Silver Nanocarriers Targeted with a CendR Peptide Potentiate the Cytotoxic Activity of an Anticancer Drug

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Silver nanoparticles (AgNP) can be tracked in cells and tissues by optical imaging and isotopic fingerprinting. AgNPs are particularly useful as endocytosis probes as they can be rapidly dissolved with a biocompatible etching solution to eliminate extracellular NPs. Here, affinity-targeted AgNPs are evaluated as therapeutic carriers of a potent cytotoxic compound, monomethyl auristatin E (MMAE). AgNPs are coated with MMAE via a lysosomal protease cathepsin B sensitive linker and functionalized with a prototypic CendR peptide (RPAPPAR) that targets neuropilin-1 (NRP-1). This gives the AgNPs dual tumor specificity as both cathepsin B and NRP-1 are overexpressed in many types of solid tumors. Cellular imaging, flow cytometry, viability assays, and high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis show that the RPAPPAR–MMAE–AgNPs are internalized and induce apoptotic cell death in NRP-1-positive PPC-1 prostate cancer cells while sparing NRP-1-negative M21 melanoma cells. Furthermore, in a mixed culture of PPC-1 and M21 cells, RPAPPAR–MMAE–AgNP treatment selectively eliminates NRP-1-positive PPC-1 cells. The study demonstrates that affinity-targeted AgNPs can be used as carriers to selectively deliver and potentiate the activity of cytotoxic compounds in vitro, and that treatment with a biocompatible etching solution can be used to control the internalization and, thus, the cytotoxicity of these AgNPs.

1. Introduction

Nanoparticles are widely used to improve the pharmacological profiles of anticancer drugs and enhance their therapeutic efficacy.[1–3] Targeting of therapeutic NPs with affinity ligands (peptides, antibodies, low molecular weight compounds) that interact with systemically accessible malignancy-associated markers can be used to further improve selectivity and the therapeutic index of anticancer nanodrugs.[4,6] Screening of peptide phage libraries in live mice (in vivo phage display) has resulted in the identification of a panel of tumor-homing peptides, some of which are also able to extravasate and penetrate deep into the extravascular tumor tissue.[7,8] Such tumor-penetrating peptides are first recruited to tumor blood vessels through interactions with primary receptors (e.g., \(\alpha v\)-integrins, p32), followed by proteolytic activation of C-terminal R/KXXR/K C-end Rule (CendR) motif to allow peptide interaction with NRP-1 overexpressed on the plasma membrane of tumor endothelial cells and cells in tumor parenchyma to trigger extravasation and a tissue penetration cascade.[7,9—11] NPs coated with RPAPPAR, a prototypic CendR peptide, are taken up in NRP-1-positive cells via an unconventional endocytosis pathway that resembles micropinocytosis (Figure 1).[12,13]

We have developed silver NPs (AgNPs) as a model platform for targeted in vitro and in vivo delivery.[11–15] The AgNPs...
have several unique features that make them well-suited for biodistribution studies: 1) AgNPs plasmonically enhance emission from coupled fluorescent dyes to allow ultrasensitive imaging of single nanoparticles; 2) AgNPs can be observed under dark-field illumination for time-lapse studies to circumvent issues related to photobleaching; 3) the AgNP signal in fixed cells and tissues can be amplified using approaches adopted from protein silver staining methods; 4) AgNPs can be barcoded using silver isotopes for ultrasensitive ICP-MS-based ratiometric multiplex studies. Finally, the critical advantage of the silver-based nanoplatform for in vitro and in vivo targeting studies is that it allows tracking of the internalized fraction of AgNPs as the extracellular nanoparticles can be instantly removed by exposure to a biocompatible etching solution.

Here, we evaluated the suitability of the AgNP carriers for targeted delivery of cytotoxic drugs. As a payload, we used monomethyl auristatin E (MMAE), a highly potent synthetic analogue of dolastatin 10. MMAE is used as a molecular warhead on a monoclonal anti-CD30 antibody (Brentuximab vedotin) approved by the US Food and Drug Administration (FDA) for the treatment of relapsed Hodgkin’s and large cell anaplastic lymphomas. Like our drug-armed AgNPs, Brentuximab vedotin utilizes a linker with a cathepsin B cleavable motif (valine-citrulline) to increase tumor specific release of the active drug. We observed that functionalization with NRP-1-targeting RPARPAR peptide renders MMAE-AgNPs selectively cytotoxic to cells that express the receptor, while sparing cells negative for NRP-1 expression, and that etching solution can be used to further reduce unwanted cytotoxicity in nontarget cells. These data suggest that conditional precision-guided nanocarriers are well suited for delivery of peptide-linked potent cytotoxic drugs, thus warranting follow-up translational studies.

