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Developing Nanoceria-Based pH-Dependent Cancer-Directed Drug Delivery System for Retinoblastoma

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Abstract

Development of a single combinatorial nano-platform technology to target cancer cells has been an unprecedented reality in boosting synergistic anti-tumor activities and in reducing off-target effects. We have designed a novel anti-tumor delivery system using a chemotherapy drug and a

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Supporting Information

Supporting information contains additional information on Experimental section, Western blotting, confocal images, and H&E histology and Figure S1-4. Supporting Information is available from the Wiley Online Library.

tumor target molecule covalently linked to cerium oxide nanoparticles (nanoceria). Nanoceria have a unique redox activity in that they possess antioxidant activity at physiological pH but have an intrinsic oxidase activity at acidic pH. Our system is integrated with (1) extracellular pH responsive functionality, (2) tumor cell targetable (CXC chemokine receptor 4, CXCR4 receptor specific) antagonist, (3) reactive oxygen species (ROS) inducible nanoceria, and (4) chemotherapeutic doxorubicin (DOX). These combinatorial nanoparticles (AMD-GCCNPs-DOX) are not only sensitive to the extracellular acidic pH conditions and targeted tumor cells but can also instantaneously induce ROS and release DOX intracellularly to enhance the chemotherapeutic activity in retinoblastoma cells (WERI-Rb-1 and Y79) and in xenograft (Y79/GFP-luc grafted) and genetic p107s (*Rb^{Lox/lox}*, *p107^{+/-}*, *p130^{-/-}*) orthotopic mice models. Together we introduce a lucidly engineered combinatorial nano-construct that offers a viable and simple strategy for delivering a cocktail of therapeutics into tumor cells under acidosis, exhibiting a promising new future for clinical therapeutic opportunities.

Short Summary

Exploiting the pH differential of the tumor microenvironment vs. the pH of non-malignant tissue is potentially useful for cancer treatment. In this study, we focused on the treatment of retinoblastoma using our nanoceria as a base-component due to its antioxidant activity at physiological pH but intrinsic oxidase activity at acidic pH. We show our development of a highly versatile, pH-responsive, and biocompatible nanoplatform that can effectively deliver a combination of therapeutics to target tumor cells.

Graphical Abstract

Keywords

Nanoceria; Doxorubicin; CXCR4 targeting; Retinoblastoma; Reactive oxygen species

1. Introduction

Several biological barriers in cancer chemotherapy often challenge the transport and accumulation of chemotherapeutics in tumor cells.^[1] Tumor microenvironment (TME),

pioneered by Rudolph Virchow in 1863,^[2] is one of the major barriers for various potential drug delivery systems (DDSs) to effectively reach tumor cells. Indeed, it is now well established that TME is responsible for tumorigenesis and metastasis through cross-talk with tumor cells.^[3] The Warburg Effect, discovered in 1920, explains how a lack of nutrients and oxygen causes tumor cells to adopt aerobic glycolysis and produce lactic acid, metabolizing glucose by an altered metabolic pathway.^[3a, 4] The lactic acidosis surrounding a solid tumor can further cause the metabolic reprogramming of tumor cells, which leads to tumor growth, proliferation, evasion, invasion, and further causes rejections and failure of cancer chemotherapeutic strategies.^[4–5] Therefore, development of a TME sensitive DDS to target the acidic TME may be an alternative for an efficient therapeutic strategy to improve cancer therapy. To this end, several stimuli (enzyme,^[6] pH,^[7] temperature,^[8] and redox^[9]) responsive DDS were developed. Most notably, pH triggered strategies were frequently utilized that showed promising improvements in cancer chemotherapeutic strategies^[9b]. Nonetheless, these cases majorly explored endosomal/lysosomal mediated drug release methodologies, and a very limited number of strategies explored TME acidosis, a driving force for tumor metastasis. Therefore, designing a TME sensitive and effective DDS would meet the need for targeting solid tumor conditions and improving the efficiency and selectivity of cancer therapeutics.

Recent studies have demonstrated irregular angiogenesis, consequent metabolic reprogramming, and an adaptive reversed pH gradient in the extracellular microenvironment of solid tumors (6.5–7.0).^[4, 10] An alkaline intracellular pH is required for the metabolic reprogramming, evasion of apoptosis, proliferation, migration, and invasion of tumor cells.^[11] Thus, tumor acidosis, an important hallmark of cancer, was exploited as a potential target in the development of cancer specific DDS. Acidosis of tumor niches leads to the reprogramming of cancer cells.^[12] In fact, a plethora of DDS were designed and developed, such as chitosan derivatives, liposomes, polyethylene glycol (PEG)-poly (β -amino ester) micelles, poly (L-histidine) micelles,^[10a, 10b] poly (methacrylic acid)-coated silica micelles, and 2,3-dimethylmaleic anhydride (DMMA) modified nanogels^[10c] to deal with the acidic TME and treat tumor conditions. However, these methodologies are challenged by complex synthetic procedures, reproducibility, and scalability. Hence, there is still an unmet need for the development of a biocompatible DDS that can integrate a targeting agent, drug, and a variety of advanced and relevant therapeutic modalities that can efficiently deliver a cocktail of therapeutics to the targeted cancer cells.

Nanoceria is a potent antioxidant that deactivates free radicals via oxidation–reduction reactions at physiological pH but behaves as an oxidase at acidic microenvironments, exhibiting cytotoxicity.^[13] C-X-C chemokine receptor 4 (CXCR4),^[14] a G-protein coupled receptor (GPCR), is activated upon attachment to its cognate ligand C-X-C chemokine ligand 12 (CXCL12), which initiates its downstream signaling pathways.^[14a–c] CXCR4 exerts various functions in different physiological and pathological conditions and is the most common chemokine receptor overexpressed in more than 23 human cancers, including breast, pancreatic, colorectal, neuroblastoma, and glioblastoma.^[15] In our current study, we found that CXCR4 was overexpressed in the eye tissues of retinoblastoma patients in comparison to those of normal eye patients. The CXCR4-CXCL12 interaction demonstrates a significant cross-talk between tumor cells and their TME.^[15–16] Therefore, it is recognized

to play a decisive role in the prognosis and metastasis of tumors. To this end, targeting the CXCR4-CXCL12 interaction and suppressing CXCR4 activity have been exploited as potential promising therapeutic strategies to inhibit tumor conditions.^[14a, 15–16] Recently, AMD11070 was discovered as an efficient and orally bioavailable inhibitor of CXCR4 with a consistent pharmacokinetic profile.^[17] A literature report demonstrated that AMD11070 could inhibit the growth, proliferation, and metastasis of gemcitabine resistant pancreatic cancer cells (GEM-R PaCa) by blocking CXCR4-CXCL12 interactions in TME.^[18] However, the improvement of a tumor targeting strategy depends not only on the primary attack to the tumor cells but also on the tumor niche-targeting efficacy of the therapeutics. Therefore, combinatorial strategies that use active targeting while considering other factors such as the cross-talk between tumor cells and their microenvironment usually show more advantages than a single strategy in cancer therapy.

