HuR-targeted nanotherapy in combination with AMD3100 suppresses CXCR4 expression, cell growth, migration and invasion in lung cancer

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The CXCR4 chemokine receptor has an important role in cancer cell metastasis. The CXCR4 antagonist, AMD3100, has limited efficacy in controlling metastasis. HuR, an RNA-binding protein, regulates CXCR4 in cancer cells. We therefore investigated whether targeting HuR using a siRNA-based nanoparticle plus AMD3100 would suppress CXCR4 and inhibit lung cancer metastasis. We treated human H1299 lung cancer cells with HuR-specific siRNA contained in a folate-targeted lipid nanoparticle (HuR-FNP) plus AMD3100, and compared this with AMD3100 alone, HuR-FNP alone and no treatment. HuR-FNP plus AMD3100 treatment produced a G1 phase cell cycle arrest and reduced cell viability above and beyond the effects of AMD3100 alone. HuR and CXCR4 mRNA and protein expression levels were markedly reduced in all treatment groups. Phosphorylated (p) AKT protein was also reduced. P27 protein expression increased with HuR-FNP and combination treatment. Promoter-based reporter studies showed that the combination inhibited CXCR4 promoter activity more than did either treatment alone. Cell migration and invasion was significantly reduced with all treatments; the combination provided the most inhibition. Reduced matrix metalloprotease (MMP)-2 and -9 expression was associated with reduced invasion in all treatment groups. Thus, we found that combined HuR and CXCR4 targeting effectively controlled lung cancer metastasis.

INTRODUCTION

Lung cancer-related death is primarily due to disease recurrence and metastasis.1,2 Although molecularly targeted therapies for lung cancer have demonstrated efficacy, the overall 5-year survival of lung cancer patients continues to be dismal.1,3 Therefore, new and improved therapies that can effectively control metastasis will reduce the incidence of mortality and increase the disease-free survival of patients diagnosed with lung cancer.

Studies have shown that cellular signaling between the chemokine C-X-C receptor type 4 (CXCR4) and its ligand, the stromal cell derived factor (SDF)-1, also known as chemokine ligand (CXCL)-12, has an important role in tumor growth, cell migration and invasion and metastases.4,5 High CXCR4 expression has previously been reported in several solid tumors, including non-small cell lung cancer (NSCLC).6,7 Further, the incidence of metastasis and increased risk of disease recurrence have been shown to be greater in high CXCR4-expressing lung tumors.8-11 Results from these studies demonstrate that CXCR4 is a molecular target for cancer therapy and that suppressing the SDF-1/CXCR4 signaling axis will effectively inhibit tumor metastasis.

Preclinical studies have demonstrated that inhibiting CXCR4 or the SDF-1/CXCR4 signaling axis reduced tumor cell migration, invasion and metastasis.12-15 On the basis of these results, a CXCR4 antagonist AMD3100 (Plerixafor, Mozobil), is being tested as a cancer therapeutic for reducing metastasis.5-18 Results from these studies have shown limited efficacy, warranting improved CXCR4-targeted therapeutic approaches.16-18

HuR is an RNA-binding protein that is ubiquitously expressed and belongs to the Drosophila embryonic lethal abnormal vision protein (ELAV) family.19 HuR is a nucleo-cytoplasmic shuttling protein that binds to AU-rich (ARE) elements on the 3′ untranslated region of target messenger (m) RNAs and transports the mRNAs from the nucleus to the cytoplasm. Apart from shuttling the mRNA to the cytoplasm, HuR has been shown to have an important role in mRNA stability and protein translation.20 mRNAs that have AREs at the 3′-end and are targets of HuR include oncopogenes, cytokines, chemokines, growth factors and other factors that influence tumor cell growth, angiogenesis and metastasis.21-23 Researchers have demonstrated high HuR expression in a variety of human cancers, including lung cancer.24-26 Furthermore, high HuR expression in cancer tissues was correlated with metastasis, drug resistance and poor survival.24-27 More recently, CXCR4 was shown to be a target of, and regulated by HuR.28 All of these reports suggest that HuR is a promising target for cancer therapy, and inhibiting HuR should produce anti-tumor and anti-metastatic effects.

