Drug Delivery

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Dual-Function CXCR4 Antagonist Polyplexes To Deliver Gene Therapy and Inhibit Cancer Cell Invasion**

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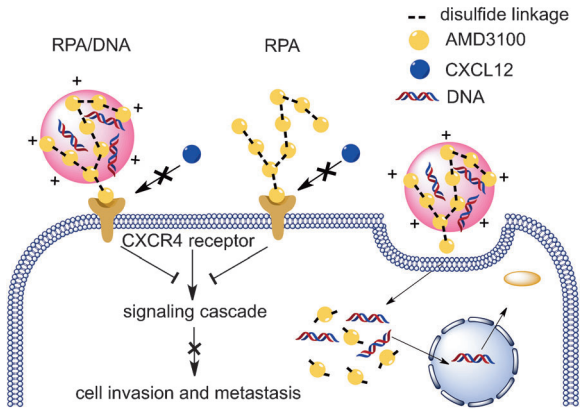
Synthetic polycations have been investigated widely as delivery vectors for nucleic acid therapeutics, such as plasmid DNA and small interfering RNA. Polycations form polyelectrolyte complexes (polyplexes) with the nucleic acids, thereby protecting them from degradation and facilitating transport across cellular membranes. Significant effort has been devoted to synthesizing polycations that have improved transfection activity and reduced toxicity.[1] Traditionally, polycations have been seen as pharmacologically inert components of delivery systems.

Herein, we propose a conceptually new approach in which polycations have both a delivery function as well as pharmacological activity, to enhance the therapeutic outcome of gene and RNA interference therapies. We describe a novel class of polycations that not only deliver plasmid DNA but also function as CXCR4 antagonists to inhibit cancer cell invasion and possibly limit metastasis (Scheme 1).



Scheme 1. Mechanism of action of dual-function polycations as CXCR4 antagonists and gene-delivery vectors.

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CXCR4 is a highly conserved transmembrane G-protein-coupled receptor that exclusively binds its ligand CXCL12. Several studies have established that cancer cells use the CXCL12/CXCR4 interaction to metastasize to distant sites.[2] Consistent with the seed-and-soil hypothesis of metastatic dissemination,[3] high levels of CXCL12 are found in sites commonly affected by metastases in cancer types known to overexpress CXCR4.[4] CXCR4 expression is often associated with poor survival and aggressive types of cancer.[5]

Among the most widely investigated CXCR4 inhibitors are cyclam derivatives,[6] including AMD3100 (Figure 1a). It is a highly specific CXCR4 antagonist, and it inhibits both

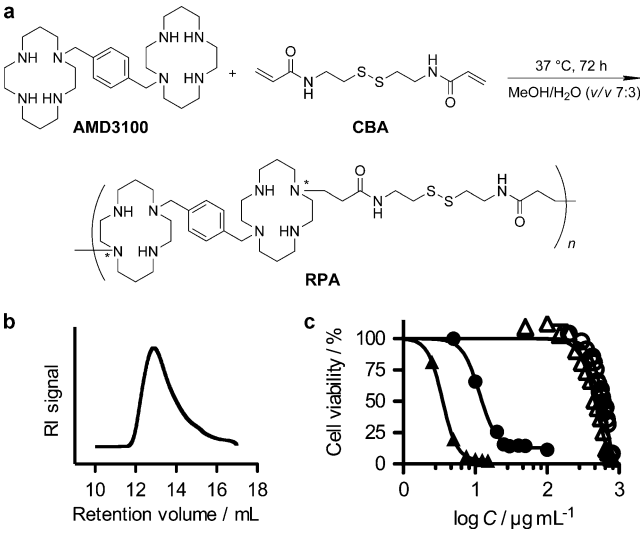

Figure 1. Synthesis and characterization of RPA. a) RPA synthesis by Michael-addition polymerization (* any secondary amine of the ring could be substituted and there could be multiple substitutions per ring); b) Size-exclusion chromatogram of RPA; c) Comparison of the cytotoxicity of RPA and PEI 25 kDa in HepG2 cells (RPA ○, PEI ●) and CXCR4$^+$ U2OS cells (RPA △, PEI ▲) determined by MTS assay.

binding and signaling of CXCL12.[7] The AMD3100 binding site on CXCR4 and its antimetastatic activity have been well characterized.[8] Not all eight amino groups of AMD3100 are required for its activity,[6a] so these groups can be used for the synthesis of the polycations described in this study while still maintaining CXCR4 inhibition.

