Am J Cancer Res 2021;11(10):4884-4899
www.ajcr.us /ISSN:2156-6976/ajcr0136630

Original Article

Sensitization of breast cancer to Herceptin by redox active nanoparticles

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Received June 22, 2021; Accepted July 30, 2021; Epub October 15, 2021; Published October 30, 2021

Abstract: Herceptin-resistant tumor relapse remains a major clinical issue responsible for the poor prognosis of HER2$^+$ breast cancer. Understanding the underlying mechanisms and finding a therapeutic solution are of paramount urgency to improve the patient management. Here we report that anticancer redox active cerium oxide nanoparticles (CONPs) can potently sensitize the cancer cells to the cytotoxicity of Herceptin. By comparing between Herceptin-sensitive and Herceptin-resistant human breast cancer cell lines under normoxic as well as hypoxic culture conditions, we found that in the presence of CONPs, Herceptin can kill the Herceptin-resistant cells equally effectively as it kills the Herceptin-sensitive cells under the hypoxic, but not normoxic, culture conditions by inhibiting the cell viability, survival and proliferation. Signaling analysis reveals that under the normoxic conditions, the levels of hypoxia induced factor 1α as well as vascular endothelial growth factor are higher in the Herceptin-resistant cells than that in the Herceptin-sensitive cells and are strongly induced once the culture is switched to the hypoxic conditions, which can be potently suppressed by CONPs. Treatment with CONPs plus Herceptin significantly slows down the primary tumor growth and lung metastasis of the Herceptin-resistant cells in a xenograft mouse model of orthotopic breast cancer through inhibiting the cell proliferation and survival as well as tumor angiogenesis. These results shed new lights on the mechanisms underlying the Herceptin resistance of the HER2$^+$ breast cancer and provide insights into introducing CONPs-like agents to Herceptin-based therapy to improve treatment outcomes.

Keywords: Breast cancer, HER2, cerium oxide nanoparticles, Herceptin resistance

Introduction

Breast cancer remains a leading cause of cancer death in women due largely to the lack of effective strategies against drug resistant tumor relapse. Nearly a quarter of breast tumors overexpress the epidermal growth factor receptor 2 (HER2) proto-oncogene [1, 2], a favorable cell surface target of Herceptin (a.k.a. trastuzumab), a HER2 antagonist monoclonal antibody drug widely used to treat HER2$^+$ breast cancer. Unfortunately, more than a quarter of early stage patients develop recurrent tumors within a year of treatment due to acquired Herceptin resistance [1, 3, 4]. Because of this, Herceptin treatment has not given rise to any significant improvement in disease-free survival [2]. On the other hand, nearly three quarters of late stage patients do not respond to Herceptin even if given in combination with chemotherapy [5, 6]. Better knowledge about the mechanisms underlying the Herceptin resistance is key to developing effective resistance-free therapeutic strategy.

The cerium oxide nanoparticles (CONPs) have emerged with promise for hypoxia-targeted anticancer therapy. The anticancer potential of CONPs results from the unique surface chemistry, ignorable side effects and effective clearance [7, 8]. CONPs are well known for its catalytic and oxygen buffering capacity [9] due to the dynamic surface oxidative status [10, 11]. In an acidic environment, CONPs act as a pro-oxidant and convert superoxide (O_2^{2-}) to hydrogen peroxide (H_2O_2) [8, 12, 13]. By contrast, in a

neutral pH environment, CONPs play an anti-oxidant role by converting H_2O_2 to H_2O [8, 13, 14]. Reactive oxygen species such as H_2O_2 can damage both tumor and normal tissues. Fortunately, the pro-oxidant role of CONPs allows CONPs to selectively target and kill tumor cells that tend to be acidic, particularly in the hypoxic tumor core, while the anti-oxidant property of CONPs allows CONPs to selectively avoid toxicity to normal tissues that tend to have a neutral pH value. Indeed, the tumor-selective anticancer role of CONPs has been demonstrated in various cancer types [8, 13, 15-18]. When used in combination with radiotherapy, CONPs protect normal tissues [13, 17, 19-24] including the mammary glands [25] from undesired radiation damage. The non-toxic feature of CONPs is also attributed to their effective clearance from the body without overt pathology regardless of administration routes [12, 26-29].

The hypoxia-induced factor 1α (HIF-1α) is highly induced under hypoxic and acidic tumor milieu. Various cancer signaling factors including cell surface receptors such as HER2 and intracellular signaling proteins such as PI3K-AKT, MEK-ERK, STAT3 and PKC can induce HIF-1α expression under hypoxia [30, 31]. HIF-1α in turn activates the transcription of a wide array of target genes such as the vascular endothelial growth factor (VEGF), insulin like growth factor binding protein (IGF-BP), transforming growth factor-α (TGF-α), multidrug resistance protein 1 (MDR1), leptin and carbonic anhydrase 9 (CAIX) [30, 31]. HIF-1α signaling strongly promotes aggressive tumor progression and is highly associated with drug resistance, tumor relapse and poor patient survival in many types of cancer [30, 31]. Therefore, targeting HIF-1α could possibly improve the outcome of existing anticancer therapy including anti-HER2 therapy [32-36]. However, there remains no active drug that can selectively target HIF-1α in tumors. In addition, whether HIF-1α is responsible for $HER2^+$ breast cancer resistance to Herceptin is unclear but an important research topic.

The concurrence of acidity and hypoxia in the drug resistant core of tumors raises a high possibility for CONPs to selectively inhibit HIF-1α induction in the Herceptin-resistant $HER2^+$ breast cancer cells and overcome the Herceptin resistance in the clinic. In this study, we discovered that application of CONPs can strongly sensitize Herceptin-resistant $HER2^+$ breast cancer cells to Herceptin-based treatment through inhibiting induction of HIF-1α and VEGF expression, reducing proliferation, survival and angiogenesis and consequently suppressing tumor growth and metastasis.

Materials and methods

Antibodies and reagents

Antibodies against HIF-1α (SC-13515), VEGFA (SC-7269), and CAIX (SC-365900) were purchased from Santa Cruz Biotechnology Inc. Antibodies for Ki67 (8D5), CD31 (#3528) and cleaved caspase 3 (#9661) were purchased from Cell Signaling Technology Inc. CONPs were described previously [37, 38]. Herceptin was purchased from Bio-Rad (MCA6092. a.k.a. Trastuzumab biosimilar). WST-1 Cell Proliferation Reagent was purchased from Clontech (PT3946-1). The VEGF-A human ELISA kit was purchased from Invitrogen (ThermoFisher Scientific BMS277-2).