In the present study, we investigated whether inhibiting HuR using a siRNA-based nanoparticle in combination with AMD3100 would effectively reduce CXCR4 and provide enhanced inhibition on lung cancer cell survival, cell migration and invasion.
Here, we demonstrate that combined targeting of HuR and CXCR4 has enhanced inhibitory effects on lung metastasis. Our results warrant further testing of the combination of HuR-FNP and AMD3100 as a means of controlling lung cancer metastasis.

MATERIALS AND METHODS

Synthesis and characterization of nanoparticles

Cationic lipid nanoparticles (NPs) were synthesized using 20 mM DOTAP: cholesterol (Chol.) (Avanti Polar Lipids, Alabaster, AL, USA) as previously described. For preparation of DNA or siRNA containing NPs, DOTAP: Chol (20 mM) stock solution and DNA or siRNA solution diluted in 5% dextrose in water (D5W) were mixed in equal volumes to give a final concentration of 4 mM DOTAP:Chol-DNA (1 μg) or siRNA (100 nM). A stock solution of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate (polyethylene glycol)-5000] (Folate-PEG5000 DSPE; 17 μM; Avanti Polar Lipids) was synthesized by the thin film hydration method. Folate-PEG5000 DSPE was inserted into preformed DNA or siRNA containing NPs by the post-insertion technique. Briefly, we mixed Folate-PEG5000 DSPE (0.03% of total lipid) with DNA or siRNA containing NP using vigorous pipetting, incubated the mixture at room temperature (RT) for 60 min. The NPs were then dialyzed against distilled water overnight at 4 °C. The next day, the NP solution was removed from the dialysis bag, transferred to sterile 1-ml screw-cap tubes, and labeled as FNP. The FNP that contained HuR siRNA were labeled as HuR-FNP. Those containing control siRNA were labeled as C-FNP.

An aliquot of HuR-FNP and C-FNP was used to determine the particle-size distribution and zeta potential using the Brookhaven ZetaPALS instrument as previously described. The shape and size of the NPs was analyzed with the transmission electron microscope at the Oklahoma Medical Research Foundation (OMRF) core facility, as previously described.

Cell culture

Human non-small cell lung cancer cells (H1299) were cultured in RPMI-1640 medium (GIBCO) as previously described. The cell line was authenticated at the Genetic Resource Core Resource Facility, Johns Hopkins University, Baltimore, MD. In all experiments, untreated cells served as controls.
Cells (1 × 10^5 cells/well) seeded in six-well plates were transfected with HuR-FNP (100 nM siRNA). At 6 h following transfection, the culture media was replaced with 2% serum-containing media, with or without AMD3100 (100 ng ml\(^{-1}\)). Incubation continued at 37 °C, 5% CO\(_2\). Untreated cells served as controls. At 24 and 48 h after treatment, cells were collected and used for RNA isolation. Total RNA was isolated using Trizol reagent (Life Technologies, Grand Island, NY, USA), as previously described.\(^{34}\) RNA quantity was analyzed using a Denovix DS11 spectrophotometer. Using a Quant script cDNA synthesis kit (Bio-Rad, Richmond, CA, USA), 2 μg of total RNA was subjected to reverse transcription, as previously described.\(^{34}\) Briefly, 3 μl of the synthesized complementary DNA (cDNA) was used to perform real-time (RT)-PCR (Bio-Rad CFX96 Touch Real-Time PCR Detection System) using the premix IQ SYBR green QRT-PCR kit (Bio-Rad) with human HuR-specific oligonucleotide primers (Forward-5′ AGTAAGACCACTAGGCGAGACT 3′-Sense; Reverse-5′ TGTGTTGATGTCTCTCCAGAT 3′-Antisense) and human CXCR4-specific oligonucleotide primers (Forward-5′ CCACCATCCTACTCCCT CATCTTC 3′-Sense, Reverse-5′ ACTTGTGCATAGTCTCTC 3′-AntiSense; Integrated DNA Technologies, Coralville, IA, USA). Thermal cycling was programmed as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 20 s, alternating with 62 °C for 20 s and 72 °C for 20 s. The crossing threshold (Ct) value assessed by RT-PCR was normalized with human GAPDH (Forward-5′ AGCTTCAAGATCAG CAATGCC 3′ and Reverse-5′ TGTGTTGATGTCTCTCCAGAT 3′). The changes in mRNA expression levels were expressed as fold change relative to control ± s.d. Each sample was run in triplicate. The experiments were repeated at least twice for reproducibility and statistical calculation.

