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Antiandrogen Gold Nanoparticles Dual-Target and Overcome Treatment Resistance in Hormone-Insensitive Prostate Cancer Cells

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Abstract

Prostate cancer is the most commonly diagnosed cancer among men in developed countries.[1] One in six males in the US[2] and one in nine males in the UK[3] will develop the disease at some point during their lifetime. Despite advances in prostate cancer screening, more than a quarter million men die from the disease every year[1] due primarily to treatment-resistance and metastasis. Colloidal nanotechnologies can provide tremendous enhancements to existing targeting/treatment strategies for prostate cancer to which malignant cells are less sensitive. Here, we show that antiandrogen gold nanoparticles – multivalent analogues of antiandrogens currently used in clinical therapy for prostate cancer – selectively engage two distinct receptors, androgen receptor (AR), a target for the treatment of prostate cancer, as well as a novel G-protein coupled receptor, GPRC6A, that is also upregulated in prostate cancer. These nanoparticles selectively accumulated in hormone-insensitive and chemotherapy-resistant prostate cancer cells, bound androgen receptor with multivalent affinity, and exhibited greatly enhanced drug potency versus monovalent antiandrogens currently in clinical use. Further, antiandrogen gold nanoparticles selectively stimulated GPRC6A with multivalent affinity, demonstrating that the delivery of nanoscale antiandrogens can also be facilitated by the transmembrane receptor in order to realize increasingly selective, increasingly potent therapy for treatment-resistant prostate cancers.

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Author Disclosures
The authors declare no competing financial interests other than those disclosed. L.D.Q. is a consultant to Amgen, KAI Pharmaceuticals and Shire; he has also been a paid speaker for Amgen.

Supporting Information
Antiandrogen ligand synthesis; nanoparticle synthesis, conjugation, and characterization; docking methods, cell culture/in vitro analysis, radioligand binding, *GPRC6A* expression/stimulation, and imaging methods. This material is available free of charge via the Internet at http://pubs.acs.org.

Androgen deprivation therapy (ADT) is currently recommended for the treatment of advanced/metastatic prostate cancer.[4] Nonsteroidal antiandrogens such as flutamide (Eulexin®), bicalutamide (Casodex®), and nilutamide (Nilandron®) are some of the most commonly prescribed ADT drugs and diminish androgenic effects by competitively inhibiting androgen-androgen receptor binding associated with prostate cancer growth, division, and survival. While most advanced or metastatic prostate cancers initially respond well to ADT, malignant cells that survive 2–3 years will typically enter an antiandrogen-resistant[5] (i.e. castration-resistant) state and subsequently exhibit chemotherapy-resistance as well.[6] Without further intervention, median survival following this period is just 18–24 months. Increasingly selective and potent drugs are urgently needed to treat these prostate cancers.

Nanoscale drug conjugates can provide improved targeting selectivity for prostate cancer treatments via multivalent ligand display (augmented affinity and avidity) and size-dependent passive accumulation;[7] they can also realize increasing potency through high drug loading capacity and enhanced intracellular transport rates (endocytosis versus passive diffusion).[8] Langer, Farokhzad, and Lippard have shown that PLGA nanoparticles targeted with aptamers toward prostate-specific membrane antigen can deliver platinum prodrug chemotherapeutics to prostate cancer cells with substantially greater drug potency than untargeted carriers or cisplatin alone.[9, 10] Folate-targeted lipid nanoparticles have also been applied in gene therapy[11] and RNA interference[12] for prostate cancer in vivo. Katti and Kannan have shown that gold nanoparticles targeted with bombesin peptides directed toward gastrin-releasing peptide receptor (overexpressed on prostate cancer cells) selectively target prostate cancer cells in vitro/vivo with multivalent affinity and can provide enhanced contrast for x-ray computed tomography (CT) imaging.[13] Neoadjuvant administration of gold nanoparticles has been further shown to sensitize prostate cancer cells towards external beam radiation therapy[14] and to facilitate in vivo laser photothermal ablation therapy in animal models of prostate cancer.[15]