Cell line generation, plasmid construction, and cell culture

The Herceptin-resistant human $HER2^+$ breast cancer cell lines HR20 and Pool2 established from the Herceptin-sensitive parental cell lines BT474 and SKBR3, respectively were described previously [3, 39, 40]. We generated HR20-nGL, BT474-nGL, Pool2-nGL and SKBR3-nGL cell lines stably expressing a GFP-Luciferase fusion reporter to allow whole animal non-invasive live bioluminescent imaging (BLI) monitoring and analysis of tumor growth and metastasis [41-43] by retroviral transduction as we previously reported [44, 45]. The cells were cultured in DMEM/F-12 (1:1) medium containing 10% FBS and a 37°C humidified atmosphere containing 5% CO_2 and 20% (normoxia) or 1% (hypoxia) O_2 supplemented with Herceptin (5 μg/ml), CONPs (75 μM) or both as needed for the experiments.

Analysis of cell viability, survival and proliferation

Cells were grown under the normoxic or hypoxic culture conditions in the presence or absence of CONPs or/and Herceptin for an appropriate time length. For viability assay, the cells that

were rounded up were collected and then stained with trypan blue by standard procedure. In the meantime, the cells that remained adherent were detached by trypsin treatment, collected and stained similarly with trypan blue. Cells were counted with a hemocytometer for analysis of cell viability. Cells that do not take the dye are considered as cells that remain alive, and normalized to mock-treated group for analysis of significant difference. Cell survival (or colony-forming) capability and proliferation potential were analyzed by clonogenic and WST-1 assays, respectively as described previously [13, 46, 47]. Briefly, the attached cells were collected and re-seeded to allow them to grow and form colonies under the same culture and treatment conditions for clonogenic assay, or be incubated with the WST-1 reagent for measuring the optical density values at 440 nm, namely, proliferative potential.

Western blotting, quantitative real-time PCR, dot blotting and ELISA

Antibodies, cell lines and reagents were described above. Cells were grown and treated as needed. Total RNA and protein were extracted from the cells or conditioned medium and processed for expression analysis by these standard assays indicated as previously described [45, 48-52]. Primers for human VEGFA were 5'-AGGGCAGAATCATCACGAAGT-3' (forward) and (5'-AGGGTCTCGATTGGATGGCA-3' (reverse).

Analysis of tumor growth and metastasis in vivo

The mice were housed and maintained in specific pathogen-free conditions in the UCF vivarium approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the National Institute of Health. The animal experiments were guided by the university-approved IACUC protocol (PROTO202000093) with thorough consideration of humane care of the mice. Female NSG mice at age of 4-6 weeks were randomly assigned into six mice per group. Orthotopic tumor growth, metastasis and response to CONPs and/or Herceptin treatments were examined and analyzed by weekly BLI analysis using a Xenogen IVIS imaging system as previously described [41, 44, 45, 48, 50, 53, 54]. Briefly, 5×10^6 cells in 50 µl phosphate buffered saline with 50% Matrigel were injected into the mammary fat pad. The next day, each group of mice received treatment with intraperitoneal injection of 100 µl of saline solution of Herceptin, CONPs, their combination or saline alone, twice a week for two consecutive weeks [13]. Mice were subject to weekly whole body live BLI analysis of primary tumor growth for up to 8 weeks. After the last whole body BLI, mice were sacrificed and immediately subject to lung-specific BLI analysis for lung metastasis [54]. After mice were sacrificed, tumors were collected and tumor weight was recorded. Tumor growth rate was determined by the change in the luminescent signal intensity over the time [44]. The length and width of each tumor were also measured using caliper to document tumor volume using the formula of volume = (length × width^2)/2.

Immunohistochemical (IHC) staining

The tissue collection and processes and the staining procedures were previously described [41, 44, 45, 48, 50, 53, 54]. Antibodies were described above. The tumor tissue samples were stained for the expression of Ki67 for proliferation, VEGF and CD31 for angiogenesis or cleaved caspase 3 for apoptosis. Optical density for IHC staining in five random areas was obtained for each treatment group by ImageJ Fiji color deconvolution analysis. Relative optical density values were normalized to that of the saline treated group and graphed.

Statistical analysis

Mean ± standard deviation was presented with a minimum of three observations per group. Student's *t*-test, or one-way ANOVA, unpaired, paired or single sample, with the Bonferroni correction for the multiple comparisons and Prism were used as appropriate. Significance was determined by the alpha level of 0.05.

Results

CONPs enhance the cytotoxic effect of Herceptin on the Herceptin-resistant cells particularly under hypoxic conditions

The Herceptin-resistant HER2$^+$ human breast cancer cell lines R20 and Pool2 were established by treating the respective Herceptin-

sensitive HER2+ cell lines BT474 and SKBR3 in a way that mimics the clinical treatment of HER2+ breast cancer patients who eventually acquired Herceptin resistance and tumor relapse [3, 39, 40]. Thus, we assessed the growth and survival of R20 and Pool2 cell lines to recapitulate the Herceptin-resistant relapse of breast cancer. To test if CONPs or its combination with Herceptin affects the viability of the HER2+ breast cancer cells, the R20 cells, paired with BT474 cells [3], were grown under the normoxic or hypoxic conditions while being treated with CONPs in combination with Herceptin, CONPs, Herceptin, or saline only (**Figure 1**).

Under the normoxic conditions, Herceptin treatment caused significant round-ups of the Herceptin-sensitive cells (**Figure 1A**, compared b with a), but not the Herceptin-resistant cells (**Figure 1A**, compare b' with a'). CONPs treatment did not seem to affect either the Herceptin-sensitive cells or the Herceptin-resistant cells (**Figure 1A**, compare c with a or c' with a'). Herceptin plus CONPs caused more round-ups than Herceptin alone did of the Herceptin-sensitive cells (**Figure 1A**, compare d with b), but not the Herceptin-resistant cells (**Figure 1A**, compare d' with b'). These results suggest that under the normoxic conditions, CONPs do not seem to be cytotoxic to either the Herceptin-sensitive or the Herceptin-resistant cells, or sensitize either of cell lines to the cytotoxic effect of Herceptin.