Western blotting

Cells (5 × 10^5 per well) seeded in six-well plates were trypsinized and collected at 24 h and/or at 48 h after HuR-FNP (100 nM), AMD3100 (100 ng ml\(^{-1}\)) or a combination. Cell lysates were prepared and protein samples were subjected to western blotting.\(^{32–34}\) Primary antibodies against HuR, p27, MMP-2 (Santa Cruz Biotechnology, Dallas, TX, USA), CXCR4 (Abcam, Cambridge, MA, USA), MMP-9, phospho-AKT\(^{S473}\) and total AKT (Cell Signaling Technology, Inc., Beverly, MA, USA) were purchased and used. Beta-actin (Sigma Chemicals, St Louis, MO, USA) was used as an internal loading control. Proteins were detected using the appropriate secondary antibodies, as previously described.\(^{32–34}\) Protein expression levels were quantified using GeneTools (Syngene) software.

Cell migration assay

Cells (5 × 10^5) suspended in 1 ml RPMI-1640 medium were seeded in the upper chambers. After 24 h, the cells in the upper chamber were transfected with HuR-FNP in serum-free medium. After 6 h of transfection, the culture medium in the upper chamber was replaced with 2% serum-containing medium, with or without AMD3100 (100 ng ml\(^{-1}\)), while the lower chamber was replaced with 20% FBS-containing medium, and incubation continued. Cells that did not receive any treatment served as control. After incubation for 48 h, the inserts were removed, processed, and the number of migrated cells were counted using an inverted bright-field microscope as previously described.\(^{34–36}\)

Cell invasion assay

The cell invasion assay was performed using six-well Matrigel invasion chambers with 8 μm pore inserts (BD Biosciences). Cells (5 × 10^5) were seeded into the upper chambers. The lower chambers were filled with serum-free medium. After 24 h, the cells in the upper chamber were transfected with HuR-FNP in serum-free medium. After 6 h of transfection, the culture medium in the upper chamber was replaced with 2% serum-containing medium with or without AMD3100 (100 ng ml\(^{-1}\)), while the lower chamber was replaced with 20% FBS-containing medium and incubation continued. After an additional 48 h of incubation, the filters were removed, processed and the number of invaded cells counted as previously described.\(^{34–36}\)
CXCR4 promoter assay

H1299 cells (1 × 10^5 cells per well) were transfected with 1 μg of reporter plasmid carrying the human CXCR4 promoter that drives the expression of Gaussia luciferase (GLuc) gene (GLuc–ON promotor reporter clone for CXCR4; GeneCopoeia, Rockville, MD, USA). Following transfection, the cells were treated with C-FNP or HuR-FNP (100 nM siRNA) in serum-free medium. At 6 h after FNP treatment, the culture medium was replaced with 2% serum-containing medium, and incubation continued. After 24 and 48 h of FNP treatment, 10 μl of the supernatant was taken out from each sample and the luciferase activity was measured using a Secrete pair.