Herein, we report a newly designed, highly versatile, broadly applicable, biocompatible, glycol chitosan-coated ceria nanoparticles (GCCNPs)^[13b] based ‘3-in-1’, pH-responsive, and controlled DDS that is integrated with a CXCR4 antagonist (AMD11070), nanoceria, and DOX for tumor targeted and TME responsive combination therapy. The resultant combinatorial AMD-GCCNPs-DOX are simple, highly reproducible, and scalable. They endorse three therapeutics together and can be internalized into the cells, recognized, and consequently cleaved by the intracellular glutathione (GSH) level (2–10 mM),^[9b] an intracellular reducing agent. The tethered AMD11070 ensures and enhances tumor cell specific targeting, blocks CXCR4 receptors, and enhances the anti-cancer effect of DOX via reducing its off-target effects. Blocking CXCR4 is also a promising approach for reducing the cross-talk between TME and tumor cells, which leads to the inhibition of tumor metastasis.^[16] Nanoceria has already established its oxidant property by inducing reactive oxygen species (ROS) under an acidic environment.^[13a, 19] Here, this oxidant property of nanoceria was exploited and integrated to provide an additional therapeutic modality for our nano-construct to kill the targeted tumor cells under extracellular acidic (extracellular pH= 6.5) conditions, mimicking acidic TME. We also demonstrate that the combinatorial AMD-GCCNPs-DOX nanoparticles (NPs) induced tumor cell apoptosis and reduced tumor growth *in vitro* in human retinoblastoma (Rb) cells and *in vivo* in mouse genetic *Rb^{Lox/lox} p107^{+/-} p130^{-/-}* (p107s) and human xenograft Rb models, while remaining biocompatible to normal physiological conditions. Taken together, this study highlights that our newly integrated, stimuli responsive, ‘3-in-1’ (AMD-GCCNPs-DOX) DDS significantly advances the anti-tumor activity of a widely used chemotherapeutic agent (DOX) by overcoming tumor barriers such as TME and the cell membrane. This provides a new combinatorial strategy for improving the therapeutic efficacy for Rb or other CXCR4-overexpressing tumors.

2. Results and Discussion

2.1. CXCR4 Overexpressed in Rb Specimens *in vitro* and *in vivo*

CXCR4 is a well-recognized regulator for tumor metastasis due to CXCR4-CXCL12 crosstalk between TME and tumor cells, and has proven to be a potential therapeutic target for cancer.^[16] In oncology, TME plays a critical role in the growth, evasion, and invasion of tumors. Hence, it has been a main focal point for exploitation as a trigger point to reduce or

manage tumor growth and metastasis. As one of the major characteristics of TME, acidosis plays a key role in tumor progression, metastasis, and drug resistance. In this study, we exploited AMD11070, a CXCR4 antagonist^[16, 18] with a very stable pharmacological profile,^[17a] as one of our DDS components to target CXCR4 overexpressing tumor cells and inhibit the progression of Rb by blocking CXCR4-CXCL12 interactions. To determine the potential significance of CXCR4 expression in conjunction with Rb progression, we first evaluated CXCR4 protein expression by western blotting analysis of Rb samples from both the human eye (Figure S1 in SI) and the p107s Rb mice model (Figure 1). We found a high level of CXCR4 protein expression in human retinoblastoma samples compared to normal human eye samples as demonstrated in Figure S1 of supporting information. At the cellular level, protein expression of human Rb cell lysates (WERI-Rb-1 and Y79; Figure 1a) demonstrated a significant (Figure 1d) enhancement in the CXCR4 expression compared to the normal human ARPE19 cells, which is consistent with our human tissue sample results (Figure S1, supporting information). A549, human alveolar basal epithelial cell, lysate was considered as a positive control (Figure 1a, d). Immunoblot analysis of lysates from postnatal day 30 (PND 30; Figure 1b) and 60 (PND 60; Figure 1c) of the p107s mice eye samples established CXCR4 overexpression in Rb cases compared to the age-matched normal C57BL/6 mice (wild type; WT) eye tissues (Figure 1b,c and Figure 1e,f). Notably, the CXCR4 overexpression pattern in the p107s mice model closely correlated with that of human Rb. The high expression of CXCR4 correlated with the tumor progression and metastasis. Taken together, our results indicated that CXCR4 overexpression could be a target for delivering therapeutics to the Rb cells and tissues.

2.2. Synthesis of Combinatorial AMD-GCCNPs-DOX

The GCCNPs were synthesized using standard bioconjugation techniques.^[13b] In this study, we integrated DOX and AMD11070 to GCCNPs using N, N'-Bis (acryloyl) cystamine (BAC) as a cross linker. As a proof of concept, we selected AMD11070, a CXCR4 antagonist, to target the CXCR4 receptor on the cell surface of Rb cells. The CXCR4 targeting strategy has been previously extensively exploited in targeting CXCR4 overexpressing tumor cells for cancer drug delivery.^[20] The GCCNPs were synthesized following our earlier protocol where nanoceria was conjugated to glycol chitosan to form a water-soluble complex with a large number of amine groups.^[13b] In the first step, DOX and AMD11070 containing a single free terminal amine group were separately conjugated to BAC by Michael addition^[14d] to form DOX-BAC and AMD11070-BAC, respectively. Next, these two independent compounds were conjugated to GCCNPs to generate a purple colored and highly water-soluble AMD-GCCNPs-DOX complex. This combinatorial DDS was purified by extensive dialysis using dialysis bag (12–14 kDa molecular weight cut-off, MWCO) and dialyzed against ultrapure water for 2 days (changing water every 8h) that could eliminate the small molecule impurities. The whole synthetic procedure was simple, reproducible, and scalable. In our construct, we considered disulfide linkages to conjugate DOX and AMD11070 to GCCNPs (nanoceria conjugated to glycol chitosan), which are disintegrated in the reducing environment of cytosol. Therefore, our present construct is composed of three cleavable linkages: (1) acid labile glycosidic linkage, (2) GSH sensitive disulfide linkage, and (3) intracellular protease sensitive amide linkage. Hence, the combinatorial AMD-GCCNPs-DOX is overall degradable and responsive to the intracellular

endogenous stimuli. Several studies have already proven the advantages of multifunctional nanoparticle platform technologies in reducing the progression of cancer.^[9b, 10b] However, the major challenge in assembling multiple components into one unite lies in their synthetic complexity and toxicity and in the difficulty of combining antitumor drugs that work by different molecular mechanisms. Thus, to circumvent these limitations, the rational approach may be to use very specific, stimuli responsive, and simple building blocks that can exert therapeutic values at the site of a disease. Here, the present multifunctional NP platform is generated by using a simple, reproducible, and scalable synthetic approach, which increases its clinical translational potentiality. To the best of our knowledge, this is the first report to integrate these three clinically important and viable functionalities into a single platform with targetability.

2.3. The Characterizations of Multifunctional AMD-GCCNPs-DOX

The size, morphology, and surface charge of a particle play decisive factors for cellular uptake of NPs.^[21] Due to the conjugations and changes in surface properties, the size of the AMD-GCCNPs-DOX (226.1 ± 18.87 nm) was significantly ($P < 0.05$) larger than the average diameter of GCCNPs (171.8 ± 3.71 nm, Figure 2a), but there was almost no variation in polydispersity index (PDI) between AMD-GCCNPs-DOX and GCCNPs (Figure 2b). The freeze-dried AMD-GCCNPs-DOX were readily solubilized in water to form a transparent suspension, which exhibited unimodal size distribution (Figure 2c) and a positive zeta potential ($+23.30 \pm 2.12$ mV, Figure 2c) as determined by DLS. Additionally, TEM analysis revealed the ellipsoidal morphology of AMD-GCCNPs-DOX with an average length and diameter of 100 and 30 nm respectively, along with entrapped ~ 5 nm (average diameter) ceria NPs (Figure 2d). The difference in size measurements as reflected by the results from TEM and DLS might be due to the loss of a hydration layer around the AMD-GCCNPs-DOX matrix (in dried condition) in TEM. However, AMD-GCCNPs-DOX ($+23.30 \pm 2.12$ mV) also did not show a significant difference in zeta potential compared to GCCNPs ($+18.47 \pm 2.89$ mV, Figure 2e), which may be due to the entrapment of hydrophobic drugs through a flexible linker in the core of the AMD-GCCNPs-DOX matrix. The charge distribution of AMD-GCCNPs-DOX NPs was demonstrated in Figure 2f.