We hypothesized that derivatives of commercially-available antiandrogen chemotherapeutics could serve as combined targeting *and* therapeutic agents for tissue-selective drug delivery of nanoscale drug carriers to prostate cancers expressing *membrane* androgen receptor[16, 17] *and/or* a recently deorphaned androgen-sensing G protein-coupled receptor, GPRC6A,[18] involved in increased prostate cancer risk, growth, and poor survival. We found that antiandrogen gold nanoparticles selectively target and engage both androgen receptor and GPRC6A with multivalent affinity and facilitate cell death in antiandrogen treatment-resistant prostate cancer cells at substantially lower concentrations than their corresponding free drugs. Per ligand, antiandrogen gold nanoparticles bound androgen receptor with affinity 5- to 11-times greater than free antiandrogens, and per particle, bound androgen receptor with affinity superior to *endogenous* androgens, providing opportunities for further increased treatment efficacy via drug co-conjugation, laser photothermal ablation, radiotherapy sensitization, and imaging-based treatment guidance/monitoring.[8, 19]

Gold nanoparticles (AuNPs, 29 ± 4 nm diameter, Figure 1a) were synthesized by Turkevich/Frens reduction of chloroauric acid and conjugated with a mixed self-assembled monolayer of 5% thiol PEGylated antiandrogen and 95% thiolated poly(ethylene glycol) stabilizer (PEG-SH, 5 kDa). Antiandrogen ligands used in these studies were employed to reflect structural homology between antiandrogens in clinical use with α- and β-Bicalutamide (α-Bic, β-Bic; Figure 1a) both bearing an aromatic α-anilide ring characteristic of flutamide, bicalutamide, and nilutamide, as well as a five-membered imidazolidinedione ring characteristic of nilutamide and the H-bonded structure of bicalutamide and/or the active metabolite of flutamide. β-Bic contains an additional β-aromatic ring characteristic of bicalutamide which binds the hydrophobic pocket formed by helix 12 residues on androgen

receptor and confers it enhanced potency[20] (Figure 1b). Antiandrogen ligands were synthesized by Cu(I)-catalyzed Huisgen cycloaddition (i.e. click, azide-alkyne coupling) with PEGylated lipoic acid. Thiol anchoring groups were used to enable stable Au surface bond formation and PEG stabilizer/spacer groups were employed to sterically stabilize the subsequent nanoparticle constructs in physiological media and to resist protein adsorption and/or diminish immunogenic response. α-Bic- and β-Bic-AuNPs contained $2.25 \pm 0.02 \times 10^3$ and $1.56 \pm 0.08 \times 10^3$ antiandrogen ligands per particle, respectively (See Supplementary Information for detailed experimental methods; Scheme S1, Figure S1).

α-Bic and β-Bic AuNPs were found to be 50 ± 1 nm in hydrodynamic diameter, which recent studies by Chan and coworkers indicate to the be within the optimal size range for both tumor accumulation and cellular internalization of AuNPs[21, 22] (Figure 1d). PEGylated control nanoparticles were found to be 49 ± 1 nm. The octanol:water partition coefficient of α-Bic- and β-Bic-AuNPs was found to be −1.4 ± 0.2 and −0.27 ± 0.03, respectively, both below that expected from an intravenously administered drug (1.92) with acceptable pharmacokinetics.[23]