Figure 3. HuR-FNP plus AMD3100 treatment reduces HuR and CXCR4 mRNA and protein expression. H1299 cells were treated with AMD3100, HuR-FNP or a combination. Cells were collected at 24 and 48 h after treatment and analyzed for mRNA and protein expression using quantitative RT-PCR and western blot analysis, respectively. (a) CXCR4 mRNA was markedly reduced in all three treatment groups, with the greatest reduction observed with HuR-FNP treatment (P < 0.05). HuR mRNA was significantly reduced with HuR-FNP and HuR-FNP plus AMD3100 treatments (P < 0.05). HuR mRNA levels were reduced in the AMD3100 group, but this reduction was not significant compared with control. (b, c) Western blotting showed that all three treatments reduced CXCR4 protein expression, while only HuR-FNP and HuR-FNP plus AMD3100 reduced HuR protein expression. A slight reduction in HuR expression was observed in the AMD3100 treatment group, but this was not significant compared with control. Change in p27 and phosphorylated (p)AKT S473 expression was also observed in all three treatments compared with controls. Asterisk denote significance (P < 0.05).
Gaussia Luciferase Assay kit (GeneCopoeia) following the manufacturer’s protocol. The results from duplicate wells for each sample were calculated and represented as a percentage of luciferase expression.

To determine the ability of HuR-FNP to suppress CXCR4 promoter activity in the presence of SDF-1, cells (1 × 10^5 cells per well) were transfected with CXCR4 promoter containing plasmid (1 μg; GLuc–ON plasmid), followed by treatment with HuR-FNP (100 nM siRNA) in serum-free medium. After 6 h of HuR-FNP treatment, the culture medium was replaced with 2% serum-containing medium and incubation continued overnight. The next day, SDF-1 (100 ng ml^{-1}) was added to the cells. Aliquots of culture supernatant were collected at 0.5, 1, 2 and 4 h after SDF-1 treatment and measured for luciferase activity.

For studies determining the inhibitory activity of the combination treatment of AMD3100 and HuR-FNP on CXCR4, cells were treated with AMD3100 (100 ng ml^{-1}), HuR-FNP (100 nM siRNA) or a combination, in the presence of CXCR4 promoter containing plasmid (1 μg; GLuc–ON plasmid). The luciferase activity was measured at 24 and 48 h after treatment, as described above.
FNP uptake was observed in H1299 cells (P positive H1299 lung cancer cells. A time-dependent increase in siRNA when compared with free siRNA that was used as a control indicated that FNP eficiently encapsulated and protected the siRNA when compared with free siRNA that was used as a control. As shown in Figure 1b, agarose gel electrophoresis fi

whether the siRNA was eficiently encapsulated and protected in the FNP. Next, we determined HuR-FNP was spherical in shape (Figure 1a). Next, we determined HuR-FNP was positively charged with a zeta potential of 4.3 mV, and Physico-chemical characterization studies showed HuR-FNP was positively charged with a zeta potential of 4.3 mV, and 300 ± 10 nm in size. Transmission electron microscopy showed HuR-FNP was spherical in shape (Figure 1a). Next, we determined whether the siRNA was eficiently encapsulated and protected in the FNP. As shown in Figure 1b, agarose gel electrophoresis indicated that FNP eficiently encapsulated and protected the siRNA when compared with free siRNA that was used as a control. We next determined the siGLO-FNP uptake by folate receptor-positive H1299 lung cancer cells. A time-dependent increase in FNP uptake was observed in H1299 cells (P < 0.05; Figure 1c). That the FNP uptake was receptor-specific and was facilitated by receptor-mediated endocytosis was demonstrated by determining the fluorescence activity in Td/tomato-FNP-treated cells incubated at 37 °C and 4 °C. As shown in Figure 1d, the fluorescence activity increased over time when the FNP-treated cells were incubated at 37 °C. In contrast, the fluorescence intensity was markedly reduced when the FNP-treated cells were incubated at 4 °C. These results demonstrate that the FNP enters the cells by receptor-mediated endocytosis. To further test the specificity, we determined the uptake of FNP containing Td/tomato plasmid DNA in the presence and absence of folic acid in the tissue culture medium. A significant increase in fluorescence was observed in FNP-treated cells growing in folic-acid-deficient RPMI-1640 culture medium compared with FNP-treated cells growing in folic-acid-containing RPMI-1640 culture medium (P < 0.05; Figure 1e). The results demonstrate that our FNP are folate targeted and can eficiently deliver plasmid DNA or siRNA into the cells.