Although several strategies of caging small hydrophobic drugs in a single drug delivery nano-platform have been extensively studied, their applications for intraocular tumors are limited considering the intraocular condition. Nanoceria has dual roles depending on its surrounding environment. At physiological pH, they behave as oxidant and can protect normal cells from the oxidative damage via scavenging free radicals. However, at acidic pH, they can automatically switch their role into oxidase and exhibit cytotoxicity.^[22] Therefore, in theory, nanoceria could produce different responses facing acidic TME and the physiological microenvironment of normal cells.

2.4. GSH Triggered DOX Release from AMD-GCCNPs-DOX

Successful conjugation of DOX to GCCNPs was first confirmed through full-length scanning. As shown in Figure 2g, the freeze-dried AMD-GCCNPs-DOX dissolved in pure water showed a characteristic absorbance peak at 510 nm. After digestion with concentrated hydrochloric acid (HCl) for 1 h at RT, this peak shifted to 490 nm, which matched well with

the characteristic absorbance peak of free DOX at 490 nm (Figure 2g). Literature demonstrates that the fragmentation of chitosan (basic construct of glycol chitosan) by hydrolysis of the glycosidic linkages occurs when using concentrated HCl. [23] This might lead to the degradation of the complex and release of AMD11070 and DOX. The characteristic absorbance peaks of AMD11070 and GCCNPs in AMD-GCCNPs-DOX were observed at 270 nm and 290 nm, respectively (Figure 2g).

During the synthesis of AMD-GCCNPs-DOX, the BAC-containing disulfide linkages (Figure 2h) were introduced intentionally to initiate the DOX release by intracellular redox-sensitive GSH, which could trigger the breakage of disulfide linkages. The intracellular concentration (~10 mM) of GSH is high compared to the extracellular level (2–10 μ M).^[9b] To ensure and evaluate DOX release from the combinatorial NPs, we incubated the AMD-GCCNPs-DOX with and without 10 mM GSH at 37°C over different time points (Figure 2h). The results demonstrated a controlled cumulative release pattern of DOX over 14 days as determined by intrinsic fluorescence of DOX. There was about 69% DOX cumulatively released from the combinatorial matrix by a high concentration of GSH. We found 7.5% of cumulative DOX release from AMD-GCCNPs-DOX without GSH.

2.5. pH Sensitive Surface Property of AMD-GCCNPs-DOX

Additionally, we also investigated the influence of pH on the size and surface charge of AMD-GCCNPs-DOX at physiological (pH 7.4, 1x DPBS) and acidic (pH 6.5, 1x DPBS) conditions. Acidic pH was used to mimic the acidosis of TME. The results demonstrated that at pH 7.4, the surface charges of AMD-GCCNPs-DOX were neutralized (0.54 ± 0.21 mV), which was further increased to 3.5 ± 0.13 mV at pH 6.5, although the size of AMD-GCCNPs-DOX remained the same (168.6 ± 0.51 and 168.8 ± 0.54 nm for pH 7.4 and 6.5, respectively) as demonstrated in Figure 3a. We anticipated that mild hydrolysis of amide bonds (in BAC linkages) was increased in surface amine groups. In the strong acid condition, the AMD-GCCNPs-DOX were degraded to small fragments, which might be due to the hydrolysis of glycoside and amide linkages in the combinatorial AMD-GCCNPs-DOX.

The most interesting feature of these AMD-GCCNPs-DOX NPs lies in their charge alteration from neutral to positive without any change in size on exposure from pH 7.4 to pH 6.5. Furthermore, addition of HCl (~pH 2.0) changes the AMD-GCCNPs-DOX solution from purple to orange, the color of free DOX, immediately. We speculate that such changes in surface charge and color of the solution are due to the core shell morphology of these NPs, where initially conjugated DOX molecules remain at the core in water. But as the pH further changes from 7.4 to 2.0, DOX and AMD11070 are disassembled and released from these NPs. In consequence, the amine groups of the glycol chitosan matrix are gradually exposed on the surface through the rearrangement of surface structure upon acidification, which results in the appearance of a positive charge. The combinatorial NPs developed here (AMD-GCCNPs-DOX) can serve as a multi-drug carrier where the core houses hydrophobic and aromatic-rich drug molecules (AMD11070 and DOX) together by covalent conjugation, π - π , and hydrophobic interactions. In essence, this new combinatorial therapeutic can respond to major stimuli synergistically. The drop in extracellular acidic pH, mimicking

TME, can alter the surface charge of AMD-GCCNPs-DOX and expose more DOX and AMD on the surface of these NPs. This enables specific targeting to CXCR4 overexpressing tumor cells, and upon internalization into the cell cytosol, the intracellular GSH can cleave the disulfide linkages. In order to kill tumor cells, this nanoparticle (AMD-GCCNPs-DOX) construct is also able to deliver a significant amount of ROS into the Rb cells at pH 6.5 compared to the physiological condition. One additional advantage of this new nanotechnology lies in its ability to substitute other drugs inside the NP core using the same bioconjugation techniques. Furthermore, the cross linking of hydrophobic drugs confirms the reduction of premature drug release from AMD-GCCNPs-DOX while still maintaining their functionality.

2.6. pH Dependent Drug Exposures on the Surface of AMD-GCCNPs-DOX

Next, we sought to evaluate the intrinsic fluorescence of DOX in AMD-GCCNPs-DOX NPs at different pH conditions (1x DPBS) using a microplate reader (96 well Corning costar plate, #3917). Here, free DOX and AMD-GCCNPs-DOX were taken in equal quantities in each of the transparent suspensions (buffers and plain water). We carried out a pH dependent study in two time points for 10 and 90 min (Figure 3b,c). Surprisingly, we observed that the DOX fluorescence from AMD-GCCNPs-DOX significantly increased with a decrease in pH (8.0 to 5.8), whereas free DOX did not show an increment in DOX fluorescence in the same extent with a lowering pH (pH 8.0 to 5.8) of free DOX solutions (Figure 3c). The DOX fluorescence of AMD-GCCNPs-DOX in plain water remained significantly low. To verify if the DOX fluorescence in AMD-GCCNPs-DOX was increased due to the protonation of glycol chitosan amine groups on the surface, we incubated the NPs with 60 mM HCl and found that the fluorescence intensities of DOX from these NPs were enhanced in a time dependent manner until 240 min (Figure 3d), whereas we did not find any change in plain water over the same time limit (240 min). Additionally, on measuring the fluorescence spectra, we found the same pH dependent behavior of DOX in AMD-GCCNPs-DOX (Figure 3e,f). We then further investigated if there was any alteration in the morphology of the AMD-GCCNPs-DOX in two different pH values (7.4 and 6.5) by TEM. We found that the average size and structures of AMD-GCCNPs-DOX as found from TEM analyses were comparable in both pH conditions (Figure 3g,h), which was also consistent with the DLS findings (Figure 3a). The results therefore demonstrate that AMD-GCCNPs-DOX have a core-shell in construct. After conjugation with the GCCNPs through the flexible BAC linker, we found that the conjugated hydrophobic drugs (DOX and AMD11070) remained in the core of the NPs in plain water, but with a decrease in the pH of the 1x DPBS, the glycol chitosan amines were protonated and the entrapped hydrophobic drugs were (e.g. DOX) exposed on the surface of NPs. So the AMD-GCCNPs-DOX tend to protect drugs inside the core from disintegrating in response to stimuli at physiological pH.

Furthermore, we evaluated the stability of this combinatorial AMD-GCCNPs-DOX in pH 7.4 and 6.5 conditions at 37°C (data not shown). We found that even after 5 days of incubation, the NP solution was firmly stable, and we did not find any precipitation even with FBS (70% v/v).