Previous studies have shown cytotoxic effect of CONPs on various types of cancer cells [8, 13, 17, 18, 54, 55], particularly under acidic conditions [8, 37, 38]. Since the acidic conditions *in vivo* are primarily associated with the hypoxic tumor microenvironment, we thought that CONPs may play a cytopathic or sensitizing role under the hypoxic conditions. Under the hypoxic culture conditions, Herceptin treatment again caused a similar degree of round-ups to the Herceptin-sensitive cells (**Figure 1A**, compared f with e). Interestingly, Herceptin treatment also caused round-ups of the Herceptin-resistant cells (**Figure 1A**, compare f' with e') albeit to a much less degree (**Figure 1A**, compare f' with f). CONPs treatment did not seem to be cytotoxic to either of the cell lines (**Figure 1A**, compare g with e or g' with e'). However, when Herceptin and CONPs were combined together, two to four times more cells became rounded up particularly in the Herceptin-resistant cells compared to treatment with Herceptin alone (**Figure 1A**, compare h' with f' or h with f).

The cell viability analysis confirmed that the attached cells and the rounded up cells echo likely viable and dead cells, respectively (**Figure 1B**). Similar results were obtained also from the independent pair of SKBR3 and Pool2 cell lines [3] (data not shown). These results suggest that in the hypoxic environment, CONPs can potently sensitize the cells to the cytotoxic effect of Herceptin regardless of their prior sensitivity to Herceptin.

CONPs sensitize the breast cancer cells to the inhibitory effect of Herceptin on cell survival and proliferation

To confirm that the cell round-up observed truly reflects cytotoxicity of the treatments, we tested the effect of the treatments on the cell survival and proliferation using the clonogenic (**Figure 2A**) and WST-1 (**Figure 2B**) assays, respectively.

We found that under the normoxic conditions, treatment with Herceptin alone dramatically reduced the colony-forming capacity (by ~70%) (**Figure 2A**, compare b with a) and proliferative capacity (by ~25%) (**Figure 2B**, compared b with a) in the Herceptin-sensitive cells, but not in the Herceptin-resistant cells (**Figure 2A** and **2B**, compare b' with a'). Treatment with CONPs alone moderately inhibited the colony-forming capacity (by ~20%) (**Figure 2A**, compare c with a) and proliferative capacity (by ~25%) (**Figure 2B**, compare c with a) in the Herceptin-sensitive cells. In the Herceptin-resistant cells, however, CONPs alone did not affect the colony-forming capacity (**Figure 2A**, compare c' with a') although CONPs alone did inhibit the proliferative capacity slightly (**Figure 2B**, compare c' with a'). Herceptin plus CONPs together caused further inhibition of both the colony-forming capacity and proliferative capacity in both the Herceptin-sensitive (**Figure 2A** and **2B**, compare d with b) and Herceptin-resistant cells (**Figure 2A** and **2B**, compare d' with b') although the Herceptin-sensitive cells remain much more sensitive than the Herceptin-resistant cells to the combined treatment (**Figure 2A** and **2B**, compare d with d').

