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Chloroquine-Containing HPMA Copolymers as Polymeric Inhibitors of Cancer Cell Migration Mediated by the CXCR4/SDF-1 Chemokine Axis

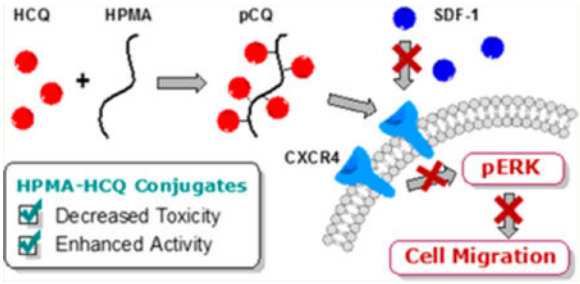
Fei Yu, **Ying Xie**, **Yan Wang**, **Zheng-Hong Peng**, **Jing Li**, and **David Oupický***

Center for Drug Delivery and Nanomedicine, Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, Nebraska 68198, United States

Abstract

Chloroquine-containing HPMA copolymers (pCQs) were synthesized for the first time by copolymerization of methacryloylated hydroxychloroquine and HPMA. The copolymers showed lower cytotoxicity when compared with hydroxychloroquine. Treatment of cancer cells with pCQ resulted in decreased surface expression of chemokine receptor CXCR4. The pCQ copolymers showed effective inhibition of CXCR4/SDF1-mediated cancer cell migration that was fully comparable with a commercial small-molecule CXCR4 antagonist AMD3100. The reported pCQ represent unique and simple polymeric drugs with potential use as part of a combination antimetastatic therapies.

Abstract



Synthetic polymers play an important role in contemporary approaches to drug delivery. Polymers are typically used as inert carriers that either physically encapsulate or covalently conjugate drugs. Alternatively, polymers can be designed and used for their inherent pharmacologic activity.[1] These so-called polymeric drugs offer multiple potential pharmaceutical benefits related to their macromolecular and multivalent nature.[2] Traditional examples of polymeric drugs include sequestrants to bind and remove harmful substances

*Corresponding Author: david.oupicky@unmc.edu.

Supporting Information

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Notes

The authors declare no competing financial interest.

from the organism.[3] Recent examples include polymeric drugs aimed at treating cancer, modulating hemostasis, or use as antiviral agents.[4]

Chemokines and chemokine receptors are actively involved in the growth and metastatic spread of multiple types of solid tumors.[5] Although tumor cells from different types of cancer have different expression profiles of chemokine receptors, CXCR4 is the most widely expressed chemokine receptor in human cancers, making it among the most promising targets within the chemokine network for novel therapies and drug delivery strategies.[6] CXCR4 and its ligand SDF-1 have been implicated in tumor aggressiveness and metastatic spread. Studies on multiple types of cancer show that inhibition of CXCR4 prevents macrophage infiltration into tumors, induces tumor growth arrest and apoptosis, and prevents metastatic spread. In hematologic malignancies, CXCR4 has a major contributing effect to chemoresistance and thus negatively affects overall survival in multiple types of leukemia, lymphoma, and multiple myeloma.[7]

Multiple small-molecule CXCR4 inhibitors are currently under clinical development either as monotherapies or in combination with other chemotherapeutics. The first FDA-approved CXCR4 inhibitor, AMD3100, is extensively tested in clinical trials for the treatment of metastatic cancer.[8] A growing number of reports also focus on development of drug delivery methods that explore inhibition of the CXCR4 axis in the treatment of cancer.[9]

Chloroquine (CQ) is a safe drug that has been in clinical use for more than 60 years.[10] In addition to its antimalarial and antirheumatoid use, CQ is known to synergistically enhance the activity of multiple anticancer drugs via its inhibitory effect on autophagy.[11] More recently, the ability of CQ and its derivatives like hydroxychloroquine (HCQ) to inhibit CXCR4 has also been recognized and successfully utilized in the treatment of several types of solid tumors.[12] For example, CQ and HCQ inhibited SDF1-mediated pancreatic cancer cell invasion and proliferation *in vitro* and contributed to the inhibition of pancreatic cancer stem cells. The CXCR4 inhibition by CQ translated into a potent antimetastatic effect *in vivo* when combined with a chemotherapeutic gemcitabine.[12b]