Although several reports have described lipid-based delivery vectors that incorporate peptide-targeting ligands to increase delivery to CXCR4-overexpressing cells,[9] no attempts have been made to synthesize polymeric CXCR4 antagonists that take advantage of their antagonistic properties to enhance the delivery of gene therapies. Herein, we

synthesized such polycations, named RPA, by direct Michael-addition polymerization of AMD3100 with a disulfide-containing bisacrylamide CBA (Figure 1). Soluble RPA was synthesized using a 1:1 molar ratio of AMD3100 to CBA in MeOH/water at 37 °C. The reaction was terminated by addition of excess AMD3100 to assure the consumption of all residual acrylamides and the presence of terminal cyclam residues in the prepared RPA. RPA was purified by precipitation and extensive dialysis. The weight-average molecular weight, M_w, of the RPA used in this study was 13.9 kDa, and the polydispersity index, M_w/M_n, was 1.26 (Figure 1b). A control bioreducible polycation that lacks CXCR4 activity, called RHB, was synthesized by copolymerization of CBA with *N*,*N*-dimethylaminodipropylenetriamine as described previously[10] and had $M_w = 11.3$ kDa and $M_w/M_n = 1.95$. Cytotoxicity of RPA was measured using an MTS assay in HepG2 liver cells and in U2OS osteosarcoma cells overexpressing CXCR4 ($CXCR4^+$ U2OS; Figure 1c). In both cell lines, RPA had remarkably low toxicity compared with the 25 kDa poly(ethyleneimine) (PEI) control. The IC_{50} of RPA was almost 50 times higher than that of PEI in HepG2 cells (599 vs. 12 $\mu g\,mL^{-1}$) and 116 times higher in U2OS cells (464 vs. 4 $\mu g\,mL^{-1}$). The IC_{50} of RHB was 57 $\mu g\,mL^{-1}$ in HepG2 cells (Supporting Information, Figure S5). RPA combined with plasmid DNA formed polyplexes that were positively charged (zeta potential = 25 mV, RPA:DNA (*w*/*w*) ratio = 5) and had a relatively small size (56 nm at *w*/*w* = 5) compared with PEI (84 nm at *w*/*w* = 1.2) and RHB polyplexes (157 nm at *w*/*w* = 5; see Supporting Information, Table S1). Glutathione treatment of the RPA/DNA polyplexes triggered DNA release owing to cleavage of the disulfide bond and depolymerization of RPA (Supporting Information, Figure S4).

When CXCL12 binds to CXCR4, it induces downstream signaling through multiple pathways, including Ras and PI3 kinase. Treatment with CXCR4 antagonists not only prevents the CXCL12-induced downstream signaling but it also inhibits endocytosis of the receptor. To evaluate CXCR4 antagonism by RPA and RPA/DNA, we used a receptor redistribution assay (Figure 2). The assay uses U2OS cells stably expressing human CXCR4 receptor fused to the N-terminus of enhanced green fluorescent protein (GFP). The assay monitors cellular translocation of GFP-CXCR4 upon stimulation with CXCL12. We observed internalization of the CXCR4 receptor into endosomes in CXCL12-stimulated cells, as suggested by the punctate fluorescence distribution (Figure 2b) away from the original diffuse pattern (Figure 2a). RPA inhibited the CXCL12-triggered CXCR4 internalization in a dose-dependent manner and full inhibition was measured above 0.5 $\mu g\,mL^{-1}$ (Supporting Information, Figure S6). To exclude the possibility that this effect was caused by nonspecific electrostatic binding of RPA to the negatively charged binding site of the CXCR4 receptor, we used the control RHB but observed no CXCR4 antagonism (Figure 2f).