2.7. AMD-GCCNPs-DOX Reduced Rb Cell Viability in a pH-Dependent Manner

Upon confirmation of AMD-GCCNPs-DOX formation and controlled drug release kinetics and stability over different pH conditions, we next assessed the cytotoxicity of AMD-GCCNPs-DOX and GCCNPs towards normal ARPE19 and two Rb (WERI-Rb-1 and Y79) cell lines by MTT or WST-1 assay (Figure 4a) at two pH conditions (7.4 and 6.5) and in a dose dependent manner between 0 and 200 μ M (equivalent cerium content). Here, the extracellular pH conditions at pH 7.4 and 6.5 were used to mimic physiological and TME conditions, respectively. As demonstrated in Figure 4a, both GCCNPs and AMD-GCCNPs-DOX showed no obvious effects on the viability of normal ARPE19 retinal cells up to 100 μ M (equivalent cerium) at either of the pH conditions (7.4 and 6.5). Importantly, AMD-GCCNPs-DOX only demonstrated a little bit of toxicity against ARPE19 cells at 200 μ M (equivalent ceria) at both pH conditions. However, GCCNPs and AMD-GCCNPs-DOX demonstrated a dramatic increase in cytotoxicity against Rb cells (WERI-RB-1 and Y79) at pH 6.5 compared to pH 7.4 conditions. Furthermore, an equal cerium amount of AMD-GCCNPs-DOX displayed a more potent anti-tumor activity than that of GCCNPs, which could be related to the conjugation of AMD and DOX. Herein, we report a pH-responsive integrated DDS that uses glycol chitosan-mediated self-assembly products of GCCNP as a base vector, the chemokine receptor CXCR4 as the target of intraocular Rb, and DOX as a tumor killing molecule in order to produce a nanoceria-induced pH-dependent effect on intracellular ROS as the auxiliary anti-tumor enhancer.

Next, we used DOX as a control to evaluate the cell cytotoxicity of AMD-GCCNPs-DOX under the same culture conditions as described above by MTT or WST-1 assay (Figure 4b and Table 1). The DOX content in AMD-GCCNPs-DOX was determined using intrinsic DOX fluorescence after complete hydrolysis in HCl for 1 h at 37°C. Free DOX between 1 to 10 μ M inhibited cell growth in a dose dependent manner. Free DOX showed more toxicity at pH 7.4 than at pH 6.5 for the entire three cell lines. AMD-GCCNPs-DOX could evidently attenuate the cytotoxicity of DOX towards these 3 cell lines at pH 7.4 (Figure 4b and Table 1). On the contrary, AMD-GCCNPs-DOX displayed more potent cytotoxicity towards the tested cell lines at pH 6.5 than at pH 7.4. Relative to free DOX, AMD-GCCNPs-DOX demonstrated a significantly low IC₅₀ value for Y79 cells at pH 6.5, indicating their stronger killing ability towards Y79 cells (Table 1). These results suggest that AMD-GCCNPs-DOX have a dual role depending on pH conditions. At physiological conditions, the combinatorial AMD-GCCNPs-DOX tend to protect cells from DOX; at acidic pH, they can release DOX to kill cells.

2.8. AMD-GCCNPs-DOX Significantly Increased Production of Intracellular ROS When the pH Dropped Below Physiological Levels

The overproduction of ROS causes cell death via oxidative damage.^[24] To validate the oxidase-like (ROS inducing) activity of GCCNPs and AMD-GCCNPs-DOX, we evaluated the intracellular ROS levels at both acidic (pH 6.5) and physiological (pH 7.4) conditions in a dose dependent way from 0 to 20 μ M (equivalent to ceria) using a cell-permeable non-fluorescent DCFH-DA probe. As shown in Figure 4c, the overall quantity of intracellular ROS was induced more at pH 6.5 compared to the pH 7.4 condition for both Rb cells. Importantly, the ROS activity induced by AMD-GCCNPs-DOX at pH 6.5 was further

enhanced for these two Rb cells based on that of GCCNPs. At pH 6.5, we found a significant increase in ROS production linked to increasing concentration of both NPs in human WERI-Rb-1 and Y79 cells but not in ARPE19 cells up to 20 μ M ceria equivalent (Figure 4c). This indicates that combinatorial AMD-GCCNPs-DOX and GCCNPs could robustly induce intracellular ROS in human Rb cells. At pH 7.4, we could not find any significant induction of ROS by either NPs in ARPE19 cells or Y79 cells, whereas a little bit of ROS increase was present in human WERI-Rb-1 Rb cells. This might be related to the sensitivity of WERI-Rb-1 to ROS. This clearly indicates that the ROS induced oxidative damage by nanoceria can be more potent under an acidic environment, which can substantially enhance the cell-killing activity of AMD-GCCNPs-DOX.

2.9. Rb Cells Took More AMD-GCCNPs-DOX in Acidic Conditions

We next sought to validate internalization behavior of AMD-GCCNPs-DOX and free DOX in the human Rb cell line (Y79, Figure 5a) and in normal ARPE19 retinal cells (Figure 5b) at two pH conditions (pH 6.5 and 7.4) using confocal microscopy (Figure 5). Surprisingly, different cellular distribution patterns were observed at two different pH conditions in these two cell lines.

The free DOX incubation at pH 7.4 on Y79 cells (Figure 5a) demonstrated a more prominent red fluorescence (intrinsic fluorescence of DOX) in the nucleus compared to that at the pH 6.5 condition. On the contrary, incubation with AMD-GCCNPs-DOX showed a stronger distribution of red fluorescence in both the cytoplasm and nucleus at pH 6.5, whereas we could not find any red fluorescence in the nucleus except a very faint red fluorescence in the cytoplasm at the pH 7.4 condition. Normal ARPE19 cells incubated with free DOX exhibited similar fluorescent distribution patterns as that of Y79 at both pH conditions. However, the DOX uptake from AMD-GCCNPs-DOX by ARPE19 cells was lower than that of Y79 cells at pH 6.5. Overall, these results demonstrated that at pH 6.5, the AMD-GCCNPs-DOX were more easily internalized into Y79 cells and could be activated to release DOX in a controlled way on demand upon interaction with the acidic TME. Furthermore, this CXCR4 receptor mediated pathway plays a major role in the internalizations of the AMD-GCCNPs-DOX into CXCR4 overexpressing Y79 Rb cells. The possible mechanisms of internalization might be as follows. Upon acidosis, AMD-GCCNPs-DOX become more positively charged, which make them tend to approach the negative-charged cell membrane and then improve their interactions with the receptor CXCR4 on the cell surface using anchored AMD11070. Indeed, this close proximity to the cell surface also strengthens their interaction with the cell membrane, an important barrier of this delivery route, and further enhances the internalizations into the cells. Moreover, upon cellular internalizations, the AMD-GCCNPs-DOX can slowly release the drug that is responsive to the intracellular reducing environment. At the pH 6.5 condition, a consistent and significant DOX accumulation was maintained inside the Y79 cells even after 48 h of incubation over the free DOX. The acidosis substantially increased the uptake of AMD-GCCNPs-DOX and enhanced the accumulations of cleaved DOX from these NPs inside the cells. Meanwhile, released DOX traveled to the nucleus and induced expected apoptosis. Overall, these data indicate that AMD-GCCNPs-DOX could partially inhibit the off-target effects of DOX via

the CXCR4 antagonist-mediated active targeting and the interattraction between positive and negative charges.