Despite its promise, CQ is a poor (mM) inhibitor of CXCR4. In this study, we proposed that using the polymeric drug concept in the synthesis of CQ-containing polymers will allow us to greatly improve CXCR4-inhibiting activity of CQ by taking advantage of the multivalency effect. We thus synthesized copolymers named pCQ based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) and methacryloyl-hydroxychloroquine (MA-CQ). We selected HPMA-based copolymers due to their hydrophilicity and established safety in drug delivery applications.[13]

To test our hypothesis, we synthesized pCQ as shown in Scheme 1. Chloroquine-containing monomer MA-CQ was prepared first by acylation of the primary hydroxyl of HCQ with methacryloyl chloride. MA-CQ was purified by silica column chromatography using dichloromethane and methanol as eluents and characterized by 1H NMR and mass spectrometry. Selective substitution of the hydroxyl group and the absence of substitution of the secondary alkyl/aryl amine in HCQ were confirmed by NMR analysis. Chemical shifts of the aromatic quinoline protons and protons adjacent to the secondary amine were

identical in HCQ and MA-CQ. In contrast, the chemical shifts of the methylene protons adjacent to the hydroxyl group changed from 3.55 to 4.21 ppm as a result of the esterification of the hydroxyl group in HCQ (Figures S1 and S2). The mass $[M + H]^+$ of MA-CQ was found to be 404.03 (calcd 404.21).

The MA-CQ and HPMA were copolymerized by free radical polymerization using azobis(isobutyronitrile) (AIBN) as the initiator. By changing the molar ratio of MA-CQ and HPMA in the feed, we were able to obtain pCQ copolymers with different MA-CQ content (Table 1). Negative control pHPMA with no expected pharmacologic activity was also synthesized and used in the study. Hydrolytic stability of pCQ was tested before proceeding to evaluate the copolymer activity to ensure the observed effects are due to pharmacologic activity of the polymers and not due to released HCQ. The copolymers were incubated at 37 °C in phosphate-buffered saline (pH 7.4) and 0.1 M sodium acetate (pH 5) for 3 days, and the amount of released HCQ was determined by HPLC. We observed negligible hydrolysis and release of HCQ (<1%) from both pCQ copolymers.

Biological activity of pCQ was evaluated in human T lymphoblastoid cells (Jurkat) and human Burkitt's lymphoma cells (Raji). Control groups were treated with HCQ or pHPMA. HCQ exhibits similar CXCR4 inhibitory activity as CQ and was selected to account for potential hydrolytic release of HCQ from pCQ.[12] First, we determined cytotoxicity of pCQ using the CellTiter-Blue cell viability assay (Figure 1). The cytotoxicity of pCQ was compared at equivalent concentrations with HCQ. Control pHPMA was tested at 0.017, 0.17, and 1.7 mg/mL, corresponding to the HPMA content in pCQ-10.0. As shown in Figure 1, HCQ exhibited significantly higher cytotoxicity in both cell lines than pCQ. The estimated IC50 values for HCQ were 44 μM in Jurkat cells and 100 μM in Raji cells. As expected, increasing the content of HCQ moieties in the copolymers resulted in increased toxicity as documented by the fact that pCQ-10.0 with lower HCQ content exhibited lower cytotoxicity than pCQ-16.7 (IC50 778 vs 358 μM in Jurkat cells and 340 vs 237 μM in Raji cells, respectively). pHPMA showed no significant cytotoxicity in both cell lines within the tested concentration range. On the basis of the cytotoxicity results, we selected pCQ and HCQ concentrations for all subsequent experiments such that cell viability was maintained above 70%.

Typical CXCR4 antagonists like AMD3100 bind the CXCR4 receptor present at the cell surface to prevent binding of the SDF-1 ligand. The antagonist binding then results in inhibition of the receptor internalization, in CXCR4/CXCL12 downstream signaling, and ultimately in the inhibition of cancer cell migration and invasion. In contrast, CQ and HCQ appear to function by promoting internalization of the CXCR4 receptor and its sequestration in the lysosomes, making it unavailable for SDF1-mediated chemotaxis.[12b] To evaluate the effect of pCQ on surface expression of CXCR4, we used flow cytometry to determine the amount of surface CXCR4 receptors in Jurkat and Raji cells. The cells were treated with AMD3100 (300 nM), HCQ (20 μM), and pCQ (20 μM, 100 μM HCQ equivalent) for 24 h prior to incubation with fluorescently labeled anti-CXCR4 antibody. AMD 3100 is a more potent antagonist than HCQ and was thus used at 300 nM in this study.[9b] The toxicity of HCQ limited its concentration used in this study to 20 μM. Consistent with its mechanism of action, the amount of surface CXCR4 receptors remained unchanged after treatment with