Next, we examined whether the polyplexes themselves exhibit CXCR4 antagonism. CXCR4 internalization was inhibited more efficiently by RPA/DNA prepared at a *w*/*w* ratio of five (2.5 $\mu g\,mL^{-1}$ total RPA) than at a *w*/*w* ratio of one

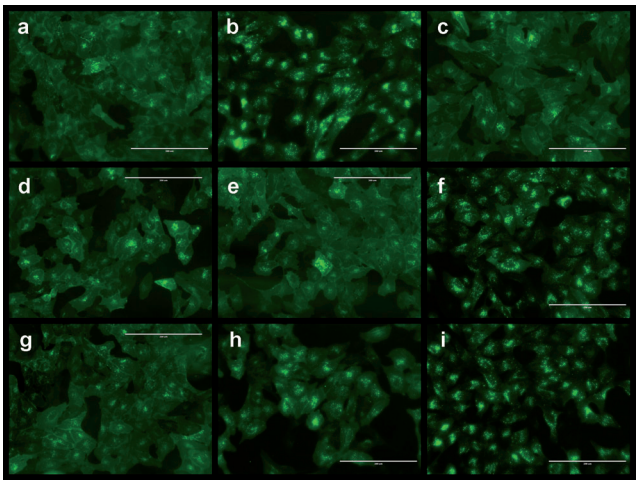

Figure 2. CXCR4 antagonism of RPA and RPA/DNA polyplexes. The CXCR4 receptor redistribution assay was conducted in U2OS cells expressing GFP-tagged CXCR4 (a). Before stimulation with 10 nM CXCL12, the cells were treated for 30 min with b) no drug; c) 0.24 $\mu g\,mL^{-1}$ AMD3100·8 HCl; d) 1.5 $\mu g\,mL^{-1}$ RPA·HCl; e) 2.5 $\mu g\,mL^{-1}$ RPA·HCl; f) 1.5 $\mu g\,mL^{-1}$ RHB·HCl; g) RPA/DNA polyplexes (*w*/*w* ratio = 5, total RPA conc. = 2.5 $\mu g\,mL^{-1}$); h) RPA/DNA polyplexes (*w*/*w* ratio = 1, total RPA conc. = 0.5 $\mu g\,mL^{-1}$); i) RHB/DNA polyplexes (*w*/*w* ratio = 5, total RHB conc. = 2.5 $\mu g\,mL^{-1}$). Scale bars = 200 μm.

(Figure 2g,h). DNA is not fully condensed in polyplexes at a *w*/*w* ratio of one (Supporting Information, Figure S3); thus, the formulation contains only a minimal amount of free RPA, suggesting that the polyplexes themselves may inhibit CXCR4. Similar to RHB, no CXCR4 antagonism was observed with RHB/DNA polyplexes (Figure 2i), confirming the specific CXCR4 antagonism of RPA and RPA/DNA.

The CXCR4/CXCL12 axis plays a critical role in cancer metastasis, because of its function in trafficking and homing of cancer cells to organs that express high levels of CXCL12. Blocking the CXCR4/CXCL12 interaction with small-molecule antagonists suppresses metastasis in a variety of cancers.[11] Using a Matrigel invasion assay, we show that RPA and RPA/DNA effectively block CXCL12-mediated invasion of $CXCR4^+$ U2OS cells (Figure 3). Both free RPA and RPA/DNA blocked invasion of cells by 71–77%, similar to that of AMD3100 (75%). The DNA dose used in the experiment with the polyplexes (1 $\mu g\,mL^{-1}$ DNA) was in the range of typical doses used in transfection experiments. The decrease in cell invasion seen with control RHB/DNA polyplexes was not statistically significant (Supporting Information).

Having confirmed CXCR4 antagonism and inhibition of cancer cell invasion by RPA, we then evaluated its gene delivery capability (Figure 4). RPA/DNA polyplexes mediated transfection that was fully comparable with that of control PEI/DNA polyplexes in B16F10 and U2OS cell lines at a DNA dose of 2.35 $\mu g\,mL^{-1}$.

