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Polymeric chloroquine as an inhibitor of cancer cell migration and experimental lung metastasis

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ABSTRACT

Chloroquine (CQ) is a widely used antimalarial drug with emerging potential in anticancer therapies due to its apparent inhibitory effects on CXCR4 chemokine receptor, autophagy, and cholesterol metabolism. This study reports on polymeric CQ (pCQ) as a macromolecular drug with antimetastatic activity. The pCQ polymers were synthesized by copolymerization of methacryloylated hydroxy-CQ (HCQ) and *N*-(2-hydroxypropyl)methacrylamide (HPMA). The results show that pCQ is significantly more effective in inhibiting cancer cell migration and invasion when compared with the parent HCQ. The proposed mechanism of action at least partially relies on the ability of pCQ to inhibit cell migration mediated by the CXCR4/CXCL12 pathway. The pCQ also demonstrates superior inhibitory activity over HCQ when tested in a mouse model of experimental lung metastasis. Lastly, pCQ shows the ability to efficiently translocate to the cytoplasm while exhibiting lower cytotoxicity than HCQ. Overall, this study supports pCQ as a promising polymeric drug platform suitable for use in combination antimetastatic strategies and potential use in cytoplasmic drug delivery.

1. Introduction

The use of polymers has made remarkable contribution in pharmaceutical discovery and development of modern drug delivery methods. Polymer applications, whether already in clinical use or in preclinical development, range from simple functions such as providing sustained drug release to more sophisticated uses such as targeted drug delivery [1,2]. In addition to the traditional use of polymers to improve safety and delivery efficacy of existing drugs, there has been a growing interest in the development of pharmacologically active polymers. These so-called polymeric drugs exhibit intrinsic therapeutic activity and have been employed in the treatment of various diseases [3]. Besides altered pharmacokinetics and biodistribution, polymeric drugs usually take advantage of multivalent interactions to achieve improved activity when compared with small molecule drugs [4]. This is often the result of amplified downstream signaling when compared to monovalent binding with small molecule drugs; leading to improved and/or prolonged therapeutic effects and outcomes [5,6].

Metastasis is the leading cause of cancer-related deaths and there is a need for the development of novel antimetastatic strategies. Metastasis is a complex multistep process during which cancer cells from primary tumor migrate to secondary sites to establish new tumors. Chemokines and chemokine receptors play a prominent role in facilitating the metastatic spread and in determining the sites to which specific cancers preferentially metastasize [7]. Among the chemokine receptors, C-X-C receptor 4 (CXCR4) is most commonly overexpressed in human cancers. Primary tumor cells that overexpress CXCR4 have increased tendency to metastasize to distant organs where the levels of the CXCR4 ligand CXCL12 are elevated [8]. Mounting body of evidence shows that inhibiting the CXCR4/CXCL12 axis by CXCR4 antagonists or silencing expression of CXCR4 by siRNA, provides significant antimetastatic effect in multiple cancer models [9]. In addition to the only clinically used CXCR4 antagonist (AMD3100, Plerixafor), which has been on the market since 2008, there are multiple small molecule and peptide CXCR4 antagonists in various stages of development [10–13]. Interestingly, CXCR4 antagonism of chloroquine (CQ) and its derivatives has been recently reported [14,15], offering a pathway to repurposing CQ for antimetastatic therapies.

CQ is a classic antimalarial drug that has been in clinical use for decades. CQ was developed from natural product quinine eighty years ago and is still widely used for the control of malaria worldwide. Besides its antimalarial properties, a broader spectrum of CQ pharmacological activities, including anti-inflammatory and anticancer activity has been discovered and explored over the years [16,17]. CQ, and its derivatives like hydroxychloroquine (HCQ), have also been recognized as effective autophagy inhibitors that exhibit beneficial anticancer properties [18,19]. Autophagy controls cellular homeostasis by lysosomal degradation of cytoplasmic components, including invading pathogens, cytotoxic proteins and damaged organelles. In cancer,