Molecular docking of the antiandrogen ligands with androgen receptor (AR) show that the contact points of their parent drugs within the AR binding pocket are maintained by the ligands and that their thiol PEGylated linker groups face outward to enable accessibility by nanoparticle-bound ligands (Figure 1c).[24] Receptor binding competition with radiolabeled androgen (Figure 2a) shows that the AR binding inhibition constant (K_i) of α-Bic and β-Bic is enhanced 11- and 5.4-fold per drug molecule, respectively, when displayed as a multivalent construct, with total nanoparticle AR binding affinity exceeding that of its endogenous hormone dihydroxytestosterone (DHT, 0.28 – 2 nM v. 0.16 – 0.24 nM nanoparticles)[20, 25] and yielding, to the best of our knowledge, the highest reported nanoparticle K_i for a non-steroidal antiandrogen (Figure 2a inset, Figure 2b). Free α-Bic- and β-Bic bound AR with affinities comparable to those previously reported for bicalutamide[26]. Because membrane AR (mAR) binds antibodies[16] and endogenous androgens[27] for intracellular AR, and because antiandrogens can diminish the effects of androgenic mAR stimulation,[28] these data suggest that antiandrogen gold nanoparticles can selectively target mAR which is preferentially overexpressed by human prostate cancer cells and whose expression levels correlate with poor prognosis (Gleason score)[29] and total AR levels[16] found in 80–90% of all prostate cancers.[30]

GPRC6A[31] is a membrane-associated C family G protein-coupled receptor recently discovered[32] through genomic homology search. We have previously shown that GPRC6A senses androgens and is a positive regulator of testosterone and a negative regulator of estrogen production in mice;[18] its expression was also found to contribute to prostate cancer growth, malignancy, and poor survival in animal models of prostate cancer.[18] Polymorphism at the *GPRC6A* gene locus was recently associated with significantly altered susceptibility to prostate cancer in a genome-wide association study among Japanese men ($P=1.6 \times 10^{-12}$).[18, 33] Here, we found that *GPRC6A* overexpression strongly correlates malignant phenotype across several prostate cell lines (Figure 2c). Although Pi et al. recently found that androgens selectively stimulate GPRC6A and subsequently promote prostate cancer cell growth,[34] we hypothesized that high affinity antiandrogens and multivalent antiandrogen gold nanoparticles may similarly engage GPRC6A, albeit with subsequently diminished cell growth (*vide supra*) rather than agonist-induced proliferation. Downstream production of the second messenger, cyclic adenosine monophosphate (cAMP), accumulated in response to stimulated GPRC6A signal transduction, was assessed using an established $AR^-/GPRC6A^-$ and $AR^-/GPRC6A^+$ transfected cell line (See Supplementary Information).[34] α-Bic- and β-Bic-AuNPs significantly stimulated GPRC6A in an androgen-competitive manner (Figure 2d), eliciting cAMP production at low μM ligand (sub-nM AuNP) concentrations. GPRC6A

stimulation by α-Bic- and β-Bic-AuNPs was 2.0- and 1.9-fold greater than that by an equivalent or greater concentration of PEGylated-AuNPs (P=0.06 & 0.15, respectively) and was 2.3- and 3.5-fold greater than their nanoparticle-equivalent concentrations of free antiandrogen ligands (P=0.003 & 0.03, respectively) (Figure 2d inset). GPRC6A stimulation by PEG-AuNPs was nearly equivalent to that observed from testosterone-abrogated α-Bic- and β-Bic-AuNPs, indicating GPRC6A ligand-enhanced activity from antiandrogen-AuNPs. Together, these data show that antiandrogen gold nanoparticles can selectively engage GPRC6A with multivalent affinity and can do so in a manner *independent* of AR/mAR expression (Figure 2d).

Binding/uptake selectivity of the antiandrogen gold nanoparticles was assessed in a membrane-AR^+ and $GPRC6A^+$ prostate carcinoma cell line[27] whose response to chemotherapy and antiandrogen therapy reflects that of castration-resistant prostate cancer,[6] DU-145 (Figure 3a). Fluorescently-labeled antiandrogen gold nanoparticles exhibited high intracellular accumulation in DU-145 cells and localized in a manner similar to that reported for AR,[35] while PEGylated nanoparticles exhibited no significant accumulation. Uptake and localization patterns of both targeted and untargeted nanoparticles in an AR null[36] squamous cell carcinoma cell line showed only non-specific cell surface binding (Supplementary Figure S2). We hypothesized that androgen-stimulated upregulation of AR in DU-145,[37] and correspondingly increased mAR expression,[16] may augment antiandrogen nanoparticle accumulation in prostate cancer cells. Particle uptake/localization was imaged using cardioid immersion optical dark-field scattering microscopy which can achieve sensitivity orders of magnitude higher than conventional fluorescence-based methods.[8, 19] Testosterone (T, 10^{-6} M) stimulation of DU-145 had no effect on PEGylated nanoparticle accumulation (Supplementary Information Figure S3), but significantly increased α-Bic- and β-Bic-AuNP accumulation. These findings are consistent with previous reports demonstrating 1.5–2 fold upregulation of androgen receptor expression following testosterone stimulation in DU-145[37] and concomitant increases in membrane-AR traslocation[16] (Figure 3b). These imaging data show that antiandrogen gold nanoparticles selectively accumulate in antiandrogen treatment-resistant AR^+/$GPRC6A^+$ prostate cancer cells and can also do so in an AR-/mAR-*dependent* manner.