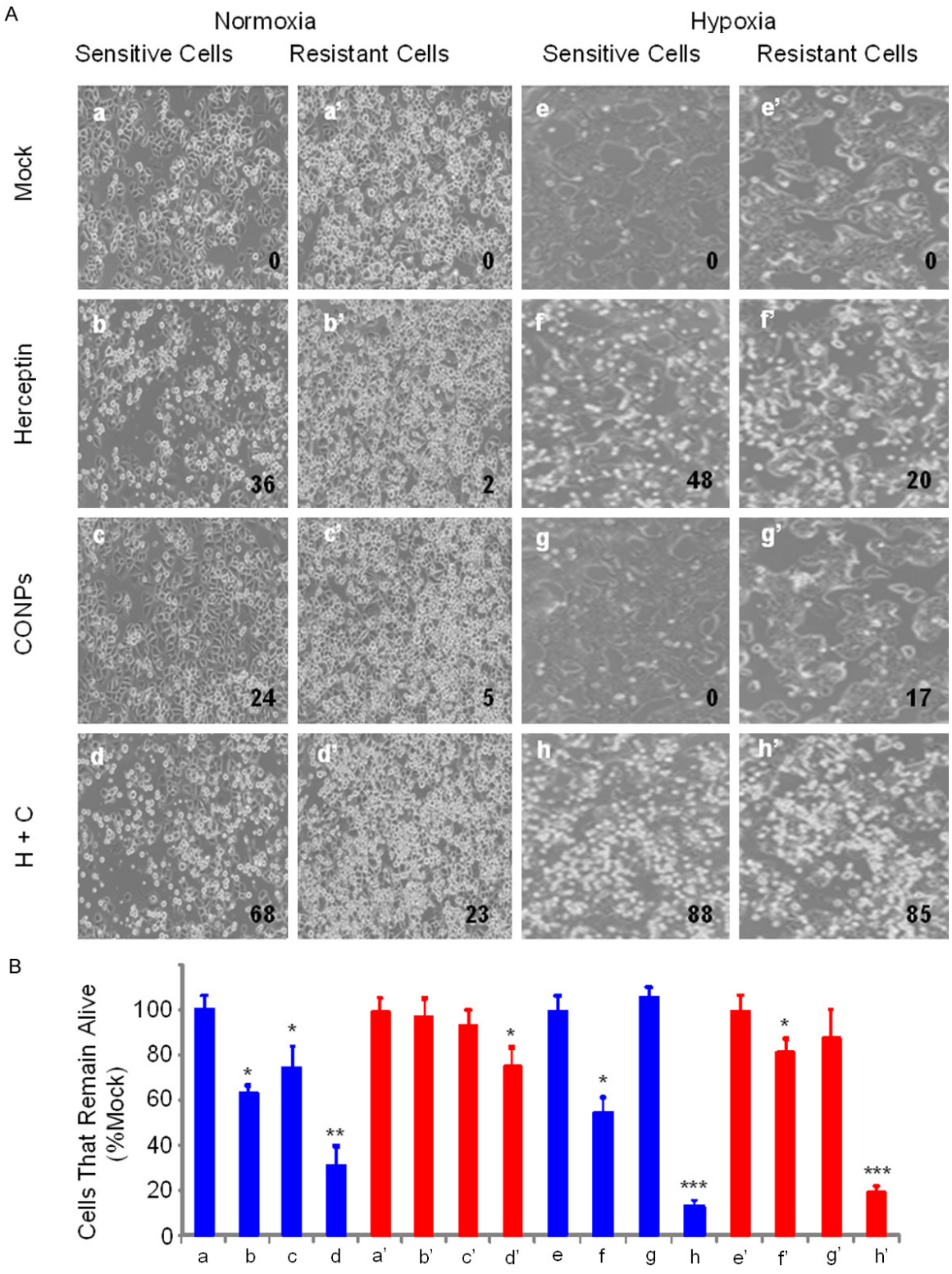

Figure 1. CONPs enhance the cell round-up effect of Herceptin particularly under the hypoxic conditions. (A) The Herceptin-sensitive (BT474) and Herceptin-resistant cells (R20) were grown under the normoxic (normoxia) or hypoxic (hypoxia) conditions. At sub-confluent density, the cells were treated with saline (Mock), Herceptin (5 μg/ml), cerium oxide nanoparticles (CONPs) (75 μM) or Herceptin in combination with CONPs (H + C) for 24 hours prior to phase contrast microscopy. (B) Cells were analyzed for viability by separately collecting and staining the adherent and round up cells with trypan blue as described in Materials and Methods. The number inside each image in (A) indicates percentage of cells that became rounded up. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to respective mock-treated group.

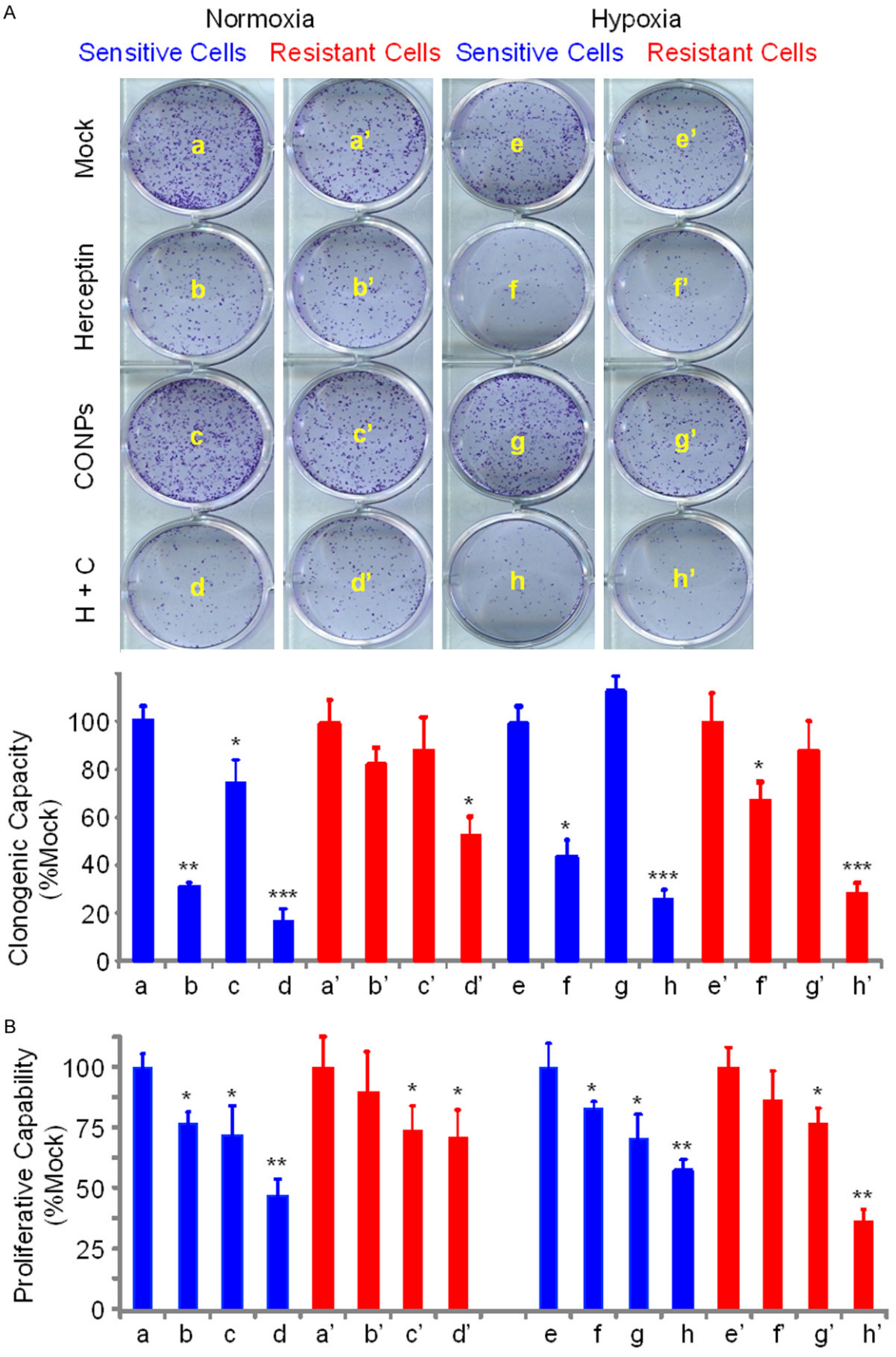
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Figure 2. CONPs sensitizes both Herceptin-sensitive and Herceptin-resistant cells to the inhibitory effect of Herceptin on cell survival and proliferative capacities more potently under hypoxia than under normoxia. The cells were grown and treated as described in **Figure 1**. The cells were then prepared for analysis of colony-forming capability by clonogenic assay (A) and proliferative capacity by WST-1 assay (B) as detailed in Materials and Methods. *P<0.05, **P<0.01 and ***P<0.001 compared to respective mock-treated group.