We next determined whether the FNP and AMD3100 treatment produced inhibitory activity (P < 0.05; Figure 2) at 24 and 48 h when compared with untreated control. However, HuR-FNP treatment produced more inhibition than the AMD3100 treatment at the two tested time points (P < 0.001). However, the combination of HuR-FNP and AMD3100 yielded no signifi

cant increase in the inhibition compared with HuR-FNP treatment alone. We next determined whether the FNP and AMD3100 treatments affected the target mRNA and protein expression. As shown in Figure 3a, CXCR4 mRNA expression was significantly reduced in all treatment groups at 24 and 48 h, compared with control (P < 0.002). HuR mRNA expression was also reduced all treatment groups at 24 and 48 h, compared with control (P < 0.02; Figure 3a). HuR-FNP and HuR-FNP plus AMD3100 demonstrated highest inhibition compared with control. However, there was no significant difference (P = 0.98) in the HuR mRNA inhibition between HuR-FNP and HuR-FNP plus AMD3100 at 24 h. Surprisingly, there was increased and significant suppression of HuR

**RESULTS**

Physico-chemical characterization of the HuR-FNP Physico-chemical characterization studies showed HuR-FNP was positively charged with a zeta potential of 4.3 mV, and 300 ± 10 nm in size. Transmission electron microscopy showed HuR-FNP was spherical in shape (Figure 1a). Next, we determined whether the siRNA was efficiently encapsulated and protected in the FNP. As shown in Figure 1b, agarose gel electrophoresis indicated that FNP efficiently encapsulated and protected the siRNA when compared with free siRNA that was used as a control. We next determined the siGLO-FNP uptake by folate receptor-positive H1299 lung cancer cells. A time-dependent increase in FNP uptake was observed in H1299 cells (P < 0.05; Figure 1c). That the FNP uptake was receptor-specific and was facilitated by receptor-mediated endocytosis was demonstrated by determining the fluorescence activity in Td/tomato-FNP-treated cells incubated at 37 °C and 4 °C. As shown in Figure 1d, the fluorescence activity increased over time when the FNP-treated cells were incubated at 37 °C. In contrast, the fluorescence intensity was markedly reduced when the FNP-treated cells were incubated at 4 °C. These results demonstrate that the FNP enters the cells by receptor-mediated endocytosis. To further test the specificity, we determined the uptake of FNP containing Td/tomato plasmid DNA in the presence and absence of folic acid in the tissue culture medium. A significant increase in fluorescence was observed in FNP-treated cells growing in folic-acid-deficient RPMI-1640 culture medium compared with FNP-treated cells growing in folic-acid-containing RPMI-1640 culture medium (P < 0.05; Figure 1e). The results demonstrate that our FNP are folate targeted and can efficiently deliver plasmid DNA or siRNA into the cells.

**Statistical analysis**

All experiments were performed at least twice. Unless otherwise stated, all data are shown as mean ± s.d. Data were analyzed using two-tailed Student’s t-test or one-way analysis of variance using SPSS 12.0 (SPSS, Inc., Chicago, IL, USA). P < 0.05 was considered statistically significant.
mRNA expression with combination treatment compared with HuR-FNP treatment at 48 h ($P = 0.01$).