2.10. CXCR4 Antagonist Inhibited the Binding Activity of AMD-GCCNPs-DOX to Rb Cells

To elucidate the CXCR4 specific targeting ability of AMD-GCCNPs-DOX in tumor cells, we next explored whether the blocking of CXCR4 on the Y79 cell surface could affect internalizations. To acquire confocal images, we cultured Y79 cells on poly-D-lysine pre-coated coverslips overnight and then blocked the receptor CXCR4 by incubating adhered Y79 cells with a CXCR4 antagonist (AMD11070) at pH 6.5 in a dose dependent manner for 1.5 h (see images for CXCR4 blocking in Figure S2, supporting information). We then incubated the AMD-GCCNPs-DOX for another 48 h (Figure 6). Interestingly, we found a gradual reduction of intracellular DOX fluorescence signal with the increase in AMD11070 doses (Figure 6). These results clearly indicate that pre-incubation with an antagonist blocks the receptor CXCR4, which further inhibits the binding site of AMD-GCCNPs-DOX. Therefore, AMD-GCCNPs-DOX internalizations in Y79 cells gradually reduce with the increase in antagonist concentration. Collectively, our data further support our hypothesis that AMD-GCCNPs-DOX are efficient at blocking the receptor CXCR4. As reported, blocking the CXCR4 receptor from CXCL12 interactions in the TME can reduce the chemokine-induced metastasis of tumors.^[14a, 14d, 15–16, 18, 20] From this study, we can also expect that these new combinatorial AMD-GCCNPs-DOX might reduce the metastasis of Rb by blocking CXCR4 receptors. We showed that the integrated AMD-GCCNPs-DOX can deliver chemotherapeutic DOX to the targeted cancer cells and subsequently release the drug in the intracellular reducing environment upon stimulation by intracellular GSH. Most importantly, this new single DDS nano-platform can be easily assembled with the therapeutically viable components mentioned above.

2.11. AMD-GCCNPs-DOX Induced Apoptosis in Y79 Cells in a pH-Dependent Way

Using flow cytometry analysis, we then examined whether the AMD-GCCNPs-DOX formulation could lead to cell death via inducing cell apoptosis using flow cytometry analysis (Figure 7a). We used free DOX treatment for a comparative study at an equivalent DOX quantity. The cell death was monitored by flow cytometry using Annexin V, a cellular marker of apoptosis. The apoptosis-inducing activity was evaluated and compared with untreated Y79 cells. Free DOX treatment (Figure 7a, b) enhanced early and late apoptosis compared to the AMD-GCCNPs-DOX treated cells at pH 7.4. On the contrary, we found that AMD-GCCNPs-DOX treatment enhanced early and late apoptosis at pH 6.5 compared to free DOX. We did not find any necrotic events. Furthermore, we observed the morphological changes of the nuclei in Y79 cells treated with AMD-GCCNPs-DOX (0–500 nM) based on DNA staining by DAPI. Interestingly, Figure 7c showed a characteristic grape-shaped nucleus morphology, a hallmark of classical nuclear apoptosis, compared to the untreated (0 nM, AMD-GCCNPs-DOX) cell nuclei. These data distinctly indicate that AMD-GCCNPs-DOX can kill tumor cells *via* initiating the apoptosis-inducing mechanism of conjugated DOX.^[25]

2.12. AMD-GCCNPs-DOX Knocked Down the CXCR4 mRNA and Protein Expressions

Next, we evaluated the CXCR4 expression in Y79 cells on exposure to free DOX and AMD-GCCNPs-DOX at two pH conditions (7.4 and 6.5). At pH 7.4, there was no such significant alteration in *CXCR4* mRNA expression in Y79 cells after 48 h of incubation with free DOX (50 nM) and AMD-GCCNPs-DOX (50 nM) relative to untreated controls (Figure 7d). On the contrary, at pH 6.5, we observed a significant ($p < 0.0001$) upregulation of *CXCR4* mRNA expression (Figure 7d) on treatment with free DOX (50 nM), which is consistent with literature.^[26] However, under the same conditions on treatment with the AMD-GCCNPs-DOX (50 nM), we found a significant downregulation ($p < 0.0001$) of *CXCR4* mRNA levels compared to the untreated controls. Furthermore, we investigated the CXCR4 protein expressions. CXCR4 protein expressions remained unaltered on treatment with free DOX (50 nM) and AMD-GCCNPs-DOX (50 nM) in the lysates of Rb cells (Y79) at pH 7.4 compared to the untreated control (Figure 7e). Conversely, at pH 6.5, we found an increase of CXCR4 protein expression in Rb cells (Y79) on treatment with free DOX and a prominent downregulation of CXCR4 expression on treatment with AMD-GCCNPs-DOX relative to the untreated control (Figure 7e). Thus, CXCR4 protein expression data correlated with the *CXCR4* mRNA expression levels. The potential induction of CXCR4 expression on treatment with free DOX, specifically at the pH 6.5 condition, definitely explores the improved response of Rb cells to AMD-GCCNPs-DOX that contain the CXCR4 antagonist (Figure 8). A number of studies reported that free DOX treatment could induce CXCR4 mRNA and protein expressions.^[26] DOX resistant human colon cancer cells also expressed CXCR4 transcript and protein expressions.^[27] The induction of CXCR4 expressions by free DOX treatment definitely indicates the significant improvement of the chemotherapeutic response of combinatorial AMD-GCCNPs-DOX in Rb cells due to integration with the CXCR4 antagonist (AMD11070).

2.13. Single AMD-GCCNPs-DOX Injection Suppressed Tumor Growth in Orthotopic Xenograft Mice Model

Although the combinatorial AMD-GCCNPs-DOX NPs were found to target Rb cells (Y79) and deliver DOX, we sought to evaluate the anti-cancer efficacy of these NPs in a xenograft mouse model of Rb. Here, we included a new control of DOX conjugated glycol chitosan (GCD) (Figure 9a), which is devoid of AMD11070 and nanoceria. At the same time, we created Y79/GFP-luc cell line by retroviral gene transfer in order to visualize the tumor growth. Before the *in vivo* study, we first evaluated the effects of AMD-GCCNPs-DOX and GCD on the growth of Y79/GFP-luc cells at both pH 7.4 and 6.5 conditions. We observed that GCD could differentially inhibit cell growth depending on its surrounding pH. Similar to GCD, AMD-GCCNPs-DOX also could suppress the growth of Y79/GFP-luc cells in a pH-dependent manner. However, the anti-tumor activities of AMD-GCCNPs-DOX (with equivalent DOX quantity) were significantly higher than that of GCD at both pH conditions with the highest prevalence at pH 6.5 (Figure 9a). These findings suggested that our combinatorial AMD-GCCNPs-DOX were more effective than monotherapy of DOX conjugates without AMD11070 and cerium at pH 6.5, leading to enhanced antitumor activity.

Next, we investigated the anti-tumor activity of AMD-GCCNPs-DOX in an Y79/GFP-luc orthotopic xenograft RB mouse model (Figure 9b, c). Saline, GCCNPs, and GCD treated animals were used as controls. A small *in vivo* imaging system was used to monitor the tumor growth in real-time. To explore this, we injected 2×10^4 Y79/GFP-luc cells into the subretinal space of one eye of the nude mice, leaving the other eye uninjected. At day 7, when *in vivo* bioluminescent imaging (BLI) showed steady photon emission (average 5×10^6 p/sec/cm²/sr), we performed intravitreal injections (2 μ l) of saline, GCCNPs (4 mg/ml; 2 μ l), GCD (0.2 μ g, 2 μ l of free DOX equivalent), and AMD-GCCNPs-DOX NPs (0.2 μ g of free DOX equivalent; 2 μ l). Thereafter, a bioluminescent signal was detected weekly by IVIS. We found that the AMD-GCCNPs-DOX treatments significantly suppressed the tumor growth by 3 weeks post injections compared to all of the controls (Figure 9b, c). GCD treated eyes also exhibited significant anti-tumor activity compared to saline and GCCNPs controls. The results indicated that GCCNPs alone could not prevent the growth of tumors *in vivo*. GCD was also not efficient enough to reduce the tumors due to the lack of a targetable entity on the surface and ceria. Overall, these observations indicated that a combination of these individual anti-tumor components in one unit (3-in-1) synergistically enhanced their anti-tumor activities.