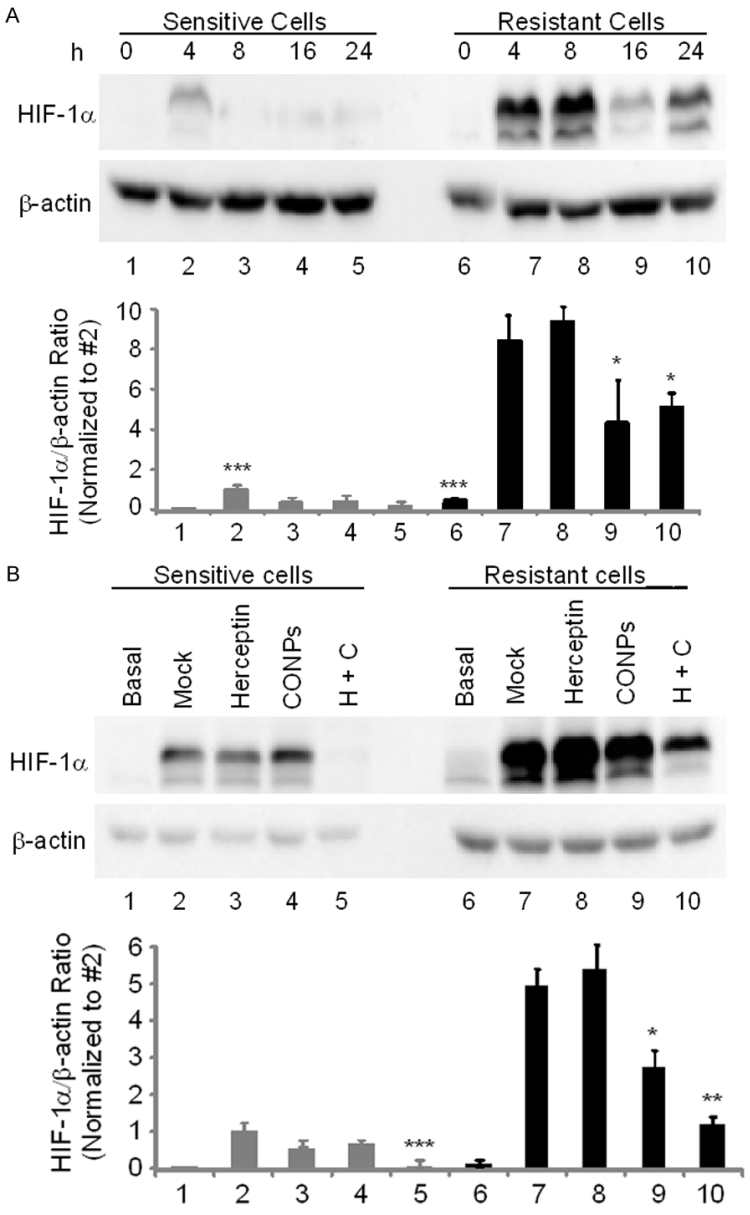

Figure 3. CONPs potently and rapidly inhibit HIF-1α induction by hypoxia particularly in the Herceptin-resistant cells. A. Time-course change in HIF-1α expression under the hypoxic conditions. After incubated for indicated periods, the cells were processed for western blotting. HIF-1α levels relative to β-actin were quantified and normalized to 4-h-treated Herceptin-sensitive cells. *P<0.05 compared to columns 7 or 8. ***P<0.001 compared to column 1 as well as columns 7-10. B. CONPs markedly inhibit HIF-1α induction by hypoxia in the cells. Indicated cells were incubated either under that normoxic conditions and mock-treated only (basal) or under the hypoxic conditions and treated with vehicle (Mock), Herceptin, CONPs or their combination (H + C) for 4 hours. The cells were then processed for western blotting and quantitative analysis. *P<0.05 compared to column 7 or 8. **P<0.01 compared to column 7 or 8. ***P<0.001 compared to columns 2-4.

Under the hypoxic conditions, treatment with Herceptin or CONPs alone did not make much difference from what were seen under the normoxic conditions in the profile of the colony-forming capacity and proliferative capacity in either of the Herceptin-sensitive (**Figure 2A** and **2B**, compare e-g with a-c) and Herceptin-resistant cells (**Figure 2A** and **2B**, compare e'-g' with a'-c'). Herceptin plus CONPs, however, caused significantly more inhibition of colony-forming capacity (by ~70%) (**Figure 2A**, compare h' with d') and proliferative capacity (by ~60%) (**Figure 2B**, compare h' with d') in the resistant cells.

Taken together with the data shown in **Figure 1**, these results suggest that under the hypoxic conditions, CONPs potently sensitize the otherwise Herceptin-resistant cells to the cytotoxic effect of Herceptin.

CONPs potently inhibit HIF-1α induction by hypoxia

HIF-1α is critical for cell survival and proliferation under hypoxia [30, 31], and the acidity associated with hypoxia is the preferential factor for CONPs to function as an anti-cancer prooxidant [8, 37, 38]. We thus tested if the expression of HIF-1α in the cells is affected by the treatment with CONPs.

We first compared the pattern of HIF-1α induction by hypoxia between the Herceptin-sensitive and Herceptin-resistant cells under the hypoxic culture conditions (**Figure 3A**). In the

beginning of the hypoxic culture, the basal levels of HIF-1α were nearly undetectable in the Herceptin-sensitive cells, but slightly detectable in the Herceptin-resistant cells (compare lane/column 6 with 1). HIF-1α expression was rapidly induced as early as 4 hours of the hypoxic culture in both the Herceptin-sensitive cells and the Herceptin-resistant cells (compared lane/column 2 or 7 with 1 or 6). While the induced HIF-1α expression quickly faded away in the Herceptin-sensitive cells thereafter, the levels of HIF-1α in the Herceptin-resistant cells not only went much higher (~8 times) after 4 hours but also remained constantly high (>24 hours) (compare lane/column 7 with 2 and 7-10 with 2-5). We then treated the cells with CONPs or/and Herceptin during the first 4 hour of the hypoxic culture (**Figure 3B**). Treatment with CONPs alone reduced HIF-1α levels by ~50% in the Herceptin-resistant cells (compare lane/column 9 with 7 or 4 with 2) but not in the Herceptin-sensitive cells. Significantly, treatment with CONPs plus Herceptin almost completely prevented the induction of HIF-1α expression in both the Herceptin-resistant cells and the Herceptin-sensitive cells (compare lane/column 10 with 7 & 6 or 5 with 2 & 1). Notably, treatment with Herceptin alone did not alter the induction of HIF-1α in either of the cells (compare lane/column 8 with 7 or 3 with 2).