AMD3100. In contrast, all pCQ-treated cells exhibited a decrease in the surface CXCR4 expression in a concentration-dependent manner. pCQ-16.7 showed more pronounced activity than pCQ-10.0 at both tested concentrations. For example, at 20 μM equivalent HCQ concentration, the surface expression of CXCR4 was reduced by 13% when treated with pCQ-10.0 and 50% when treated with pCQ-16.7 in Jurkat cells. HCQ alone had no detectable effect at this concentration. Increasing the concentration to 100 μM HCQ equivalent, pCQ-10.0 and pCQ-16,7 treatment led to 40% and 70% decrease of surface CXCR4, respectively. No significant toxicity was observed in the cells treated with pCQ, while HCQ caused extensive cell death at 100 μM. We have validated that the decrease in surface expression of CXCR4 is due to receptor internalization and not pCQ blocking of antibody binding by observing changes in CXCR4 distribution in cells expressing GFP-CXCR4 fusion protein. Our results showed redistribution of CXCR4 from the surface of the cells to lysosomes as a result of pCQ treatment (not shown).

Next, we evaluated if a pCQ-induced decrease in cell surface CXCR4 expression results in a decrease in SDF1-induced cell migration. As discussed above, the CXCR4/SDF-1 axis plays an important role in migration of multiple cancer cells. Binding of SDF-1 to cell surface CXCR4 triggers activation of various signaling cascades, leading to cancer cell migration. We used a Boyden chamber assay to determine the effect of pCQ on CXCR4/SDF1-mediated cell migration. Cell culture inserts with 8 μm pore size were used for Jurkat cells, and 5 μm pore size inserts were used for Raji cells. The cells were treated with pCQ and allowed to migrate through the porous insert membrane to the bottom compartment in response to SDF-1 concentration gradient (20 nM). As shown in Figure 3, SDF-1 stimulation triggers a substantial increase in cell migration (13.4-fold in Jurkat and 3.7-fold in Raji cells). HCQ at 20 μM exhibited no significant inhibition of cell migration in both cell lines. In contrast, pCQ-16.7 at the same equivalent CQ concentration demonstrated significant migration inhibition (55% in Jurkat and 15% in Raji). At 100 μM CQ equivalent, pCQ-16.7 was able to completely inhibit the cancer cell migration. This outcome was fully comparable to the clinically used CXCR4 antagonist AMD3100. The copolymer with lower CQ content, pCQ-10.0, only showed significant decrease in cell migration at 100 μM (72% in Jurkat and 46% in Raji). Not surprisingly, control polymer pHPMA showed no inhibitory activity in either cell line. These results further confirm that the pCQ mechanism of action differs from the common CXCR4 antagonists. These results suggest that pCQ or HCQ do not bind to the CXCR4 receptor on the cell surface to any significant extent and that pCQ-induced CXCR4 internalization is critical for its inhibitory activity. As in Figure 2, the lack of activity of HCQ alone points to the importance of the pCQ multivalency and decreased toxicity in achieving the observed effects.

We further investigated the mechanism of action of pCQ by examining its effect on phosphorylation of ERK 1/2 (pERK), which is one of the most important downstream signaling targets upon CXCR4 activation with SDF-1.[14] Jurkat cells were treated with pCQ for 6 h, followed by 10 min incubation with SDF-1, and pERK levels were determined by Western blot. As shown in Figure 4, the levels of pERK were significantly increased after SDF-1 stimulation of the cells. Treatment with the commercial antagonist AMD3100 completely blocked the phosphorylation. pCQ-16.7 showed a marked decrease in pERK at both concentrations tested, while pCQ-10.0 only exhibited efficient phosphorylation

inhibition at a higher concentration. HCQ alone, on the other hand, showed only limited inhibitory activity at 20 μM.