Cytotoxicity of the antiandrogen nanoparticles and their ligands to chemotherapy- and antiandrogen-resistant[6] mAR^+/$GPRC6A^+$ DU-145 prostate carcinoma cells was investigated by tetrazolium assay (24 h, See Supporting Information). AuNP-bound α-Bic- and β-Bic induced half maximal cytotoxicity (IC_{50}) at 21 and 2.5 μM ligand concentrations, respectively, exhibiting per-molecule potency 6.4- and 13-fold greater than their corresponding free drugs, respectively (Figure 3c–e). Free α-Bic- and β-Bic cytotoxicity was comparable to that previously reported for bicalutamide and OH-flutamide with DU-145,[38] while nanoparticle-equivalent concentrations of PEGylated gold nanoparticles exhibited no significant toxicity over therapeutically-relevant antiandrogen-AuNP concentrations. Unlike androgenic GPRC6A agonists which we have previously shown induce cell proliferation,[34] antiandrogen-AuNPs stimulate GPRC6A while diminishing cell proliferation (Fig 3c–e) and also maintaining antagonist activity toward androgen receptor (Fig 2a, b). Together, these data correlate selective AR and GPRC6A engagement (*vide infra*) with enhanced drug potency and cell death by antiandrogen gold nanoparticles.

In summary, we found that antiandrogen gold nanoparticles selectively engaged two distinct receptors involved in prostate cancer growth and progression. These particles selectively accumulated in castration- and chemotherapy-resistant prostate cancer cells and induced cell death at nanomolar particle concentrations, and enhanced the potency of antiandrogens currently in clinical use by roughly one order of magnitude. Further, antiandrogen gold nanoparticles antagonized androgen receptor with multivalent affinity and selectively

engaged a newly discovered transmembrane G-protein coupled receptor, GPRC6A, involved in prostate carcinogenesis and disease risk. These platforms provide opportunities for increasingly potent and selective therapy for treatment-resistant prostate cancers and may exhibit further enhanced therapeutic efficacy via drug co-conjugation, image-based treatment guidance/monitoring, concurrent laser photothermal ablation therapy, and/or high-Z enhanced radiotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

α-Bic	α-Bicalutamide ligand
β-Bic	β-Bicalutamide ligand
ADT	androgen deprivation therapy
AR	androgen receptor
AuNP	gold nanoparticle
cAMP	cyclic adenosine monophosphate
DAPI	4′,6-diamidino-2-phenylindole
DHT	dihydrotestosterone
IC_{50}	half maximal concentration
mAR	membrane androgen receptor
PEG	poly(ethylene glycol)
T	testosterone