CXCR4 antagonism by RPA does not require the polymer to be internalized, which is advantageous because no intracellular barriers need to be overcome.[13] However, because the CXCR4 receptor is not internalized in the presence of an antagonist like RPA, binding of RPA/DNA to this receptor

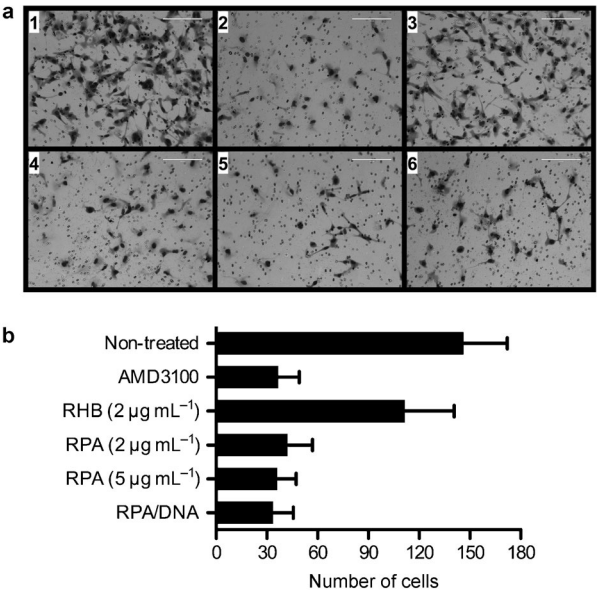

Figure 3. Inhibition of cancer cell invasion by RPA and RPA/DNA polyplexes. a) Cell invasion assay with CXCR4+ U2OS cells treated with 1) no drug; 2) 0.24 μg mL^{-1} AMD3100·8 HCl; 3) 2 μg mL^{-1} RHB·HCl; 4) 2 μg mL^{-1} RPA·HCl; 5) 5 μg mL^{-1} RPA·HCl; 6) RPA/DNA polyplexes (w/w ratio = 5, total RPA conc. = 5 μg mL^{-1}). Cells were seeded in Matrigel-coated inserts and allowed to invade towards CXCL12-containing medium for 16 h before fixation and imaging. Scale bars = 200 μm b) Quantitation of the average number of invaded cells in the 20× imaging area.

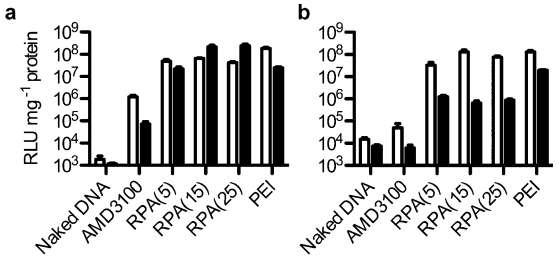

Figure 4. Transfection activity of RPA/DNA polyplexes prepared at w/w ratios of 5, 15, and 25 in the absence (white) and the presence (black) of 10% FBS in a) B16F10 and b) CXCR4+ U2OS cells. (Results are shown as mean luciferase expression in RLU mg^{-1} protein ± SD, $n = 3$).

would be unproductive for mediating transfection. Polyplex transfection by CXCR4-mediated uptake was found to be unsuitable in experiments that attempted to use AMD3100 to target PEI polyplexes to CXCR4-overexpressing cells.[12] This study found no significant increase in transfection unless phorbol myristate acetate (PMA) was used to trigger CXCR4 receptor endocytosis.[12] We found that RPA/DNA polyplexes use an alternative uptake pathway that does not require CXCR4. This result was determined by the lack of colocalization of the signal from RPA/DNA polyplexes with fluorescently labeled DNA with the membrane-anchored CXCR4 receptor (Supporting Information, Figure S9). Further support for the lack of involvement of the CXCR4 receptor in the transfection activity of RPA/DNA came from experiments showing that PMA did not enhance transfection efficiency, despite its ability to trigger internalization of the CXCR4 receptor by an alternative pathway from CXCL12 (Supporting Information, Figure S7, S8). Important for the application of dual-function polycations, we also showed concurrent CXCR4 inhibition and transfection with RPA/DNA polyplexes (Supporting Information, Figure S10). These findings support our proposed mechanism of action in which a small part of the free RPA inhibits CXCR4 while the rest of the RPA/DNA polyplex formulation participates in transfection, most likely through nonspecific charge-mediated uptake.

In summary, we have described the preparation of a synthetic polycation that functions as a CXCR4 antagonist capable of blocking cancer cell invasion while simultaneously delivering plasmid DNA and mediating transfection. Such dual-function delivery vectors could enhance antimetastatic efficacy of a variety of cancer gene therapy methods.

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