In summary, we report successful synthesis of copolymers containing antimalarial drug chloroquine as polymeric drugs capable of inhibiting migration of cancer cells. The results show favorable cytotoxicity profile and the unique ability to inhibit cancer cell invasion mediated by the CXCR4/SDF-1 chemokine axis. We propose that these polymeric inhibitors of CXCR4-mediated cancer cell migration will find use as part of a combination of antimetastatic therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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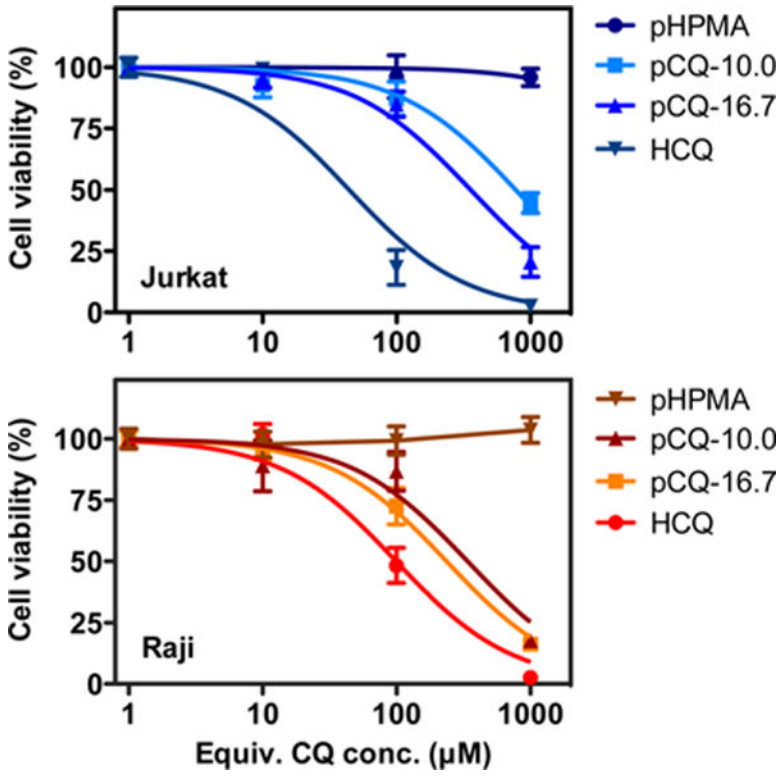


Figure 1.

Cytotoxicity of the pCQ copolymers in Jurkat and Raji cells. pCQ concentrations are plotted as equivalent molar concentrations of CQ moieties in the copolymers. pHPMA concentrations are corresponding to HPMA content in pCQ-10.0 (mean ± SD, $n = 3$).

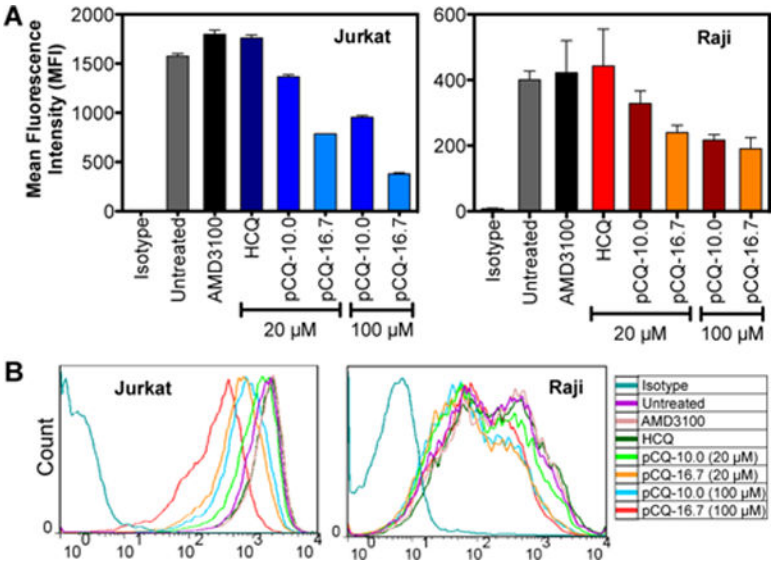


Figure 2.

Blockade of surface CXCR4 on Jurkat and Raji cells measured by flow cytometry, as shown by both (A) mean fluorescence intensity ($n = 3$) and (B) representative histogram.