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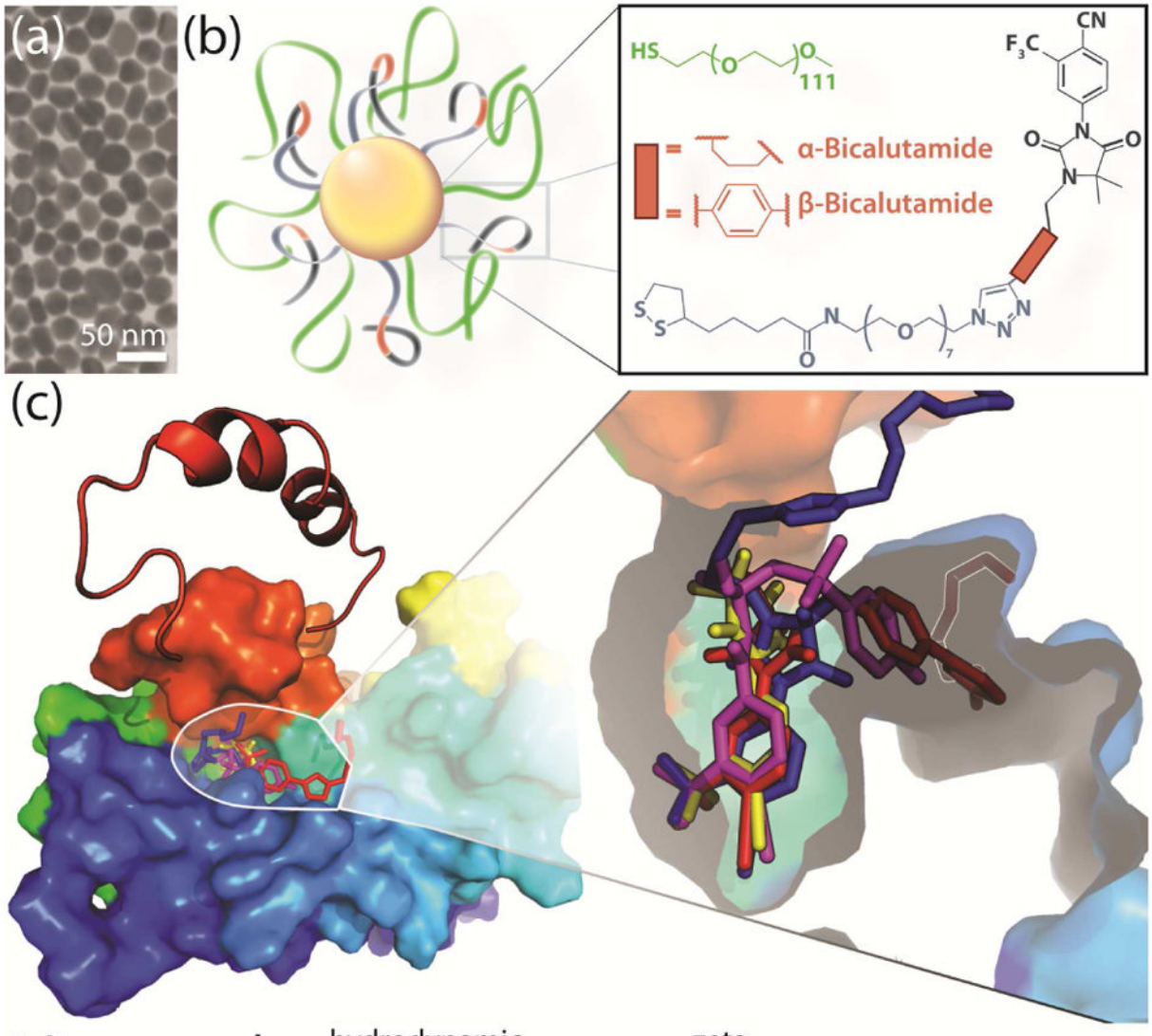


(d)

	hydrodynamic diameter (nm)	zeta potential (mV)	log *P*
PEG-AuNPs	49 ± 1	-17.2 ± 0.9	-1.5 ± 0.3
α-Bic-AuNPs	50 ± 1	-13.7 ± 0.6	-1.45 ± 0.2
β-Bic-AuNPs	50 ± 1	-4.3 ± 0.4	-0.27 ± 0.03

Figure 1. Multivalent antiandrogen gold nanoparticles for the treatment of castration-resistant prostate cancer

a, Electron micrographs of the as-synthesized 29 ± 4 nm diameter gold nanoparticles. **b,** Illustration of the antiandrogen nanoconjugates with receptor binding groups shown in gray/red. **c,** Molecular docking of the antiandrogen ligands with androgen receptor showing outward orientation of the thiol PEGylated nanoparticle linker groups and maintenance of contact points within the androgen receptor binding pocket by the bicalutamide ligand (β-Bic, red) and its β ring-deficient analogue (α-Bic, blue), as compared to their precursor drugs bicalutamide (magenta) and its nilutamide analogue (yellow). **d,** Physiochemical properties of the antiandrogen gold nanoparticles.