Moreover, the mice were euthanized, and histopathology test was performed by hematoxylin and eosin (H&E) staining (Figure S3 in SI) of the whole eye. Our results illustrated that the AMD-GCCNPs-DOX injection induced more apoptosis in the tumor compared to the saline, GCCNPs, and GCD treated controls. Additionally, we observed that retina layers and lens structures were more preserved in AMD-GCCNPs-DOX treated eyes compared to the GCCNPs and GCD controls. Remarkably, we found the lens and other retinal structures were fully damaged in the saline treated control. The lens was greatly shrunk after GCCNPs treatment due to the rapid expansion of tumor cells. In general, these data indicate that the combinatorial AMD-GCCNPs-DOX possess more advantages in inhibiting tumor growth and protecting the mice eyes from the damage induced by tumors compared to the control groups.

2.14. Single AMD-GCCNPs-DOX Injection Reduced Tumor Growth in Genetic p107s Mice Model

Rb, p107, and p130 are the pocket proteins in the retinoblastoma family that suppress tumor growth.^[28] Strong evidence has established that knocking out these three genes (*Rb*^{-/-} *p107*^{+/-} *p130*^{-/-}) in p107s mice can lead to the development of Rb.^[28] A literature report also highlighted that the p107s mice model closely matches with the human Rb condition.^[28] Therefore, we chose this model to further evaluate the anti-tumor activity of combinatorial AMD-GCCNPs-DOX.

Fundus fluorescein angiography (FA) was performed to capture live images of the tumor growth events in p107s mice at PND 30 (before treatment) and PI-30 (after treatment), along with untreated controls (Figure 10). After fluorescein was intraperitoneally injected into the mice, the FA was captured to monitor the retinal vasculatures. As shown by three representative FA and bright field (BF) images, the tumor growth for GCCNPs treated eyes was comparable to that of the uninjected controls. The FA images demonstrated the leakages

in retinal blood vessels in the tumor environment. The tumor size and retinal blood vessels leakages were not reduced by the treatment with free DOX relative to pre-treatment conditions as demonstrated by BF and FA imaging, respectively. Excitingly, the AMD-GCCNPs-DOX treatments demonstrated a substantial reduction in intraocular tumor size and blood vessel leakages relative to their pre-treatment conditions as demonstrated by BF and FA funduscopy, respectively.

The *in vivo* evaluations in both the human orthotopic xenograft and genetic p107s Rb mice models clearly indicate the therapeutic potential of the combinatorial AMD-GCCNPs-DOX in inhibiting the Rb by targeting the A single AMD-GCCNPs-DOX injection was efficient enough to inhibit the growth of the tumors in both Rb mice models *in vivo*. Earlier studies find that NPs with a neutral surface charge remain highly mobile and can penetrate through the vitreous matrix more easily than the positively charged particles.^[29] Therefore, the neutral surface charge of this combinatorial AMD-GCCNPs-DOX at physiological pH can help these NPs diffuse through the vitreous to the target site in the retina where they can make their selection on targets by reversing to a slightly positive surface charge. This might be one of main reasons for AMD-GCCNPs-DOX showing better efficacy *in vivo*.

2.15. AMD-GCCNPs-DOX Were Biocompatible and Showed No Toxicity to Normal Retina

Lastly, the safety of AMD-GCCNPs-DOX was evaluated *in vivo* in nu/nu nude mice. We injected a single dose of AMD-GCCNPs-DOX into the intravitreal space of nu/nu mice eyes at PND30. The dose was the same as the above treatments. From the H&E staining, we found that retinal layers were not affected by AMD-GCCNPs-DOX injections (Figure S4a, b in SI) even after 3 days post injection and were comparable to that of the nu/nu retina. The H&E staining showed no evidence of retinal structure damage (Figure S3d in SI). Thus, these results indicate that AMD-GCCNPs-DOX are biocompatible to the retina as found in our earlier study with GCCNPs.^[13b]

A flexible platform that effectively and specifically delivers multi-drugs to the targeted cells is an unmet need and therefore has remarkable therapeutic research and clinical potential. Our current integrated and novel nano-construct demonstrates a promising simple strategy with versatile nature to manage tumor conditions that overexpress CXCR4. We believe that this therapeutic strategy will fuel new opportunities in the development of a combinatorial drug delivery regime for the treatment of cancer or other diseases.

3. Conclusion

In summary, we designed and developed a new yet versatile nano-platform that rationally integrated therapeutic features to transport through different biological barriers such as TME. Its therapeutic effectiveness was shown in both Rb xenograft and in genetic p107s orthotopic mice models. The combinatorial AMD-GCCNPs-DOX are pH-responsive and developed using a simple, reproducible, and scalable synthetic strategy to overcome the limitations associated with the reproducibility of potential multifunctional NPs. Herein, we hypothesize that neutral and positive surface charges of NPs at physiological conditions (pH 7.4) and acidosis (pH 6.5) respectively may improve their smooth transport through the negatively charged vitreous and TME and further enhance their internalizations into the

tumor cells. Indeed, AMD-GCCNPs-DOX NPs could efficiently and specifically target tumor cells by inducing elements like DOX and nanoceria (inducing ROS) to the CXCR4-overexpressing human Rb cells *in vitro*. It is encouraging that this study demonstrated a synergistic strategy to improve the therapeutic efficacy and reduce the off-target effects of traditional chemotherapeutics (e.g. DOX) in combination with a potential chemokine receptor (e.g. CXCR4) antagonist. This new construct demonstrated significant inhibition of tumor growths as well as biocompatibility to normal retinal cells *in vivo*. In addition, this integrated AMD-GCCNPs-DOX could overcome the inherent complications of certain tumor conditions where drug resistance reduced the overall therapeutic outcomes of traditional chemotherapy. With further modification, we believe our strategy will provide new opportunities to anti-cancer drug delivery of precision medicine that can be advanced for a future human clinical trial. Besides establishing the treatment of Rb, our work will develop into an investigation of the relationship of GCCNPs with novel drugs and formulations for the treatment of other diseases.

4. Experimental Section

Synthesis of AMD-GCCNPs-DOX

GCCNPs were prepared using NH_4OH precipitation method according to our previous report^[13b]. In order to generate DOX-loaded targeted GCCNPs, 15 mg of BAC were first dissolved in 10 ml of 70% methanol-water solution, and the mixture was then sub-packaged into two little glass bottles. Subsequently, 7.5 mg of DOX and 5 mg of AMD11070 were added to each glass bottle (separately), respectively. Each reaction was performed on the magnetic stirrer at 50°C with the stirring speed at 800 rpm for 5 days in the dark with a cap. Thereafter, 120 mg of GCCNPs were dissolved in 12 ml of deionized water, and the above-mentioned two mixtures were slowly added to the GCCNPs solution. The whole reaction was completed on the magnetic stirrer at 50°C with the stirring speed at 800 rpm for an additional 5 days in the dark (with cap). Next, we dialyzed the reaction mixture for 2 days against large volume of distilled water, and then the DOX and AMD11070 loaded targetable AMD-GCCNPs-DOX (purple) solution was lyophilized and stored at -80°C for further use. The cerium content in AMD-GCCNPs-DOX was determined by ICP-MS. The DOX loaded glycol chitosan (GCD) was synthesized following the same procedure as above without AMD11070 and using glycol chitosan to replace GCCNPs.

pH adjustment

To determine the effects of pH value on the viability of the cells and ROS generation, pH 6.5 and pH 7.4 were chosen. The pH 6.5 served as the approximate pH value of TME, and the pH 7.4 represented the physiological pH condition for normal cell growth. To adjust the pH values, 1 M or 10 M acetic acid (Sigma-Aldrich, USA) was used to reduce the pH value according to the need. An aqueous solution of 1 M or 10 M sodium hydroxide (Sigma-Aldrich, USA) was also used to adjust the pH values. The pH value of the solution was measured by a pH meter pre-calibrated with standards.

