformulation) or just as a drug carrier, have contributed immensely to enhancing the therapeutic efficacy of drugs in resistant tumors. This is because of their prolonged systemic circulation lifetime, increased intratumoral drug accumulation (enhanced permeation and retention effect), sustained drug release kinetics, targeted delivery (lower adverse effects), and through bypassing the drug efflux mechanism [33–36].

Hence, we focused on overcoming the multidrug resistance in epidermoid cancer cell lines through ROS-scavenging polymeric micelles, pH-sensitive redox nanoparticles (RNP^N) and pH-insensitive redox nanoparticles (RNP^O) (Fig. 1). RNP^N and RNP^O are nitroxide radical-containing nanoparticles, composed of self-assembling amphiphilic block copolymers, poly(ethylene glycol)-b-poly[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)amino-methylstyrene] (MeO-PEG-*b*-PMNT) and methoxy-poly(ethylene glycol)-b-poly(4-[2,2,6,6-tetramethylpiperidine-1-oxyl]oxy-methylstyrene)] (MeO-PEG-*b*-PMOT), respectively [37,38]. Antioxidant-mediated therapeutic applications of RNP^O and RNP^N have been confirmed in various oxidative stress-induced *in vivo* disease models, such as ischemia reperfusion injuries, intracerebral hemorrhage, ulcerative colitis, and many cancer models [39–43]. One of the most important features of RNPs is their exceptionally low toxicity as they do not enter healthy cells and, therefore, maintain normal redox reactions; for example, with the electron transport chain to maintain a healthy mitochondrial level [44,45]. Diverse therapeutic applications of RNPs are not only restricted to being drug carriers like various other nanoparticles, but they also possess additional therapeutic effects [46]. Furthermore, combination therapy involving RNP^N and Dox in *in vivo* cancer models has proven effective at inhibiting tumor growth with negligible adverse effects [47]. Overall, the *in vivo* therapeutic application of ROS scavenger RNPs either alone, as drug carriers, or in combination have been effective owing to their stable nature, increased bioavailability, and biocompatibility [48].

Considering the dependence of cancers on ROS signaling and ROS-mediated regulation of drug efflux transporters, P-gp and MRP1, we studied the therapeutic application of pH-sensitive RNP^N and pH-insensitive RNP^O (40 nm in diameter) in epidermoid cancer cell lines, drug-sensitive KB-3-1, drug-resistant P-gp-expressing KB-C2, and MRP1-expressing KB/MRP in combination with the conventional chemotherapeutic drug, Dox (Fig. 1). KB-C2 and KB/MRP epidermoid cancer cell lines have been reported to be resistant to Dox by effluxing the drug out with active ABC transporters, P-gp and MRP1, respectively. In this study, we confirmed an increased Dox uptake in resistant cancer cell lines with the treatment of ROS-scavenging RNPs, which further enhanced cancer cell cytotoxicity. The abrogation of the crucial ROS signaling was also confirmed with the combined treatment of RNPs and Dox by evaluating ROS levels. This, in turn, affected the drug efflux protein regulation resulting in an increased sensitivity to Dox. Interestingly, we also observed a therapeutic effect of RNPs individually in resistant cancer cell lines, hence implicating the vital role of the ROS signaling pathway in these cell lines. Thus, these results establish ROS-scavenging RNPs not only as an adjunct to sensitize resistant cells for chemotherapy but also as a potential therapeutic candidate to overcome drug resistance.

2. Experimental methods

2.1. Materials

Dox and colchicine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM; 1000 mg/L glucose, L-glutamine, and sodium bicarbonate) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St Louis, MO, USA). A Penicillin-Streptomycin-Neomycin (PSN) antibiotic mixture and Hoechst 33258 were procured from Invitrogen (Eugene, OR, USA). Sodium chloride, potassium chloride, tris(hydroxymethyl)aminomethane (Tris base), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), and glycine were purchased from Wako Pure Chemical Industries.

2.2. Nanoparticle preparation

RNP^O and RNP^N were prepared by self-assembling block copolymers, MeO-PEG-*b*-PMOT (MW = 8.8 kDa) and MeO-PEG-*b*-PMNT (MW = 10 kDa), respectively, as previously reported [37,38]. Briefly, methoxy-poly(ethylene glycol)-b-poly(-chloromethylstyrene) (MeO-PEG-*b*-PCMS) was synthesized by the radical reversible addition fragmentation chain transfer (RAFT) polymerization of chloromethylstyrene (CMS) in the presence of MeO-PEG-SC(=S)Ph as a RAFT agent. Then, the chloromethyl groups were converted from CMS to TEMPO with 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) for RNP^O and 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino-TEMPO; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) for RNP^N. Micelles, RNP^O and RNP^N, were prepared by dialysis using dimethylformamide (DMF) (against water) of amphiphilic block copolymers, MeO-PEG-*b*-PMOT and MeO-PEG-*b*-PMNT, respectively. The size distribution of the nanoparticles was determined with a dynamic light scattering measurement (DLS; Zetasizer Nanoseries ZEN3600, Malvern Instruments Ltd., Worcestershire, UK). The efficiency of the introduction of TEMPO to the polymer chain was determined using electron spin resonance (ESR). PEG-b-PCMS was also synthesized, as previously reported [37], and was used as a control polymer, without TEMPO moieties, to confirm the antioxidant-driven cytotoxic effect of RNP^O and RNP^N containing TEMPO.

2.3. Cell culture

Human epidermoid cancer cell lines, KB-3-1 cells (drug-sensitive) and KB/MRP cells (overexpressed MRP1) were cultured in DMEM containing 10% FBS and 100 ng/mL PSN antibiotic mixture at

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