2. Results and Discussion

2.1. Synthesis and Characterization of RPARPAR–MMAE–AgNPs

In a series of studies, we have optimized the AgNP platform for affinity targeting of cultured cells and for systemic in vivo targeted delivery. We developed peptide-guided AgNPs that bind to target receptor-positive cells in vitro and have favorable in vivo circulation and affinity targeting properties. Importantly, we...
also demonstrated that extracellular AgNPs can be rapidly and safely dissolved by exposure to a hexacyanoferrate/thiosulfate redox-based etching solution both in vitro and in vivo.\(^4\)

Our goal here was to evaluate the suitability of affinity-targeted AgNPs as nanoscale carriers for targeted cellular delivery of a potent cytotoxic compound, MMAE (Figure 1). We synthesized AgNPs with an average core size of 62 \(\pm\) 20 nm (Figure 2A inset) and hydrodynamic size of 103 \(\pm\) 40 nm (Figure 2B) using the citrate method developed by Lee and Meisel.\(^5\) The size and shape of the AgNPs showed some heterogeneity (Figure 2A inset and 2B), as expected of this synthesis method. The UV–vis peak absorbance of AgNPs at 415 nm (Figure 2A) and extinction coefficient of 8.83 \(\times\) 10\(^{-9}\) M\(^{-1}\) cm\(^{-1}\) were used to calculate the concentration of AgNPs. AgNPs were coated with NeutrAvidin, functionalized with a biotinylated RPARPAR peptide, and loaded with MMAE, a potent inhibitor of tubulin polymerization that is three to five orders of magnitude more toxic than doxorubicin.\(^25,26\) To allow for the release of MMAE upon cellular internalization, we conjugated MMAE to the AgNPs through a lysosomal cathepsin B sensitive valine-citrulline linker, which is also used in the clinical MMAE–antibody conjugate.\(^19\)

The amount of MMAE conjugated to the AgNPs, determined by HPLC-MS, was 15 \(\pm\) 2 MMAE molecules per AgNP. The number of peptides per AgNP was determined indirectly by using a fluorescent reporter (biotin-fluorescein) instead of RPARPAR—there were 280 \(\pm\) 20 available biotin sites per AgNP. The zeta potential of the AgNPs (\(-0.6\) \(\pm\) 0.9 mV) falls in the range considered optimal for in vitro and in vivo delivery applications of nanoparticles.\(^27,28\) Functionalizing the AgNPs with RPARPAR and MMAE had very little effect on the zeta potential, possibly due to the stabilizing effect of the bulky NeutrAvidin and PEG molecules.

We also tested the storage stability of the AgNPs (Figure S1, Supporting Information). For this, the AgNPs were conjugated with fluorescent reporters (NHS-CF647 instead of NHS-MMAE and biotin-fluorescein instead of biotin-RPARPAR) and stored for up to 35 d at 4 °C in the dark as a solution in PBST. First, we monitored aggregation over time by spectrophotometrically measuring peak absorbance of the AgNPs; aggregation red-shifts the peak toward higher wavelengths due to the conduction electrons becoming shared between neighboring particles. After \(\approx\) 1 week the peak absorbance of the AgNPs started to gradually shift, indicating a slow but steady aggregation (Figure S1A, Supporting Information). Second, we investigated if the AgNPs release the peptide (fluorescein used as model) and/or cargo (CF647 used as model). For this, an aliquot of the AgNPs was diluted to a concentration of 0.1 \(\times\) 10\(^{-9}\) m with PBST and centrifuged in 100 kDa filters to dryness. The eluate was used for fluorescence measurements. There was no significant loss of CF647 (cargo) up to day 35, but loss of fluorescein (peptide) from the AgNPs did occur with minor spikes around days 6 and 15 (Figure S1B, Supporting Information). These results indicate that the AgNPs are stable at 4 °C for 1 week before they start to aggregate and lose their targeting moity.

Lastly, by dissolving the AgNPs with the etching solution and comparing the fluorescence signal to that of nonetched AgNPs, we determined the extent of the surface plasmon resonance (SPR) effect, which enhances the signal of surface-bound fluorophores.\(^18\) These AgNPs enhanced the signal of fluorescein and CF647 by about 3.8-fold and 2.8-fold, respectively. The SPR effect is dependent on distance, which could account for the difference in enhancement as the two fluorophores were conjugated through different conjugation chemistries.

### 2.2. RPARPAR–MMAE–AgNPs Target NRP-1-Positive Cells

Nanoparticles functionalized with RPARPAR peptide bind NRP-1 on the surface of cells, and are taken up through an unconventional endocytosis/transcytosis pathway.\(^29\) The interaction between CendR peptides, e.g., RPARPAR, and the b1b2 domain of NRP-1 has been previously characterized.\(^10\) The conjugation of MMAE had no effect on the ability of RPARPAR–AgNPs to bind the purified recombinant histidine-tagged b1b2 domain of NRP-1 immobilized on the Ni-NTA magnetic beads (Figure 3A). The interaction between RPARPAR–MMAE–AgNPs and NRP-1 was specific, and mediated by the CendR binding pocket, as no binding of the RPARPAR–MMAE–AgNPs to the mutant b1b2 domain of NRP-1 was observed.