Animals

p107s genetic (*Chx10-Cre; Rb^{Lox/Lox}; p107^{+/+}; -p130*) Rb mice develop retinoblastoma and were provided by Dr. Michael Dyer in St. Jude Children's Research Hospital (Memphis, TN, USA) under a material transfer agreement (MTA).^[28] All of the parent Rb mice were maintained and bred in the AAALAC-accredited Center for Experimental Animal under sterile environments at the University of North Carolina at Chapel Hill (UNC-CH). After genotyping via PCR, the mice were separated and reserved in cages. All p107s mice (1-month old) were kept in the sterile experimental facility at UNC-CH for therapy. C57BL/6 WT mice were also bred by ourselves. Nu/nu nude immunocompromised mice that do not reject human cells were purchased from Animal Studies Core at UNC-CH and were maintained in the nonsterile Center for Experimental Animal at UNC-CH. All animal experiments including experimental animals were managed in accordance with the principles approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee and were handled following the Guide for the Care and Use of Laboratory Animals (NIH publication no. 86–23, revised 1985). The genotyping was carried out with the primers using mice tail genomic DNA as the template; *Chx10-F*: 5'-GGGCACCTGGGACCAACTTCACGA-3'; *Chx10-R*: 5'-CGGCGGCGGTACGAAGTCC-3'; *Rb^{Lox}-F*: 5'-GGCGTGTGCCATCAATG-3'; *Rb^{Lox}-R*: 5'-AACTCAAGGGAGACCTG-3'; *p107 WT*: 5'-TGTCCTGAGCATGAACAGAC-3'; *p107 COM*: 5'-TCGCTGGCAGTCTGAGTCAG-3'; *p107 NEO*: 5'-ACGAGACTAGTGAGACGTGC-3'; *p130 WT*: 5'-TACATAGTTTCCCTTCAGCGG-3'; *p130 PGK*: 5'-GAAGAACGAGATCAGCAGC-3'; *p130 C1*: 5'-ACGGATGTCAAGTGTACG-3'; *RD3*: 5'-TGACAATTACTCCTTTTCCCTCAGTCTG-3'; *RD4*: 5'-GTAAACAGCAAGAGGCTTTATTGGGAAC-3'; *RD6*: 5'-TACCCACCCTTCCTAATTTTCTCACGC-3.

Anti-tumor activity in vivo

The Rb xenograft model was established by subretinal injection of bioluminescent Y79/GFP-luc cells into the right eye of each nu/nu nude mouse according to the method used by another research group.^[30] The 5 to 6-week old mice were anesthetized by intraperitoneal injection with ketamine-xylazine (kx) solution (25 mg/ml) at a dose of 100 µl/20 g mouse. The pupil was dilated with 1% Tropicamide Ophthalmic Solution (Henry Schein Inc., Melville, NY, USA) and moisturized with a drop of GenTeal® lubricant eye gel (Alcon Laboratories, Inc., South Freeway, Fort Worth, TX, USA). A subretinal injection was performed under a binocular surgical microscope. An initial puncture behind the limbus was first made using a 24-gauge beveled needle. 2×10⁴ Y79/GFP-luc cells in 1 µl of 1×DPBS were injected under the retina of the mouse's right eye using a 10 µl micro-syringe with a 33-gauge needle.

Bioluminescence imaging was performed to monitor the growth of Rb according to the manufacturer's instructions after cell injection. D-luciferin (Pierce, #88294) was dissolved into 1×DPBS and then an intraperitoneal injection was carried out to each mouse at a dose of 150 mg/kg. Mice were anesthetized via 1–3% isoflurane (Henry Schin Inc., Melville, NY, USA) inhalation in a sealed chamber with oxygen supply. After 8 min, the anesthetized mice

were transferred onto the warmed stage inside the camera box, and 1–2% isoflurane was continuously supplied to keep the mice sedated during imaging. The exposure time was set to 1 min and 5 min. The light from the ATP-dependent oxidation of D-luciferin by luciferase was detected *in vivo* by the IVIS-Imaging System (IVIS-Kinetic). The light was digitized and then electronically displayed as a bioluminescent signal with the radiance unit of p/sec/cm²/sr. Pictures were captured and analyzed using Living Image®4.5.4 software.

When the average radiance of Rb reached about 5×10⁶ p/sec/cm²/sr, the Rb-bearing mice were randomized into 4 groups (n=5 per group) and treated with the following formulations via an intravitreal route: (1) saline; (2) GCCNPs (4 mg/ml); (3) GCD (0.2 µg of free DOX equivalent); (4) AMD-GCCNPs-DOX (0.2 µg of free DOX equivalent). The bioluminescent signal was measured twice per week. At 3 weeks after drug treatment, all mice were euthanized. The eyes treated with drugs were collected for H&E staining. The tumor growth inhibition (TGI) for each group was calculated according to the following equation (1):

$$\%TGI = \left(1 - \frac{\text{Mean of treated bioluminescent signal intensity}}{\text{Mean of control bioluminescent signal intensity}}\right) \times 100$$

Statistical analysis

The results were shown as means ± s.e.m. from at least 3 independent experiments. The figures and data analysis were performed using GraphPad Prism 6.0 software (La Jolla, CA, USA) and Photoshop CS5 software. The results were analyzed by either a Student *t*-test between two independent groups or by One-way and Two-way ANOVA among multiple group comparisons as necessary. Differences were measured statistically significant at *P*<0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. High expression of CXCR4 in human Rb cell lines and eye tissues from Rb p107s mice analyzed by Western blotting.

a) Representative CXCR4 protein levels in various human cell lines. b, c) Differential CXCR4 expression in eye tissues from C57BL/6 (WT) and p107s Rb mice at PND 30 and PND 60, respectively. d) Quantification of CXCR4 showed that CXCR4 expression levels in human cancer cells were significantly higher than that in human normal ARPE19 cells. The error bars represent mean $\pm$ s.e.m. ($n=3$). ** $p < 0.001$ and *** $p < 0.0001$ (one-way analysis of variance, ANOVA, and Tukey's multiple comparison test). e, f) Quantification of CXCR4 in eye tissues demonstrated that there was a statistical difference in CXCR4 expression level between WT and p107s Rb mice at PND 30 and PND 60, respectively. CXCR4 expression level in eye tissues from p107s Rb mice was significantly increased compared to that from C57BL/6 WT mice ($P < 0.05$). Densitometric band analyses (mean $\pm$ s.e.m.) were presented from four independent experiments and were analyzed with unpaired Mann-Whitney test; * $p = 0.02$, (t -test) $p < 0.05$ was considered significant.

Figure 2. Characterizations of AMD-GCCNPs-DOX NPs.

a) Diameter of GCCNPs and AMD-GCCNPs-DOX was determined by DLS. The data (mean $\pm$ s.e.m.) were analyzed using a two-tailed unpaired Student's *t*-test. * $P < 0.05$, $n = 3$. b) Polydispersity index (PDI) of GCCNPs and AMD-GCCNPs-DOX was analyzed by DLS. c) Representative histogram of size distribution of AMD-GCCNPs-DOX. d) Representative TEM image of AMD-GCCNPs-DOX. Scale bar: 500 nm. e) Zeta potentials of GCCNPs and AMD-GCCNPs-DOX NPs. f) Representative surface charge image of AMD-GCCNPs-DOX. g) Full-length UV-Vis scanning of GCCNPs, AMD, DOX, AMD-GCCNPs-DOX/water and AMD-GCCNPs-DOX/HCl. h) *In vitro* release of DOX from AMD-GCCNPs-DOX in water with or without 10 mM GSH at 37°C. Only the water group at pH 7.4 was measured as the control. Bars correspond to mean $\pm$ s.e.m ($n = 3$).