These results suggest that treatment with CONPs can remarkably sensitize the cells, especially the Herceptin-resistant cells, to the cytotoxicity of Herceptin under hypoxia by, at least partially, downregulating the expression of HIF-1α.

CONPs rapidly reduce extracellular VEGF levels although slowly reduced intracellular VEGF levels under hypoxia

VEGF and CAIX are among the important targets of HIF-1α transcriptional activation under hypoxia. We next examined if their expression levels change upon treatment with CONPs alone or in combination with Herceptin (**Figure 4**). Western blot revealed that the basal levels of intracellular VEGF protein appeared much higher in the Herceptin-resistant cells than the Herceptin-sensitive cells (**Figure 4A**, **4B**, compare lane/column 6 with 1). By contrast, the basal levels of intracellular CAIX protein appeared unaltered, if not lower, in the Herceptin-resistant cells than that in the Herceptin-sensitive cells (**Figure 4A**, compare lane 6 with 1), suggesting that unlike VEGF, CAIX may not be a primary factor among HIF-1α target genes that contribute to the Herceptin-resistance. Levels of VEGF protein inside the cells were not affected within 8 hour treatment with CONPs and/or Herceptin (**Figure 4A**, **4B**, compare lanes 3-5 with 2 or 8-10 with 7). After 24 hours, however, treatment with CONPs alone or in combination with Herceptin dramatically reduced VEGF protein expression, especially in the Herceptin-resistant cells (**Figure 4B**, compare lanes/columns 9 and 10 with 6 or 7). Indeed, levels of VEGF mRNA were not affected, either within 8 hour treatment with CONPs and/or Herceptin (**Figure 4C**).

In order for VEGF protein to function, it must first be secreted out of the cells. To test if the levels of functional VEGF outside the cells are affected upon treatment with CONPs and/or Herceptin, extracellular VEGF protein from the culture medium was analyzed by dot blotting after 4 hours of treatment (**Figure 5A-D**) as well as by ELISA after 24 hours of treatment (**Figure 5E**). Surprisingly, treatment with CONPs (but not Herceptin), particularly in combination with Herceptin, caused a significant decrease in extracellular VEGF levels in the Herceptin-resistant cells under the hypoxic culture conditions (**Figure 5C**, **5D**, compare dots/columns 4 or 5 with 1-3; **Figure 5E**, compare column 7' or 8' with 5' or 6').

These results suggest that the hypoxia-associated HIF-1α-to-VEGF signaling axis serve as a critical factor responsible for the Herceptin resistance, and as a major inhibitory node of CONPs in combination with Herceptin.

Treatment with CONPs plus Herceptin inhibits the Herceptin-resistant tumor progression in vivo by interfering with the cell survival, proliferation, and tumor angiogenesis

We next sought to determine the effect *in vivo* of CONPs plus Herceptin on tumor progression of the Herceptin-resistant Pool2-nGL cells. As early as 4 weeks after the cells were implanted into the mammary fat pads of the mice that subsequently received treatment with CONPs in combination with Herceptin, the tumor