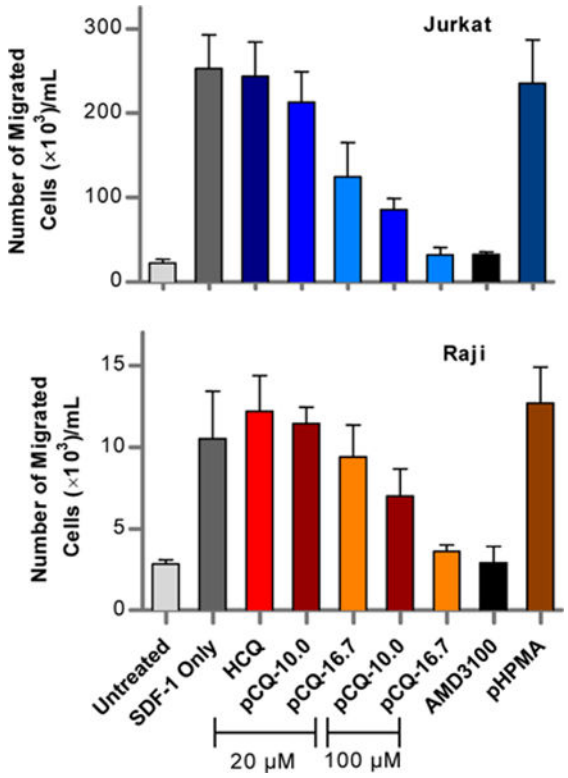


Figure 3.

Inhibition of SDF1-induced cell migration by pCQ. Jurkat and Raji cells were treated with pCQ and allowed to migrate through porous insert membranes toward 20 nM SDF-1. The cells were allowed to migrate for 4 h in the case of Jurkat cells and 18 h in the case of Raji cells. AMD3100 (300 nM) was used as a positive control. Results shown as mean number of cells that migrated through the membrane ± SD ($n = 3$).

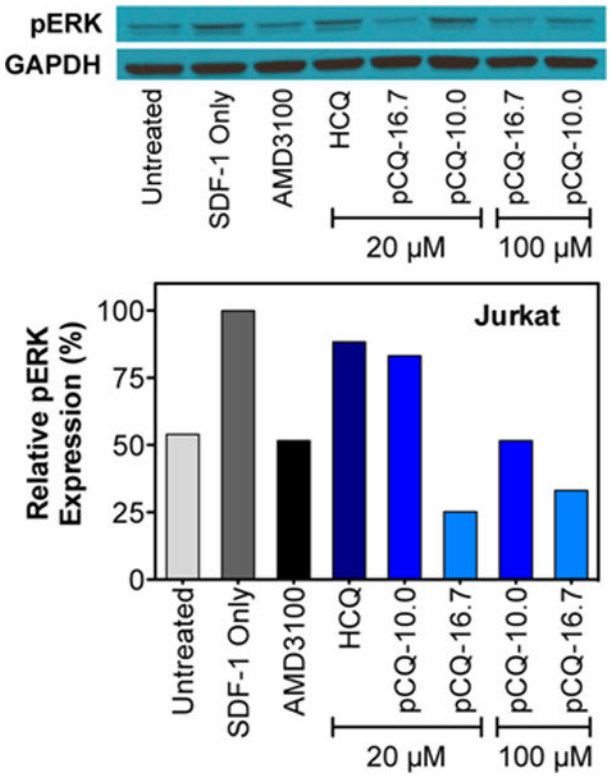
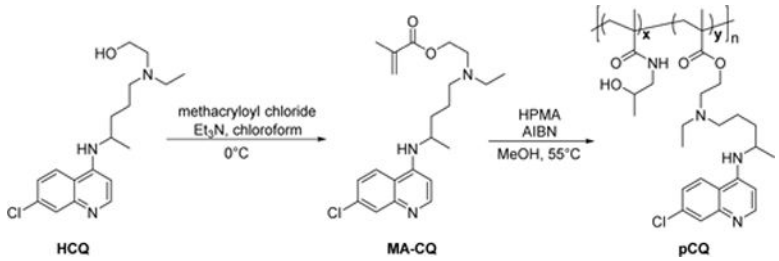


Figure 4.

Western blot analysis of pERK expression in Jurkat cells. Data are shown as relative pERK expression (%) relative to cells stimulated with SDF-1.



Scheme 1.
Synthesis of pCQ

Table 1

Polymer Characterization

	MA-CQ content (mol %)				
	in feed	in copolymer	M_n	M_w/M_n	P_n
pHPMA	0	0	47 630	2.3	333
pCQ-10.0	9.1	10.0	18 400	1.5	109
pCQ-16.7	20.0	16.7	18 900	1.8	101