Two cell lines were chosen for testing the NRP-1-dependent binding and internalization of RPARPAR–MMAE–AgNPs on cultured cells: NRP-1-positive PPC-1 prostate carcinoma cells...
Figure 3. NRP-1-dependent binding and internalization of RPARPAR–MMAE–AgNPs. A) RPARPAR–MMAE–AgNPs bind to the b1b2 domain of NRP-1. Recombinant wild type (wt) and mutant (mut) b1b2 domains of NRP-1 with a 6xHis-tag were coupled to Ni-NTA magnetic agarose beads. After 1 h incubation with AgNPs \( (1.5 \times 10^{-9} \text{m}) \) at 37 °C the unbound AgNPs were removed by washing; bound AgNPs in complex with NRP-1 were released from the beads with imidazole, and absorbance of the eluate in the UV–vis range was measured. Representative data are shown \( (n = 3) \); a.u. = absorbance units. B) RPAR PAR functionalization renders AgNPs and MMAE–AgNPs selective for NRP-1 binding. PPC-1 cells in suspension were incubated with \( 1.5 \times 10^{-9} \text{m} \) AgNPs at 37 °C for 1 h, washed, and resuspended in PBS for flow cytometry to measure AgNP uptake in the cells. Representative data are shown \( (n = 3) \). See Figure S3 (Supporting Information) for data on the NRP-1-negative control cell line. C) Representative dark-field image (with DAPI overlay) showing internalization of RPARPAR–MMAE–AgNPs in PPC-1 cells. Attached PPC-1 cells were incubated with \( 1.5 \times 10^{-9} \text{m} \) RPARPAR–MMAE–AgNPs (green) at 37 °C for 1 h. Extracellular AgNPs were removed by exposure to \( 10 \times 10^{-3} \text{m} \) etchant for 5 min. The cells were fixed with -20 °C MeOH, counterstained with DAPI (1 µg mL⁻¹; blue) and imaged using an inverted microscope. The image is a composite of dark-field and DAPI images; dotted lines outline the cells, arrows point to internalized AgNPs; scale bar: 20 µm. D) NRP-1-dependent internalization of RPARPAR–MMAE–AgNPs by PPC-1 cells. PPC-1 cells in suspension were incubated with \( 1.5 \times 10^{-9} \text{m} \) AgNPs at 37 °C for 1 h, washed, and analyzed by flow cytometry. Optional 5 min incubation with etchant prior to flow cytometry was used to remove extracellular surface-bound AgNPs. To study the role of the NRP-1 in the cellular interaction of particles, the cells were incubated with \( 10 \mu \text{g mL}^{-1} \) function-blocking polyclonal rabbit \( \alpha \)-NRP-1 antibody at 37 °C for 15 min prior to incubation with AgNPs. Representative data are shown \( (n = 3) \).
Table 1. Quantitation of PPC-1 cell-associated MMAE after incubation with RPARPAR–MMAE–AgNPs or controls. 5.7 × 10^5 cells in suspension were incubated with AgNPs (1.5 × 10^-9 M) or MMAE (22.5 × 10^-9 M) at 37 °C for 1 h, washed, lysed, and the lysate was treated with cathepsin B to release the detectable active form of MMAE. The lysate was cleared of cell debris by centrifugation, and the supernatant was used in the HPLC-MS analysis (n = 3 or 4).

| Compound                  | C_MMAE ± SD [× 10^-9 M] | Uptake [%] |
|---------------------------|------------------------|------------|
| MMAE                      | 4.5 ± 3                | 20         |
| MMAE-linker               | 2.4 ± 0.5              | 11         |
| MMAE–AgNPs                | 1.2 ± 0.2              | 5          |
| RPARPAR–MMAE–AgNPs        | 19.1 ± 4               | 85         |

*Standard deviation; †Percentage of input.

RPARPAR–AgNPs for which MMAE was replaced with CF555 fluorophore (Figure S6, Supporting Information).

To determine if MMAE is internalized along with the RPARPAR–AgNPs, we used HPLC-MS analysis to determine the concentration of MMAE in PPC-1 cells after incubation with RPARPAR–MMAE–AgNPs, nontargeted control MMAE–AgNPs, MMAE-linker or free MMAE (Table 1). Cathepsin B treatment was used to convert all of the MMAE in the cell lysate to a detectable active form. After 1 h incubation with RPARPAR–MMAE–AgNPs (1.5 × 10^-9 M) the concentration of MMAE in the cleared cell lysate was 19.1 ± 4 × 10^-9 M, ≈85% of the input of MMAE. In contrast, after incubation with the nontargeted MMAE–AgNPs, the MMAE concentration in the cell lysate was ≈16-fold lower than for RPARPAR-guided nanoparticles (1.2 ± 0.2 × 10^-9 M). Furthermore, the uptake for free MMAE was 4.5 ± 3 × 10^-9 M (≈20% of input) and for MMAE-linker 2.4 ± 0.5 × 10^-9 M (≈11% of input). Thus, conjugating MMAE to a linker or onto AgNPs reduced its cellular uptake, and affinity targeting of MMAE–AgNPs with RPARPAR increased drug uptake by peptide receptor-positive PPC-1 cells.

All in all, these studies show that conjugating cargo such as drugs and fluorophores to peptide-functionalized AgNPs does not interfere with the ability of the AgNPs to interact with the target receptor. Importantly, the entire RPARPAR–MMAE–AgNP complex, including the MMAE payload, effectively internalized into cells in a NRP-1-dependent mechanism suggesting that peptide-functionalized AgNPs can serve as targeted drug carriers.

2.3. RPARPAR–MMAE–AgNPs Are Selectively Cytotoxic to PPC-1 Cells

Next, we studied whether RPARPAR functionalization can be used to potentiate cytotoxic activity of MMAE–AgNPs. We used the xCELLigence real-time cell viability assay to study the effects of RPARPAR–MMAE–AgNPs and control compounds (RPARPAR–AgNPs, MMAE–AgNPs, AgNPs, free RPARPAR peptide, and MMAE-linker) on the viability of PPC-1 and M21 cells. To find an effective concentration window, PPC-1 and M21 cells were first incubated with RPARPAR–MMAE–AgNPs (0.1 × 10^-9, 0.45 × 10^-9, or 0.8 × 10^-9 M by Ag). A cytotoxic effect on PPC-1 cells was observed at 0.45 × 10^-9 M (Figure 4A); the AgNPs were
not toxic to NRP-1 negative M21 cells at this concentration, but some toxicity to M21 cells was observed when the concentration was increased to 0.8 × 10⁻⁹ M (Figure S7, Supporting Information). Whereas RPARPAR–MMAE–AgNPs at 0.45 × 10⁻⁹ M were highly cytotoxic on PPC-1 cells, the control compounds (MMAE–AgNPs, AgNPs, RPARPAR, MMAE–linker) had no effect at this concentration (for the MMAE-linker and RPARPAR controls, a dose equivalent to 0.45 × 10⁻⁹ M of AgNPs was used; Figure 4B and Figure S8, Supporting Information). RPARPAR–AgNPs had a modest (statistically insignificant) effect on the cell index, possibly due to antiadhesive effect of CendR peptides,[24] potentiating by the multivalent presentation on the AgNPs. When target PPC-1 cells were pre-incubated with a blocking α-NRP-1 antibody, a trend toward reduction of cytotoxic effects of RPARPAR–MMAE–AgNPs was seen, whereas IgG control antibody had no effect (Figure S9, Supporting Information).