As AMD3100, HuR-FNP, and the HuR-FNP and AMD3100 combination reduced CXCR4 and HuR mRNA expression, we next examined whether protein expression was also altered by the treatments. HuR-FNP treatment reduced HuR and CXCR4 protein expression at the two tested time points ($P < 0.01$). AMD3100 treatment reduced CXCR4 protein expression at 48 h ($P < 0.01$) but not at 24 h ($P = 0.21$) and did not demonstrate significant reduction of HuR ($P = 0.21$; Figures 3b and c). HuR-FNP plus AMD3100 treatment also reduced CXCR4 expression at 24 and 48 h ($P < 0.01$). However, the reduction in CXCR4 expression was greatest at 48 h with the combination treatment compared with individual treatments ($P < 0.001$). HuR expression, although reduced, was slightly lower after the combination treatment than observed following HuR-FNP alone (Figures 3b and c). Analysis for molecular downstream targets of HuR and CXCR4 showed a marked increase in p27 expression and reduced expression of phosphorylated (p) AKT$^{S473}$ in HuR-FNP and HuR-FNP plus AMD3100 treatment groups compared with control ($P < 0.001$; Figures 3b and c). In AMD3100 treatment group, no significant change in p27 expression was observed compared with control ($P > 0.5$). However, pAKT$^{S473}$ expression was reduced in AMD3100 treatment group compared with control ($P < 0.05$; Figures 3b and c).

HuR-FNP alone and in combination with AMD3100 induce cell cycle arrest in the G1 phase

To determine whether HuR-FNP-, AMD3100- and HuR-FNP plus AMD3100 altered the cell cycle phases, cells were subjected

Figure 6. HuR-FNP plus AMD3100 treatment inhibits cell migration and invasion. H1299 cells were treated with AMD3100, HuR-FNP or a combination, and were observed for cell migration and invasion. Untreated cells served as controls. (a) Tumor cell migration and (b) invasion was significantly inhibited by all three treatments compared with controls ($P < 0.05$). However, combination treatment produced a greater inhibitory effect on both cell migration and invasion than any individual treatment. Bars denote s.d. Asterisk denote significance ($P < 0.05$).
to flow cytometric analysis. HuR-FNP and HuR-FNP plus AMD3100 markedly induced cell cycle arrest in the G1 phase at 24 and 48 h after treatment (Figure 4). AMD3100 treatment alone produced a slight increase in the G1 phase compared with untreated control, but this difference was not statistically significant.

HuR-FNP and AMD3100 suppress CXCR4 at the promoter level
As HuR-FNP and AMD3100 treatment suppressed CXCR4 mRNA and protein expression levels, we next used a CXCR4 promoter-based reporter plasmid to investigate whether the inhibition occurred at the promoter level. First, we determined the specificity of HuR-FNP inhibitory activity by comparing with an FNP-carrying control siRNA (C-FNP). HuR-FNP, but not C-FNP, significantly reduced luciferase expression at 24 and 48 h after treatment compared with control (P < 0.05; Figure 5a). Next, we tested the ability of HuR-FNP to inhibit CXCR4 in the presence of SDF-1. HuR-FNP significantly inhibited CXCR4 expression in both the presence and absence of SDF-1 at all tested time points, compared with cells that expressed CXCR4 and were stimulated with SDF-1 (P < 0.05; Figure 5b). These results showed that HuRsiRNA specifically inhibited CXCR4 expression and could override SDF-1-mediated CXCR4 activation. Finally, we tested the inhibitory activity of the combination of HuR-FNP and AMD3100, and compared this combination with AMD3100 and HuR-FNP alone (Figure 5c). CXCR4 activity was suppressed by all treatment groups compared with control. However, maximal inhibition was observed with the combination of HuR-FNP and AMD3100 (P < 0.05), followed by HuR-FNP alone (P < 0.05) and AMD3100 alone (P < 0.05) when compared with control. In summary, our results demonstrated that both AMD3100 and HuR-FNP can inhibit CXCR4. However, combining HuR-FNP with AMD3100 provided the most inhibition of CXCR4.