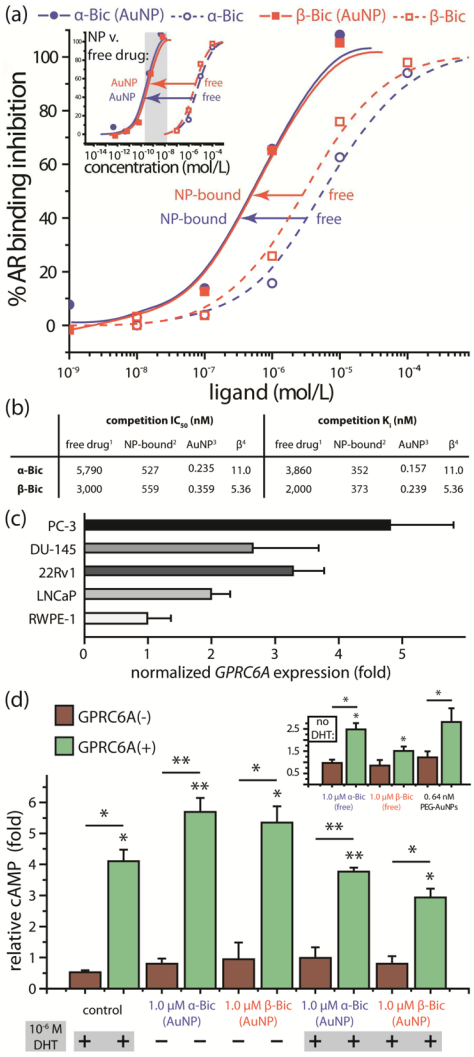
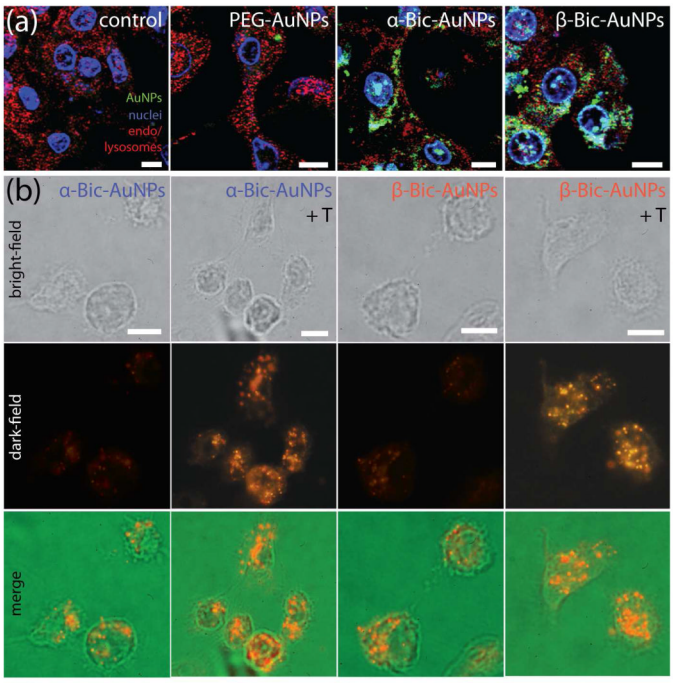