The IC₅₀ values for both PPC-1 and M21 cells were determined with a CellTiter Glo Luminescent Cell Viability Assay (Table 2). Although the active form of MMAE demonstrated a slightly higher IC₅₀ value for M21 cells (2.14 × 10⁻⁹ M) compared to PPC-1 cells (8.4 × 10⁻⁹ M), this difference disappeared when MMAE was conjugated to the AgNPs, possibly due to the limited internalization properties of untargeted AgNPs (2.9 × 10⁻⁹ M for PPC-1, 2.4 × 10⁻⁹ M for M21). Importantly, targeting the drug-loaded nanoparticles with RPARPAR peptide lowered the IC₅₀ value for the PPC-1 cells considerably (0.9 × 10⁻⁹ M), while the IC₅₀ value for M21 cells increased (15.9 × 10⁻⁹ M). In other words, RPARPAR–MMAE–AgNPs are 18-fold more cytotoxic to NRP-1-positive PPC-1 cells than NRP-1-negative M21 cells. Low IC₅₀ values of MMAE–AgNPs are potentially attributable to high concentrations of AgNPs used to conduct the experiment, which could lead to aggregation and “stickyness,” effectively increasing background binding. The MMAE could then be released from noninternalized MMAE–AgNPs, as has been observed for noninternalizing extracellular matrix targeting antibody–MMAE conjugates.[122]

It should be noted that due to the toxic solvent, dimethyl sulfoxide (DMSO), needed to dissolve the MMAE-linker, high enough concentrations for a dose-response curve could not be obtained without the effect of DMSO becoming evident. Nevertheless, it was still clear that addition of the cleavable linker greatly mitigates the cytotoxic effects of MMAE. These data show that loading of MMAE on peptide-guided AgNPs potentiates the cytotoxic activity of MMAE in a target-specific manner.

Dissolution with a biocompatible etching solution can be used to modulate in vitro cellular uptake and in vivo biodistribution of extracellular AgNPs to improve specificity and signal to noise ratio, which is similar to ionic etching (cathod-exchange) of quantum dots.[124,133] Therefore, we next studied the effect of etching on the cytotoxicity of RPARPAR–MMAE–AgNPs. PPC-1 and M21 cells were incubated with 0.45 × 10⁻⁹ M RPARPAR–MMAE–AgNPs for 30 min, 1 or 3 h, followed by washing, and etching to remove extracellular particles. The cells were cultured for an additional 48 h and subjected to flow cytometry-based Annexin V/7-AAD apoptosis assay (Figure S10, Supporting Information). The duration of incubation of PPC-1 cells with RPARPAR–MMAE–AgNPs prior to etching had a profound effect on the extent of late apoptosis. Whereas only ≈5% of cells showed uptake of the late apoptosis marker 7-AAD when they were etched after a 30 min incubation with RPARPAR–MMAE–AgNPs, ≈40% of cells became 7-AAD-positive when etched after 3 h (Figure S10A, Supporting Information). In nonetched PPC-1 cells, ≈40% of cells treated with RPARPAR–MMAE–AgNPs were 7-AAD positive regardless of the duration of incubation with the particles. These data suggest that cellular internalization and, possibly, drug release in the endocytic compartment, but not plasma membrane recruitment of NPs, are the rate limiting steps for RPARPAR–MMAE–AgNP-mediated cytotoxic effects in NRP-1-positive cells. In contrast, and in agreement with the real-time viability assay, the RPARPAR–MMAE–AgNPs had only a negligible effect on the apoptosis of M21 cells (Figure S10B, Supporting Information).

To further investigate the ability of etching to decrease off-target toxicity, we compared the incidence of apoptosis in target PPC-1 versus nontarget M21 cells by calculating the cytotoxicity selectivity index (ratio of PPC-1/M21 cells in late apoptosis). Cells were incubated with free MMAE, MMAE–AgNPs, or targeted RPARPAR–MMAE–AgNPs for 1 h, subjected to optional etching, cultured for 72 h, and assessed for the markers of late apoptosis (Figure 4C). Treatment with RPARPAR–MMAE–AgNPs caused 3.1-fold more PPC-1 cells to enter late apoptosis than M21 cells. However, etching increased the selectivity index to 7.2, a marked improvement in the ability to kill target cells while decreasing off-target toxicity in cells negative for peptide receptors. As expected, etching had no statistically significant effect on the PPC-1/M21 selectivity of untargeted MMAE–AgNPs or the free drug (MMAE). These results show that removal of extracellular (free and plasma membrane-bound) particles by mild