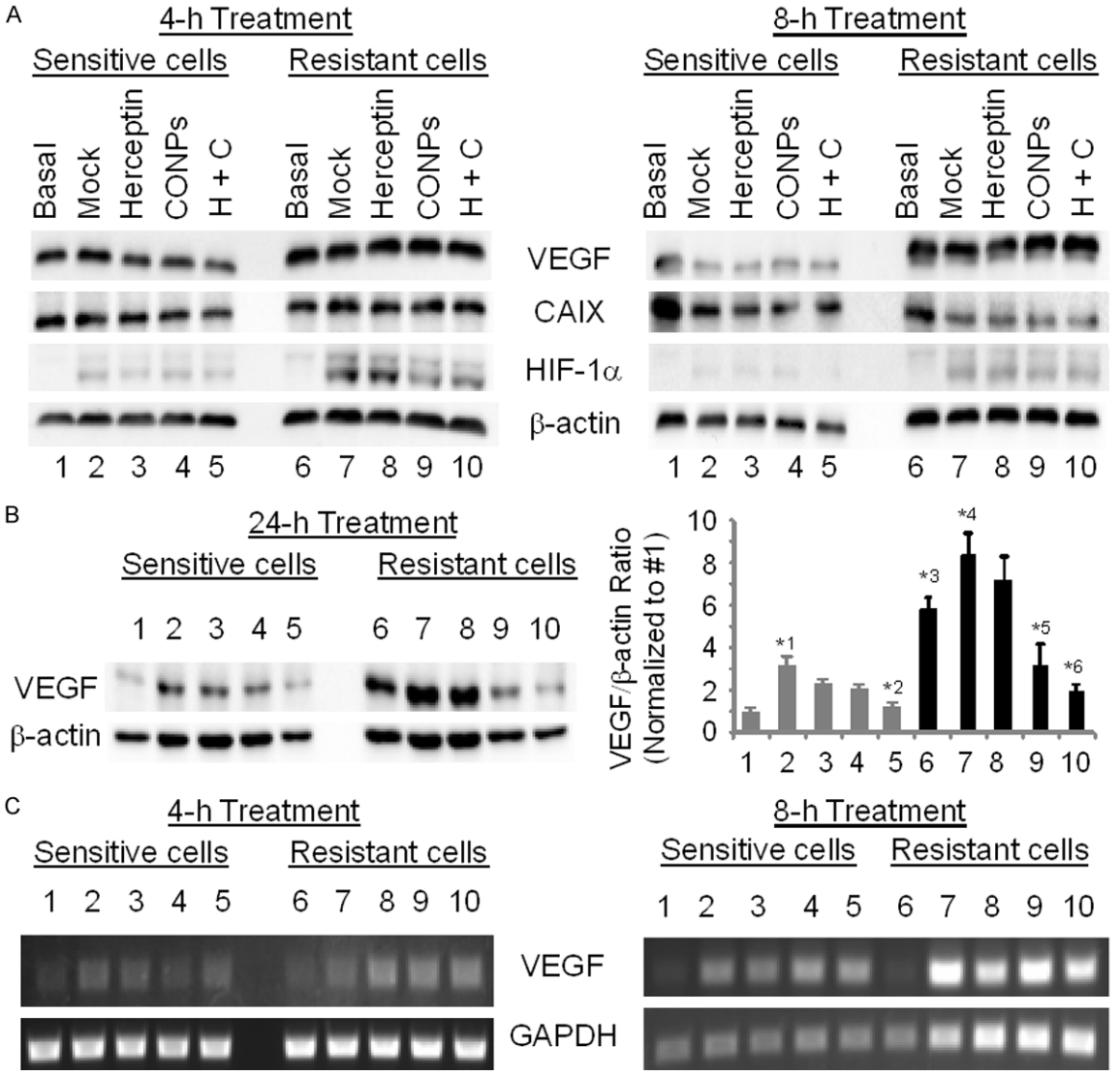

Figure 4. Treatment with CONPs causes slow reduction in intracellular levels of VEGF under the hypoxic conditions. A. Levels of VEGF and CAIX proteins were not affected within 8 hour treatment with Herceptin and/or CONPs. Indicated cells were incubated either under the normoxic conditions and mock-treated only (basal) or under the hypoxic conditions and treated with vehicle (Mock), Herceptin, CONPs or their combination (H + C) for 4 or 8 hours. The whole cell extracts were then processed for western blotting. B. Treatment with CONPs alone or in combination with Herceptin leads to severe decrease in levels of VEGF protein particularly in the Herceptin-resistant cells after 24 hour treatment. The cells were treated similarly as described above in *A* for 24 hours prior to western analysis. $^{*1}P<0.01$ compared to column 1; $^{*2}P<0.01$ compared to column 2; $^{*3}P<0.001$ compared to column 1; $^{*4}P<0.05$ compared to column 6; $^{*5}P<0.05$ or 0.01 compared to column 6 or 7; $^{*6}P<0.01$ or 0.001 compared to column 6 or 7. C. Levels of VEGF mRNA were not affected within 8 hours treatment with Herceptin and/or CONPs. VEGF mRNA was analyzed by semi-quantitative PCR shown here and quantitative real-time PCR (not shown).

growth in the primary site was significantly slowed down compared to that in the group treated with Herceptin, CONPs or saline alone (**Figure 6A-D**). IHC staining analysis showed that the CONPs plus Herceptin treatment resulted in significantly less expression of the proliferation marker protein Ki67 (**Figure 6E**, **6F**), the angiogenic marker protein VEGF (**Figure 7A**), and the vascular endothelial marker protein CD31 (**Figure 7B**), whereas expression of the apoptotic marker protein cleaved caspase 3 was significantly increased (**Figure 7C**), when compared to the other three treatment groups, suggesting that the combination of CONPs with

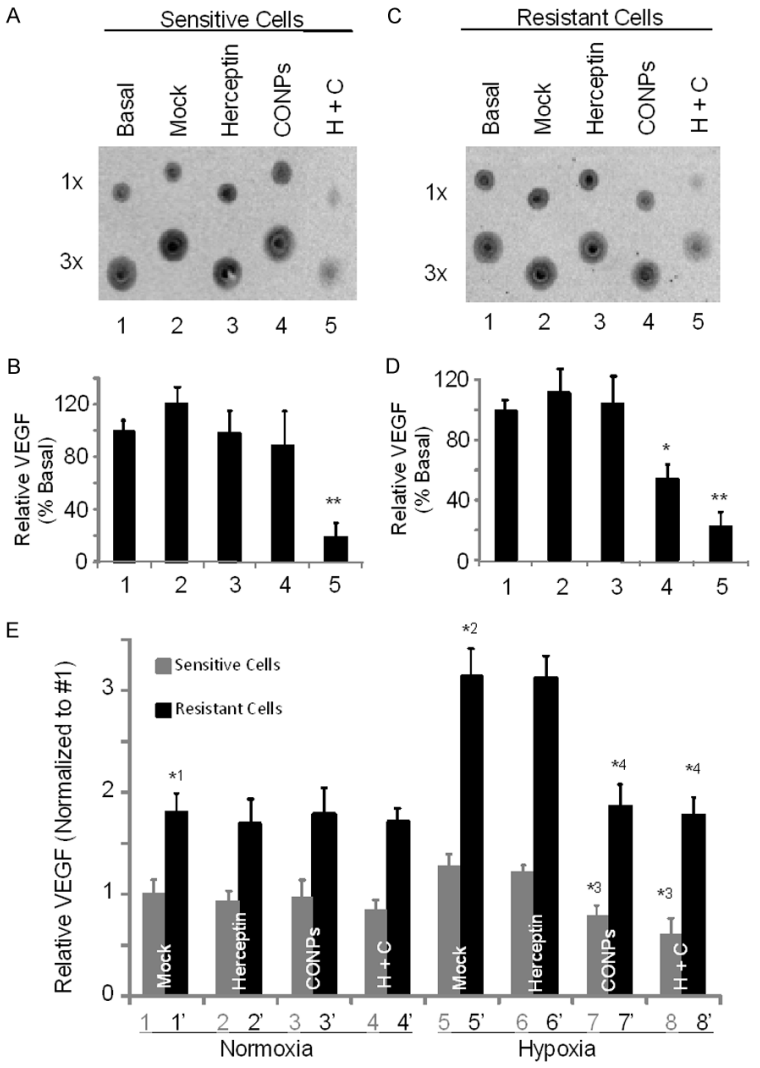

Figure 5. Treatment with CONPs causes rapid and significant decrease in extracellular VEGF levels under hypoxic conditions. (A-D) Dot blotting analysis of VEGF protein in the culture media of the cells. Cells were incubated either under the normoxic conditions and mock-treated (basal) or under the hypoxic conditions and treated with vehicle (Mock), Herceptin, CONPs or their combination (H + C) for 4 hours. The medium of the cells was then collected and processed for dot blotting (A, C) and quantitative (B, D) analysis of extracellular VEGF levels. *P<0.05 and **P<0.01 compared to column 2. (E) ELISA analysis of VEGF protein in the media of the cells. The cells were incubated under either the normoxic or hypoxic conditions for 24 hours. The media of these cells were then collected and processed for ELISA analysis. *1P<0.01 compared to column 1; *2P<0.01 compared to column 1'; *3P<0.05 and *4P<0.01 compared to column 5'.