Figure 2. Antiandrogen gold nanoparticles selectively engage androgen receptor (AR) and G protein-coupled receptor GPRC6A targets

a, AR binding competition between radiolabeled androgen and nanoparticle-bound antiandrogens (solid) or free antiandrogens (dashed) showing multivalency-enhanced AR binding affinity (K_i) from the nanoparticle constructs. Nanoparticle-bound antiandrogens displaced [^{3}H]androgen from AR with 5.4–11 fold greater per-ligand affinity than an equivalent concentration of free antiandrogens. Inset, Nanoparticle and free drug concentrations. **b,** Half maximal inhibitory concentration (IC_{50}) and binding inhibition constant (K_i) for free[1] and nanoparticle-bound[2] antiandrogens, nanoparticles[3], and their corresponding (per-drug-molecule) multivalency-enhanced values (β^4). **c,** Upregulated *GPRC6A* mRNA expression levels measured from various prostate cancer cell lines relative to non-malignant RWPE-1 prostate cells (See Supporting Information). **d,** Androgen-competitive downstream production of cyclic adenosine monophosphate (cAMP) second messenger accumulated in response to overnight stimulation of GPRC6A signal transduction by α-Bic-and β-Bic-AuNPs in an $AR^-/GPRC6A^-$ and $AR^-/GPRC6A^+$ transfected cell line. Inset, Fold-change in GPRC6A signal transduction (cAMP accumulation) in response to GPRC6A stimulation by nanoparticle-equivalent concentrations of free α-Bic/β-Bic and PEGylated control nanoparticles showing significantly lower GPRC6A engagement/

stimulation by free α-Bic (2.3-fold, P=0.003), β-Bic (3.5-fold, P=0.03), and PEGylated gold nanoparticles (2.0-fold v. α-Bic-AuNPs, P=0.06; 1.9-fold v. β-Bic-AuNPs, P=0.15) relative to their respective particle constructs. cAMP levels from PEG-AuNPs were comparable to those observed from DHT-abrogated α-Bic/β-Bic-AuNPs indicating antiandrogen ligand-enhanced GPRC6A engagement. Note that GPRC6A is a promiscuous receptor for androgenic ligands and that the cell lines in (d) are AR null. DHT, dihydrotestosterone. Grayscale area in (a) denotes the lower and upper limits previously reported for AR's endogenous high affinity ligand, dihydrotestosterone (DHT). Error bars represent SEM. P for individual values relative to untreated controls or as indicated; *P<0.05, **P<0.01.



(e)

	toxicity IC_{50}(μM)			
	free drug[1]	NP-bound[2]	AuNP[3]	β[4]
α-Bic	138 ± 6	21 ± 0.2	0.0095 ± 0.0001	6.4 ± 0.3
β-Bic	33 ± 2	2.5 ± 0.1	0.0016 ± 0.0001	13 ± 1

Figure 3. Antiandrogen gold nanoparticles selectively accumulate in chemotherapy- and antiandrogen-resistant prostate cancer cells expressing membrane-androgen receptor (mAR) and G protein-coupled receptor GPRC6A and induce cell death with multivalency-enhanced drug potency

a, Confocal fluorescence images of selective antiandrogen nanoparticle intracellular localization (green) in mAR+/GPRC6A+ DU-145 prostate cancer cells. Endo/lysosomes were labeled with dextran (red) and nuclei were stained with DAPI (blue). **b,** Optical dark-field scattering microscopy of DU-145 cells showing augmented antiandrogen gold nanoparticle accumulation in response to androgen-stimulated mAR-upregulation by testosterone (T, 10^{-6} M). Note that images in (a) and (b) were obtained using different instruments. **c,** Dose-dependent cell viability (%) of antiandrogen treatment-resistant DU-145 prostate cancer cells incubated with free antiandrogens and ligand-equivalent concentrations of antiandrogen gold nanoparticles (24 h). **d,** Total nanoparticle concentrations represented in c. **e,** Figures of merit for cytotoxicity of the free drug[1], nanoparticle-bound ligand[2], and multivalent nanoparticle [3], as well as the multivalency enhancement factor β[4] exhibiting 6.4- and 13-fold enhanced potency per drug molecule. Scale bars represent 10 μm. Error bars represent SD.