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**Table 2.** IC₅₀ values. PPC-1 (11 000 cells per well) or M21 (3000 cells per well) cells were incubated with active MMAE, MMAE–linker, MMAE–AgNPs or RPARPAR–MMAE–AgNPs in seven different concentrations (n = 3) for 1 h in suspension at 37 °C, washed, plated onto 96-well plates, and left to attach and grow for 72 h. Results for MMAE-linker were excluded due to solubility issues. CellTiter Glo luminescence cell viability assay was used to determine cell viability; GraphPad Prism 6 was used for dose-response curve-fitting and IC₅₀ calculations. Concentrations are by MMAE; there are 15 ± 2 MMAE molecules per AgNP.

| Compound                          | PPC-1 [× 10⁻⁹ M] | M21 [× 10⁻⁹ M] |
|----------------------------------|------------------|----------------|
| MMAE                             | 8.4              | 21.4           |
| MMAE–linker                      | NA               | NA             |
| MMAE–AgNPs                       | 2.9              | 2.4            |
| RPARPAR–MMAE–AgNPs              | 0.9              | 15.9           |

*NA, not applicable.*
Figure 5. RPARKR functionalization renders MMAE–AgNPs selectively cytotoxic to NRP-1 positive PPC-1 cells in a co-culture of PPC-1-GFP and M21 cells. A) Microscopy-based assay: PPC-1-GFP (green) and M21 cells were pooled in suspension at a 1:1 ratio, plated on coverslips in a 24-well plate and left to attach for 24 h at 37 °C. The next day, the cells were incubated with targeted or nontargeted MMAE–AgNPs (0.45 × 10^{-9} μg by Ag) or MMAE (9 × 10^{-9} μg) at 37 °C for 1 h, followed by optional incubation with 10 × 10^{-3} μM etchant for 5 min, washing, and growing at 37 °C for an additional 72 h. For microscopy, the cells were fixed with MeOH at −20 °C, counterstained with DAPI (1 μg mL⁻¹; blue), and mounted on microscopy slides. Representative confocal images are shown. Scale bar: 500 μm. B) The flow cytometry-based assay was performed as in (A), except that the cells were grown without coverslips and no counterstaining with DAPI was done; instead of mounting on slides the cells were detached for analysis. One-way ANOVA with post-hoc Tukey HSD test was used to calculate p-values: ** p < 0.01; error bars show standard deviation (n = 3).

biocompatible etching can be used to reduce nonspecific activity of affinity-targeted therapeutic AgNPs.

Finally, we studied the effect of RPARKR–MMAE–AgNPs on a mixed culture of GFP-expressing PPC-1 (PPC-1-GFP) and non-fluorescent M21 cells (Figure 5). The cells were mixed in a 1:1 ratio. After a 1 h incubation with RPARKR–MMAE–AgNPs followed by a 72 h culturing period, we observed a significant reduction in the GFP-positive PPC-1 cell population (Figure 5A). Quantitative flow cytometry analysis revealed that the GFP-positive cell fraction dropped from 50% to 9.6 ± 0.8% when the cells
were treated with RPARPAR–MMAE–AgNPs, whereas the GFP-positive fraction remained at ≤50% (50.5 ± 3.3%) when the cells were left untreated (Figure 5B). The removal of extracellular RPARPAR–MMAE–AgNPs by etching reduced the cytotoxic effect on PPC-1-GFP cells (36.0 ± 1.9%). Control compounds did not have a significant impact on the PPC-1-GFP/M21 ratio. These results suggest applications for affinity-targeted therapeutic AgNPs for selective elimination of target receptor-positive cells in complex systems.

3. Conclusions

We show that the peptide-targeted silver-based nanocarrier system potentiates the cytotoxic activity of a potent anticancer drug, MMAE. Cytotoxicity experiments with a mixed culture carried out in the present study indicate that therapeutic AgNPs can be used to improve targeting selectivity in complex systems, such as 3D cell culture and in vivo models. Furthermore, treatment with a biocompatible etching solution can be used to control the colloidal status of the extracellular AgNPs in order to improve the targeting selectivity and signal-to-noise ratio.

Compared to the FDA-approved antibody–drug conjugate Brentuximab vedotin (anti-CD30 antibody carrying 3–5 molecules of MMAE), the AgNPs carry more drug molecules (≥15) and display more targeting moieties (≥280 vs 2 in the case of antibody) for increased avidity. One possible limitation of AgNPs as carriers of anticancer drugs that needs to be addressed in future studies relates to their potential off-target in vivo toxicity.[34] In addition, degradation of targeting peptides on AgNPs by serum peptidases and proteases can result in a time-dependent loss of active targeting of the nanoparticles and uptake of the particles in the organs of reticuloendothelial system. Potentially, after tumor homing of peptide-AgNPs has taken place, the dissolution of circulating/non-internalized background MMAE-AgNPs by biocompatible etchant can be used for in vivo disassembly of AgNPs to trigger renal elimination over hepatic uptake.[35]

Our studies here using AgNPs coated with NRP-1 targeting CendR peptide, RPARPAR, show the ability of peptide to target therapeutic AgNPs to the receptor-positive cells for increased cytotoxicity. However, upon systemic injection RPARPAR also targets lungs and heart in addition to tumor tissue.[32] Cryptic CendR peptides that are proteolytically activated in malignant tissue, such as iRGD and lntT1,[17,36,37] are likely better suited as targeting moieties in future in vivo studies.