Figure 3. AMD-GCCNPs-DOX demonstrated pH sensitive size, charge distributions, and intrinsic DOX fluorescence.

The results were determined by DLS, and zeta potential measurements. a) DLS measurements of AMD-GCCNPs-DOX NPs in different pH conditions. Error bars represent mean $\pm$ s.e.m (n=3). *** $p < 0.0001$ (One-way ANOVA, Bonferroni's multiple comparison test). b, c, d) Measurement of DOX fluorescence (Ex=490nm, Em=590nm) in different pH conditions and in plain water over different time points. Error bars represent mean $\pm$ s.e.m (n=3). *** $p < 0.0001$, and ns= $p > 0.05$ (one-way ANOVA, Bonferroni's multiple comparison test). e, f) Determination of intrinsic DOX fluorescence (Ex= 490 nm, Em= 590

nm) of free DOX and AMD-GCCNPs-DOX respectively in different pH conditions and in plain water. Representative TEM images of AMD-GCCNPs-DOX g) at the pH 7.4 (1x DPBS), scale bar: 100 nm, and h) at the pH 6.5 (1x DPBS), scale bar: 200 nm.

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Figure 4. The pH dependent anti-proliferative and ROS inducing activities of AMD-GCCNPs-DOX with controls.

AMD-GCCNPs-DOX showed enhanced a) anti-proliferative activity, b) cytotoxicity and c) ROS generation towards human Rb cells at acidic pH compared to GCCNPs by MTT, WST-1 or DCFH-DA assays. Data were analyzed using one-way ANOVA followed by Tukey's post hoc multiple comparison test; * $P < 0.05$; *** $P < 0.001$, $n = 3$.

Figure 5. Cellular uptake of free DOX and AMD-GCCNPs-DOX at both pH 7.4 and 6.5 conditions.

The representative confocal images of a) human Y79 and b) human ARPE19 cells were treated with free DOX and AMD-GCCNPs-DOX NPs for 48h. The red color represents the intrinsic fluorescence of DOX. The cell membrane was stained with AF647-labeled WGA (white, cell membrane marker), and cell nuclei were counter-stained with DAPI (blue). Untreated cells were considered as negative controls. The enhanced cellular uptake was observed when the Y79 cells were treated with AMD-GCCNPs-DOX at pH 6.5 as demonstrated by the intense red fluorescence from DOX.

Figure 6. CXCR4 receptor targeting activity of AMD-GCCNPs-DOX in Y79 cells.

Here representative confocal images acquired under the same magnification are presented. The Y79 cells were pre-treated with AMD11070 drug (0.6 and 4.8 $\mu\text{g/mL}$) for 1.5 h and were further exposed to 200 nM AMD-GCCNPs-DOX for an additional 48 h. Untreated and AMD11070 (0 $\mu\text{g/mL}$) treated cells remained as negative controls. AMD11070 inhibited cellular uptake of DOX from AMD-GCCNPs-DOX at acidic pH in human Y79 Rb cells. The cell membrane was marked with AF647-labeled WGA (white), and cell nuclei were stained with DAPI (blue). The red color showed the intrinsic fluorescence of DOX from AMD-GCCNPs-DOX. Scale bar: 20 μm .

Figure 7. Flow cytometry analysis, gene, and protein expression studies of free DOX and AMD-GCCNPs-DOX treated human Y79 Rb cells.

a) Representative dot plots of flow cytometric analysis of Y79 cells treated with free DOX and AMD-GCCNPs-DOX NPs for 48 h. The Y79 cells were subjected to CF-Blue labeled Annexin V and 7-AAD that detect the DOX induced apoptosis. At the pH 6.5, the DOX induced apoptosis was enhanced upon treatment with AMD-GCCNPs-DOX compared to the equivalent amount of free DOX (200 nM DOX) treated samples. Untreated cells were considered as negative controls. b) The statistical analysis of Annexin V positive cells (Apoptosis) is presented for free DOX and AMD-GCCNPs-DOX. The data shows a dose

dependent enhancement of apoptosis (%) of Y79 cells upon treatment with AMD-GCCNPs-DOX at pH 6.5. Error bars represent mean $\pm$ s.e.m. *** P <0.001, n=3. c) The representative confocal images of apoptotic bodies. AMD-GCCNPs-DOX triggered the formation of apoptotic bodies at pH 6.5. White-colored arrows indicate the representative apoptotic bodies. (d) Quantitative PCR (qPCR) analysis of CXCR4 expression in Y79 cells treated with 50 nM free DOX and AMD-GCCNPs-DOX NPs for 48 h at both pH 6.5 and 7.4 conditions. Error bars correspond to mean $\pm$ s.e.m. *** p <0.001, and **** p <0.0001 (two-way ANOVA, Tukey's multiple comparison test). (e) The representative Western blotting images. AMD-GCCNPs-DOX prominently suppressed the CXCR4 protein expression in Y79 cells at the pH 6.5 condition.

Figure 8. Schematic illustration of a potential “3-in-1” nanomedicine composed of CXCR4 antagonist (AMD11070), DOX, and nanoceria against cancer.

ECM: Extracellular matrix, BAC: N, N'-bis (acryloyl) cystamine.

Figure 9. Enhanced tumor cell susceptibility to chemotherapy in human Rb Y79/GFP-luc cells and xenografts in nu/nu athymic mice.

For *in vitro* cytotoxicity, the acidic and physiological environment of tumor cells were simulated; for *in vivo* therapy, all formulations were intravitreally injected into the right eye of each mouse. During the whole therapy, only one injection was administered for each mouse. a) Cell cytotoxicity of AMD-GCCNPs-DOX towards Y79/GFP-luc cells was determined by WST-1 assay. GCD served as the control. The experiments were done in triplicate, and the data were analyzed using two-tailed unpaired Student's *t*-test. For GCD, ***P*<0.01 at pH 6.5 versus pH 7.4; For AMD-GCCNPs-DOX, #*P*<0.05 and ##*P*<0.01 at pH

6.5 versus pH 7.4. b) Rb growth curve in each group. The data were shown as mean $\pm$ s.e.m. (n=5). Significant difference between two groups was determined with two-tailed unpaired Student's *t*-test. For GCD group, **P*<0.05 and ***P*<0.01 *versus* saline group, #*P*<0.05 and ##*P*<0.01 *versus* GCCNPs group; for AMD-GCCNPs-DOX group, ^*P*<0.01 *versus* saline group, ^δ*P*<0.01 *versus* GCCNPs group, ^λ*P*<0.05 *versus* GCD group. c) *In vivo* bioluminescence images of Y79/GFP-luc xenografts. Pictures were captured and analyzed using Live Imaging@4.5.2 software. For each group, the images were shown at day (D) 0, 7, 14, and 21 after intravitreal injections of different materials including saline.

Figure 10. AMD-GCCNPs-DOX inhibited the growth of Rb in p107s Rb mice.

BF and FA represent bright field and fluorescein angiography, respectively. 2 μ l of GCCNPs, DOX and AMD-GCCNPs-DOX were intravitreally injected into each eye at PND 30, and the observation period was 4 weeks. The representative figures were presented in triplicates.

Table 1.

Cytotoxicity of AMD-GCCNPs-DOX towards human normal ARPE19 and Rb cells (WERI-Rb-1 and Y79).

Groups		IC ₅₀ (nM)		
		ARPE 19	WERI-Rb-1	Y79
pH 6.5	DOX	8.3	76.65	283.4
	AMD-GCCNPs-DOX	625.3	703.7	192.3
pH 7.4	DOX	4.5	42.3	109.0
	AMD-GCCNPs-DOX	1234.0	7986	1805