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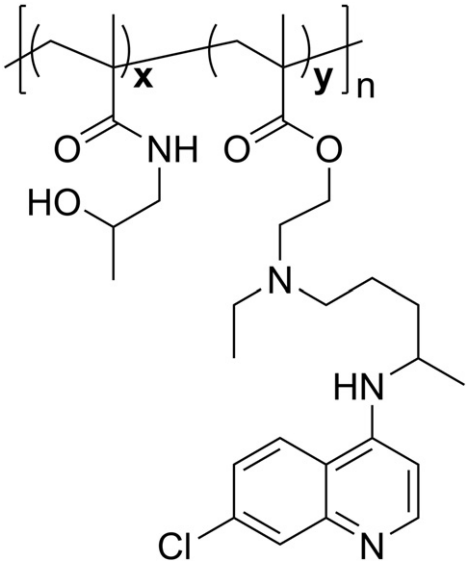
autophagy provides a survival mechanism to allow cancer cells to support proliferation during metabolic stress [20]. Inhibition of autophagy by CQ can reverse the process and suppress the proliferation of cancer cells. Although CQ and HCQ were initially tested in cancer treatment due to their ability to inhibit autophagy, we now know that their therapeutic effect depends on other mechanisms as well [21–23]. For example, a recent report revealed that CQ-induced cancer cell death was related to its inhibitory effects on cholesterol metabolism [24]. Taking advantage of its recently uncovered CXCR4 antagonism, CQ was able to inhibit CXCR4/CXCL12-mediated pancreatic cancer cell invasion and proliferation in vitro, to eliminate established tumors, and to improve overall survival when combined with gemcitabine in vivo [15]. Taken together, CQ is a promising multi-functional agent that is well-suited for development of novel combination anticancer strategies.

In this study, we report on the properties of polymeric CQ (pCQ) as a macromolecular inhibitor of cancer metastasis and a potential carrier to improve cytoplasmic drug delivery. The pCQ polymers were synthesized by a copolymerization of *N*-(2-hydroxypropyl)methacrylamide (HPMA) with methacryloylated HCQ (MA-CQ). We present data evaluating pCQ as inhibitor of cancer cell migration and invasion in vitro and its antimetastatic activity in vivo in experimental lung metastasis model of breast cancer. Intracellular trafficking of pCQ was evaluated to determine the ability of the polymers to translocate to the cytoplasm.



Scheme 1. Chemical structure of pCQ.

2. Materials and methods

2.1. Materials

Hydroxychloroquine (HCQ) sulfate (98%), triethylamine, crystal violet, DMSO-d_6 (99.8%) and chloroform-d (99.8%) were obtained from Acros Organics (Fisher Scientific, Pittsburgh, PA). Sodium acetate, dichloromethane (DCM), chloroform, methanol (MeOH), acetonitrile (HPLC grade) were from Fisher Scientific. Rhodamine isothiocyanate mixed isomers (RBITC), trifluoroacetic acid (TFA) and 2,2′-azobisisobutyronitrile (AIBN) were purchased from Sigma Aldrich (St. Louis, MO). *N*-(2-hydroxypropyl)methacrylamide (HPMA) and *N*-(3-aminopropyl)methacrylamide hydrochloride (APMA·HCl) were purchased from Polysciences (Warrington, PA). Hoechst 33,258, nitrocellulose membrane, Novex 10% Tris-Glycine Midi Protein Gels and 12% Tris-Glycine Midi Protein Gels were purchased from Invitrogen (Carlsbad, CA). Phosphate buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), sodium pyruvate, essential amino acids and non-essential amino acids were from Hyclone (Logan, UT). Fetal bovine serum (FBS) was from Atlanta Biologicals (Flowery Branch, GA). Eagle's Minimum Essential Medium (EMEM) was from ATCC (Manassas, VA). Gentamicin, enzyme-free cell dissociation buffer and F-12K medium were purchased from Gibco (Life Technologies, Grand Island, NY). Protease and phosphatase inhibitor cocktail, Pierce bicinchoninic acid (BCA) protein assay, RIPA buffer and Pierce ECL Western Blotting Substrate were purchased from ThermoScientific (Waltham, MA). LC3B antibody, phospho-p44/42 MAPK (pERK) rabbit antibody, p44/42 MAPK (ERK) rabbit antibody, GAPDH rabbit antibody and anti-rabbit IgG, HRP-linked antibody were purchased from Cell Signaling Technology (Beverly, MA). Allophycocyanin (APC) mouse B anti-human CD184 and APC mouse IgG2a, κ isotype control were purchased from BD Biosciences (San Jose, CA). Human and mouse CXCL12 were purchased from Shenandoah Biotechnology (Warwick, PA). Laemmli sample buffer and 2-mercaptoethanol were purchased from Bio-rad (Hercules, CA).