4. Experimental Section

**Synthesis and Functionalization of AgNPs:** The AgNPs were synthesized according to the Lee and Meisel citrate method.[18] Surface functionalization of AgNPs was based on the method published by Braun et al.[14] wherein biotin-Ahx-RPARPAR-OH (RPARPAR; TAG Copenhagen A/S, Fredriksberg, Denmark) was used as the targeting moiety and OSA-Clu-Val-Cit-PAB (monocyclic auristatin E (MMAE-linker; Concors Biotherapeutics, California, USA) as the cargo. Briefly, AgNO3 (360 mg; #209 139, Sigma-Aldrich Co., LLC, Darmstadt, Germany) was added to ultrapure Milli-Q (MQ) water (2 L; resistivity 18 MΩ cm⁻¹) in a flask cleaned with a piranha solution (H2SO4/H2O2). Next, trisodium citrate hydrate (400 mg; #25 114, Sigma-Aldrich Co., LLC) was dissolved in MQ water (40 mL) and added to the vessel. The solution was boiled for 30 min in the dark. The resulting Ag-citrate was used directly in the next step.

Next, NeutrAvidin (NA; #31055, Thermo Scientific Inc., Washington, USA) was modified with an OPSS-PEG(5K)-SCM linker (OPSS; JenKem Technology USA Inc., Texas, USA) according to the procedure described by Braun et al.[14] Subsequently, NeutrAvidin-OPSS (3.9 mL, 2.9 mg mL⁻¹) was added to Ag-citrate (500 mL). After 2 min, 4-morpholinoneethanesulfonic acid hemisodium salt (5 mL, 0.5 M in MQ water; #MO164, Sigma-Aldrich Co., LLC) was added. The pH of the solution was adjusted to 6.0, and the solution was kept at 37 °C for 24 h. The solution was brought to room temperature (RT) and 10x phosphate buffered saline (50 mL; PBS, Naxo OU, Tartumaa, Estonia) was added, followed by Tween 20 (250 µL; #P9416, Sigma-Aldrich Co., LLC). The solution was centrifuged at 12 200 x g for 1 h (4 °C), the supernatant was removed, and the particles were resuspended in PBS (0.05% Tween 20 in PBS). Next, tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP; #646 547, Sigma-Aldrich Co., LLC) was added to a final concentration of 1 × 10⁻³ M, followed by a 10 min incubation at RT. Then, the solution was incubated at 4 °C overnight incubation at 4 °C. The particles were washed three times by centrifugation at 3500 x g for 10 min at 4 °C, followed by resuspension of the particles in PBS. Next, biotinylated peptides were coupled to the particles by adding peptide (10 µL, 2 × 10⁻⁹ M in MQ water) to AgNPs (500 µL), followed by incubation at RT for 30 min. The AgNPs were washed, 0.2 µm filtered and stored at 4 °C in the dark.

**Characterization of AgNPs:** For transmission electron microscopy (TEM), the AgNPs were diluted in MQ water, dropped onto the carbon film-covered side of a copper Cu-300 TEM grids (Agar Scientific, Ltd., Essex, UK), air-dried, and imaged with a Tecnai-10 transmission electron microscope (FEI, Oregon, USA) at 80 kV. The core size measurements were based on TEM images and the Analyze Particle Function of ImageJ (v. 1.5.1u, National Institutes of Health, USA) was used for the analysis. For size and zeta potential measurements, the AgNPs were diluted in MQ water and measured with a Zetasizer Nano ZSP (Malvern Instruments Ltd, Worcestershire, UK); refractive index of 1.33 and absorbance value of 0.2 was used, each sample was run 3 times with 12 sub-runs. The ultraviolet–vis (UV–Vis) absorbance spectrum of the AgNPs was measured with a NanoDrop 2000c spectrophotometer (Thermo Scientific Inc.).

The concentration of AgNPs was calculated according to the Beer-Lambert law by measuring the UV–Vis absorbance of AgNPs at 415 nm and using a molar attenuation coefficient of 8.83 × 10⁻⁵ cm⁻¹ M⁻¹. The number of MMAE molecules per AgNP was determined by high performance liquid chromatography-mass spectrometry analysis for a detailed protocol see the HPLC-MS Analysis section below). The number of biotin sites available for peptide conjugation per AgNP was determined fluorometrically using biotin-fluorescein (#53 608, Sigma-Aldrich Co., LLC). For this, a standard curve was constructed with 7 serial dilutions of biotin-fluorescein with known concentrations, and a linear trend line was fitted. The fluorescence signal of etched biotin-fluorescein-coupled AgNPs was used to calculate the number of biotin sites per AgNP. A Victor X5 Multilabel Plate Reader (PerkinElmer, Massachusetts, USA) with 488/515 nm filters was used for the fluorescence measurements, and all measurements were done in triplicates.

For stability studies, AgNPs conjugated with fluorescent reporters were used: NHS-CF647 (#92 135, Biotium Inc., California, USA) instead of NHS-MMAE and biotin-fluorescein (#53 608, Sigma-Aldrich Co., LLC) instead of biotin-RPARPAR. The AgNPs in PBS were stored at 4 °C in the dark. Prior to sampling, the AgNPs were dispersed briefly (≤2 min)
by sonorex

Prostate carcinoma cells (PPC-1 and its GFP-expressing sub-line PPC-1-GFP) and M21 melanoma cells were grown as attached cultures in tissue culture-treated flasks in high-glucose Dulbecco's modified Eagle medium (DMEM; Lonza Ltd, Basel, Switzerland) with added 10% fetal bovine serum (FBS; Thermo Scientific Inc.), penicillin (Thermo Scientific Inc.) and streptomycin (Thermo Scientific Inc.). Prior to use, cells were rinsed with PBS, dissociated with a PBS-based nonenzymatic dissociation buffer (Thermo Scientific Inc.), counted with a TC20 Automated Cell Counter (Bio-Rad Laboratories AB, Sundbyberg, Sweden), and diluted with supplemented DMEM as needed.