DISCUSSION
Lung cancer-related deaths are primarily due to metastasis. The CXCR4 chemokine receptor has an important role in cancer metastasis and is highly expressed in lung cancer cells. Thus, inhibiting CXCR4 interaction with its ligand SDF-1 is likely to reduce metastasis. Among the several CXCR4-targeted drugs that are being developed and tested, only AMD3100 has been approved by the FDA for cancer treatment. Studies using AMD3100 have shown that the SDF-1/CXCR4 axis can be effectively inhibited, resulting in anti-metastatic activity. However, clinical study results indicate that AMD3100 is not effective in controlling tumor metastasis, which warrants testing of innovative treatment approaches such as combination therapy that can effectively inhibit CXCR4 and reduce metastasis.

In the present study, we tested the combined inhibitory activity of HuR-FNP and AMD3100 on lung cancer growth, migration and invasion. The rationale to target HuR was based on the report that showed that HuR regulated CXCR4. Thus, we hypothesized that suppressing HuR using a siRNA nanoparticle-based approach in
combination with AMD3100 should produce greater inhibition on lung cancer metastasis. Furthermore, using a tumor-targeted nanoparticle would offer selectivity and reduce cytoxicity to normal cells. Our present study showed that HuR-FNP and HuR-FNP plus AMD3100 markedly inhibited cell viability and induced G1 phase cell cycle arrest compared with AMD3100 treatment alone (P < 0.05). However, the inhibition produced by the combination treatment was comparable to that of HuR-FNP treatment alone. This observation is not surprising. A plausible explanation is that the inhibitory activity observed with the combination treatment is primarily due to HuR inhibition, as silencing HuR has previously been shown to inhibit cell proliferation and induce G1 arrest. AMD3100 is not known to produce direct cytotoxic activity. Molecular studies showed that all three treatments reduced HuR and CXCR4 protein expression to varying degree (P < 0.05). Further, expression of p27 and pAKT proteins, which are downstream targets of HuR and CXCR4, respectively, were also regulated in all three treatments demonstrating that the individual and combination treatments effectively inhibited the downstream signaling that support cell survival.

HuR-FNP plus AMD3100, when tested in cell migration and invasion assays, demonstrated a profound inhibitory activity that was greater than that observed with individual treatments. Molecular studies indicated that the enhanced inhibition on cell migration and invasion was likely due to greater inhibition of CXCR4 promoter activity and reduced MMP-2 and MMP-9 expression. Our data are consistent with previous reports in which HuR silencing was shown to inhibit CXCR4 and MMPs. Similarly, AMD3100 has been demonstrated to inhibit AKT and CXCR4 expression. Our data also indicated that both HuR-FNP and AMD3100 inhibited the promoter activity of CXCR4. Thus, the inhibition of CXCR4, AKT and MMPs that was produced by the combination of HuR-FNP and AMD3100 explains the significant reduction in cell migration and invasion. Our results clearly demonstrate that combination therapy for the treatment of metastatic lung cancer is likely to be more effective than individual treatments. However, additional combination therapy-based in vitro and in vivo studies are warranted to determine their efficacy in controlling metastasis.

In conclusion, we have demonstrated that HuR-targeted nano therapy combined with AMD3100 produced an enhanced suppression of cell growth, migration and invasion in lung cancer, and is an attractive treatment approach to control cancer metastasis.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS
RM and JP conducted the experiments. RM synthesized and characterized the nanoparticle (HuR-FNP and C-FNP). RM, JP, AC, YDZ, AM and RR conceived and designed the experiments. RM and RR wrote the paper. AC and YDZ performed statistical analysis. Together, RM, JP, AC, YDZ, AM and RR did data collection, analysis and interpretation. RM, JP, AC, YDZ, AM and RR critically reviewed, revised for intellectual content, and provided suggestions. RR supervised the project.

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