2.2. Synthesis of polymers

2.2.1. Synthesis of pCQ

The pCQ copolymers with different CQ content were synthesized as previously reported (Scheme 1) [25]. Briefly, HCQ·HCl was converted to a base form using ammonium hydroxide and HCQ was extracted into DCM and dried. MA-CQ was prepared by dropwise addition of methacryloyl chloride in chloroform to a mixture of ice-cold HCQ and triethylamine in chloroform under vigorous stirring. The reaction mixture was washed with saturated sodium bicarbonate solution and concentrated. MA-CQ was purified by flash chromatography with DCM:MeOH (10:1). The pCQ were prepared by polymerization of different molar ratios of MA-CQ and HPMA with AIBN as initiator in MeOH at 55 °C under N_2 overnight. The polymers were precipitated twice in cold diethyl ether, followed by dialysis against water for 3 days (membrane molecular weight cut-off 8000). The final polymers were obtained by lyophilization.

2.2.2. Synthesis of fluorescently labeled poly(HPMA) (F-pHPMA)

HPMA (143 mg, 1 mmol), APMA·HCl (3.8 mg, 0.02 mmol) and AIBN (8.4 mg, 0.05 mmol) were dissolved in MeOH (1 mL), purged with N_2 for 30 min, and the reaction mixture was stirred at 55 °C overnight. After double precipitation in cold diethyl ether, dialysis, and lyophilization, the polymer was obtained as white solid (pHPMA-amine, 78 mg, 53%). RBITC (10.7 mg, 0.02 mmol), pHPMA-amine (29 mg) and triethylamine (30 μL, 0.2 mmol) were dissolved in DMSO (0.5 mL) and stirred at room temperature for 48 h. The resulting solution was dialyzed against MeOH for 2 days and then against water for 5 days to remove unreacted RBITC. The F-pHPMA was obtained as dark red solid (25 mg) after lyophilization.

2.2.3. Synthesis of F-pCQ10.0

HPMA (123 mg, 0.86 mmol), APMA·HCl (3.6 mg, 0.02 mmol), MA-CQ (48.5 mg, 0.12 mmol) and AIBN (8.2 mg, 0.05 mmol) were polymerized, purified and conjugated with RBITC following the F-pHPMA procedure above to obtain fluorescently labeled pCQ10.0 (F-pCQ10.0).

^{1}H NMR (Bruker Avance-III HD 500 MHz) was used to analyze the composition of all polymers and the data were analyzed by Topspin 3.5 and MestReNova 9.0 software. The molecular weights of all polymers were analyzed by gel permeation chromatography (GPC) operated in 0.1 M sodium acetate buffer (pH 5.0) using Agilent 1260 Infinity LC system equipped with a miniDAWN TREOS multi-angle light scattering (MALS) detector and a Optilab T-rEX refractive index detector (Wyatt Technology, Santa Barbara, CA). The column used was TSKgel G3000PWXL-CP (Tosoh Bioscience LLC, King of Prussia, PA) at a flow rate of 0.5 mL/min. Results were analyzed using Astra 6.1 software from Wyatt Technology. The degree of polymerization was calculated from the GPC and ^{1}H NMR data. High-performance liquid