Cell Culture: Prostate carcinoma cells (PPC-1 and its GFP-expressing sub-line PPC-1-GFP) and M21 melanoma cells were grown as attached cultures in tissue culture-treated flasks in high-glucose Dulbecco's modified Eagle medium (DMEM; Lonza Ltd, Basel, Switzerland) with added 10% fetal bovine serum (FBS; Thermo Scientific Inc.), penicillin (Thermo Scientific Inc.) and streptomycin (Thermo Scientific Inc.). Prior to use, cells were rinsed with PBS, dissociated with a PBS-based nonenzymatic dissociation buffer (Thermo Scientific Inc.), counted with a TC20 Automated Cell Counter (Bio-Rad Laboratories AB, Sundbyberg, Sweden), and diluted with supplemented DMEM as needed.

HPLC-MS Analysis: PPC-1 cells in suspension (5.7 × 10^6 cells per sample) were incubated with AgNPs (500 µL per sample, 1.5 × 10^9 µM in DMEM) or MMAE (500 µL per sample, 22.5 × 10^8 µM in DMEM) at 37 °C for 1 h, washed with fresh medium, and lysed with three cycles of freeze/thaw. The cellular lysate was treated with recombinant cathepsin B (2 µL, 3.08 mg mL⁻¹; in-house expressed in E. coli) followed by depleting the active form of MMAE, and the lysate was cleared by centrifugation. An aliquot of the supernatant (150 µL) was mixed with PBS, centrifuged, and resuspended in [13C6]phenylalanine (50 µL; Cambridge Isotope Laboratories, USA) used as an internal standard, and dried with a SpeedVac (RVC 2-25 CD-plus, Micros Corporation, Washington, USA) was used for plotting graphs and calculating standard deviation.

Flow Cytometry: Cells were detached with a nonenzymatic cell dissociation buffer, followed by incubation with AgNPs (1.5 × 10^9 µM) with end-over-end mixing at 37 °C for 1 h. Finally, the cells were washed with PBS, and analyzed with a BD Accuri C6 Plus flow cytometer (BD Biosciences, New Jersey, USA).

Staining for Cathepsin B and NRP-1 Expression: After incubation with cell membraneous and intracellular AgNPs, the cells were exposed to a cell membrane impermeable staining solution (10 × 10^3 µM), 1:1 solution of tripotassium hexacyanoferrate(III) (K3Fe(CN)6; CAS# 13746-62-2, Sigma-Aldrich, Co., LLC) and sodium thiosulfate pentahydrate (Na2S2O5; CAS# 10102-17-7, Sigma-Aldrich, Co., LLC) in PBS. The staining solution was prepared and used as described earlier. During staining, the AgNPs were dissolved by oxidation with hexacyanoferrate, and the Ag⁺ ions formed a complex with the thiosulfate. After the treatment, only membrane-protected intracellular AgNPs remained intact and detectable.

FITC Annexin V / 7-AAD Apoptosis Assay: Cells were seeded onto 12-well plates at a concentration of 3–5 × 10^4 cells per well (in 1 mL) or 24-well plates at a concentration of 1–5 × 10^4 cells per well (in 0.5 mL), and left to attach at 37 °C and 5% CO₂ for 24 h. The cells were washed once with...
prewarmed complete DMEM medium, incubated with RPARRP–MMAE-AgNPs (0.45 × 10^5 μM by Ag), MMAE–AgNPs (0.45 × 10^5 μM by Ag), or MMAE (9 × 10^5 μM; MedChemExpress, Eollentuna, Sweden) diluted in medium at 37 °C for 1 h, washed twice with medium, and grown for 48 or 72 h. In experiments with etching, the cells were washed once with medium, incubated with etchant (300 μL, 10 × 10^3 μM in medium), and washed two more times prior to the growth period. For apoptosis assay, the cells were dissociated by adding PBS-based nonenzymatic dissociation buffer (250 μL) incubated for 10 min at 37 °C, and suspended with a pipette. The resulting suspension of cells was transferred to Eppendorf tubes, centrifuged at 250 g for 5 min (RT), and resuspended in 1× Annexin V Binding Buffer (300 μL; Biolegend, California, USA) or PBS. Next, FITC Annexin V (3 μL, 90 μg ml^−1; Biolegend) and/or 7-AAD containing Cell Viability Solution (3 μL, 1 mg ml^−1; BD Biosciences) was added, followed by 5 min incubation at RT. Data acquisition and analysis were carried out with a BD Accuri C6 Plus flow cytometer and the complementary software.

**Cell Viability Assay**: Real-time cytotoxicity experiments were run on the xCELLigence RTCA DP instrument (ACEA Biosciences, Inc., California, USA). Experiments were carried out in disposable 16-well xCELLigence E-Plates (ACEA Biosciences, Inc., California, USA) with microelectrodes attached to the bottom of the wells for impedance measurements. First, complete medium (50 μL) was added to each well and background impedance was measured for each well. Subsequently, complete medium (50 μL) containing 2 × 10^4 cells was added to each well and the E-plates were incubated in the RTCA DP device at 37 °C and 5% CO_2 for 24 h. The next day cells were incubated with the compounds for 1 h (where appropriate, 0.2 μg of polyclonal rabbit α-NRP-1 or rabbit IgG antibodies were added per well 15 min before compound addition), washed once, and fresh medium was added. The impedance data were automatically acquired every 30 min and expressed as the cell index (CI) value. The measurements were terminated after 63 h. All the data were recorded using RTCA software version 1.2.1. CI data from the experiments were normalized to the last data point before the addition of compounds.

**IC_{50} Determination**: A suspension of PPC-1 (11 000 cells per well) or M21 (3000 cells per well) cells in 96-well V-bottomed plates (#651 180, Greiner Bio-One GmbH, Kremsmünster, Austria) was incubated with active MMAE, MMAE-linker, MMAE–AgNPs or RPARRP–MMAE–AgNPs in suspension at 37 °C and 5% CO_2 for 1 h in suspension. Each compound was tested in seven different concentrations (3-fold serial dilutions), which were empirically chosen to include the minimum and maximum effects of the compounds, and in triplicates. After the 1 h incubation cells were washed three times with DMEM by spinning them down at 200 g for 5 min (RT) and replacing the medium. The washed suspension of cells was transferred into flat-bottomed 96-well plates with opaque walls (#655 088, Greiner Bio-One GmbH), and left to attach and grow at 37 °C and 5% CO_2 for 96 h. Thereafter, the cellTiter Glo Luminescent Cell Viability Assay (G7570, Promega Biotech AB) was used according to the manufacturer’s instructions, and luminescence signal was measured with a VICTOR Multilabel Plate Reader (PerkinElmer Inc.) using an integration time of 0.5 s. GraphPad Prism 6 (v. 6.01, GraphPad Software Inc., California, USA) software was used to analyze the data, construct a cell viability versus log of concentration (dose-response) curve and calculate the IC_{50} values. The IC_{50} values were presented as the concentration of MMAE, in the case of AgNPs with MMAE, it was estimated that there are ~15 MMAE molecules per AgNP (according to previous HPLC-MS characterization).

**Coculture Cytotoxicity Assay**: For cytotoxicity analysis of a coculture of GFP-expressing PPC-1 cells and unlabeled M21 cells, cells were plated onto 24-well plate (with coverslips for microscopy) at a concentration of 1–3 × 10^5 cells per well (0.5 mL), and left to attach at 37 °C and 5% CO_2 for 24 h. The cells were washed once with warm DMEM medium, incubated with RPARRP–MMAE–AgNPs (0.45 × 10^5 μM by Ag) or MMAE–AgNPs (0.45 × 10^5 μM by Ag) or MMAE (9 × 10^5 μM) in complete medium for 1 h at 37 °C and washed 1 to 3 times with medium. Optionally, etching solution (300 μL per well, 10 × 10^3 μM in DMEM) was added and after 5 min incubation all the wells were washed two more times with medium. The cells were grown for 72 h in DMEM (750 μL) and subjected to analysis with flow cytometry or microscopy.

For flow cytometry-based cytotoxicity analysis of the mixed culture, cells were dissociated by adding PBS-based nonenzymatic dissociation buffer (250 μL), incubated for 10 min at 37 °C, and suspended with a pipette. The suspension of cells was transferred to Eppendorf tubes, centrifuged at 250 × g at RT for 5 min, and resuspended in PBS (300 μL). Next, 7-AAD containing Cell Viability Solution (3 μL, 1 mg ml^−1; BD Biosciences) was added, followed by a 5 min incubation at RT. Measurements were carried out with a BD Aria flow cytometer (BD Biosciences) and data analysis with the complementary software.

For microscopy-based cytotoxicity analysis of the mixed culture, cells were washed two times with prewarmed medium (500 μL) and fixed with –20 °C of MeOH for 1 min. Next, the cells were washed twice with PBS, counterstained with DAPI (500 μL, 1 μg ml^−1), and washed once with PBS. Microscopy slides (76 × 26 mm) were coverslipped using an aqueous mounting medium (Fluoromount-G). Confocal imaging was performed with an Olympus FV1200MPE confocal microscope and images were analyzed with Fluoview FV10-ASW 4.0 software.

**Statistical Analysis**: Unless otherwise stated, all experiments have three replicates per group. All quantification data are expressed as mean ± standard deviation (SD). No outliers were excluded in any of the experiments. For the cytotoxicity selectivity index and early/late apoptosis experiments the data was normalized by untreated cells. The significant differences between groups were analyzed by one-way ANOVA test and Tukey’s post-hoc test using GraphPad Prism 6 (v. 6.01, GraphPad Software Inc., USA). The differences were considered statistically significant when p < 0.05; following significance symbols were used: n.s. = not significant, * p < 0.05, ** p < 0.01.

The AgNP core size measurements were based on TEM images and the Analyze Particles function of ImageJ (v. 1.51u, National Institutes of Health, USA) was used for the analysis. For determining IC_{50} values, GraphPad Prism 6 (v. 6.01, GraphPad Software Inc., USA) software was used to construct a cell viability versus log of concentration (dose-response) curve and analyze the data. The IC_{50} values were presented as the concentration of MMAE, in the case of AgNPs, it was estimated that there are 15 MMAE molecules per AgNP (according to previous HPLC-MS characterization).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

K.N.S., E.R., and T.T. are inventors of patents on CendR peptides and shareholders of CendR Therapeutics Inc., a company that holds a license for the CendR peptides and is developing the peptides for clinical use.
Keywords

homing peptides, monomethyl auristatin e, neuropilin-1, silver nanoparticles, C-end Rule