Herceptin targets proliferation, angiogenesis and survival for inhibition of tumor growth. The lung-specific BLI analysis revealed drastically less burden of lung metastasis in the mice treated with CONPs plus Herceptin than that in the mice treated with either of them alone (**Figure 8**). These results strongly indicate that CONPs can sensitize the otherwise Herceptin-resistant breast cancer to the cytotoxicity of Herceptin *in vivo* by inhibiting the cell proliferation, survival and angiogenesis, resulting in suppression of tumor growth and metastasis.

Discussion

In this study, we demonstrate two previously uninvestigated aspects regarding Herceptin resistance by the HER2$^+$ human breast cancer cells: (1) there is a higher basal level of the HIF-1α-VEGF signaling activity in the Herceptin-resistant HER2$^+$ breast cancer cells than that in the Herceptin-sensitive breast cancer cells under the normoxic conditions, and this difference becomes even larger and lasts even longer once the cells are placed under the hypoxic conditions; (2) treatment with CONPs dramatically enhance the cytotoxicity of Herceptin to the Herceptin-resistant HER2$^+$ breast cancer cells displaying a significant decrease in the expression of HIF-1α and VEGF especially under the hypoxic conditions in culture, as well as in the xenograft tumors *in vivo*.

As described previously, the Herceptin-resistant human breast cell lines (HR20 and Pool2) were established through selecting the Herceptin-sensitive parental cell lines (BT474 and SKBR3, respectively) by treatment with Herceptin [3, 39, 40]. In other words, the cell capacity of resistance to Herceptin was acquired gradually over the course of Herceptin resistance development in the laboratory that mimics the clinical course in patients. For this reason, the higher levels of HIF-1α must have been gained during the selection process by Herceptin treatment in the laboratory as well as in the clinic. It

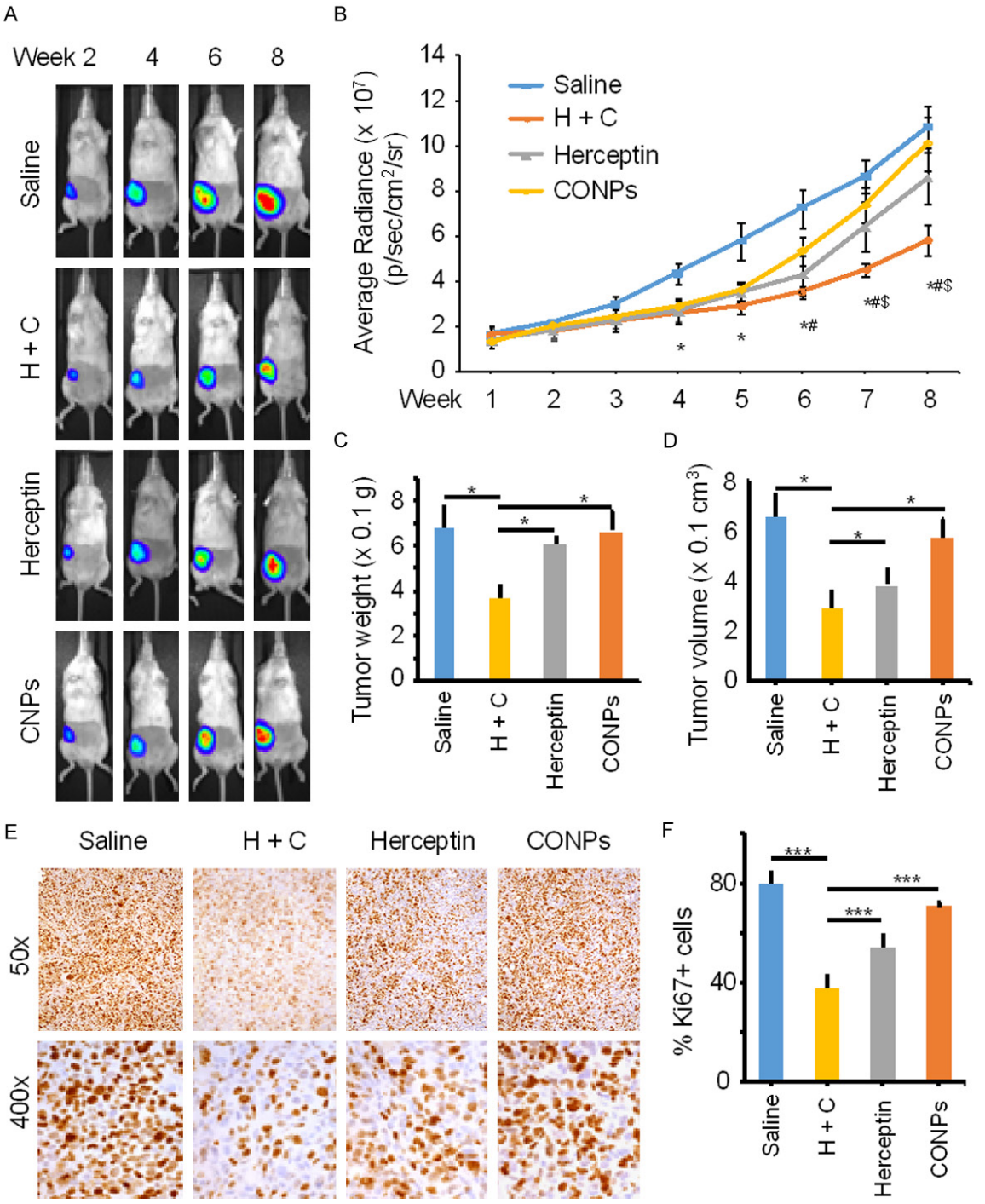

Figure 6. CONPs sensitize Herceptin-resistant cells to the inhibitory effect of Herceptin on primary tumor growth. The HR20-nGL breast cancer cells were injected into the mammary fat pad of 4-6-week-old female NSG mice. The next day mice were randomly grouped into four groups and received intraperitoneal injection of 100 μl saline solution of Herceptin (2 mg/kg) in combination with CONPs (0.1 mg/kg), Herceptin, CONPs or saline alone, twice a week for two consecutive weeks. Primary tumor progression was monitored and examined by weekly BLI analysis for 8 weeks (A, B). After sectioned, the tumors were measured for weight and volume (C, D), and the tissues were further processed for IHC staining for expression of the proliferative marker Ki67 (E, F). $P<0.05$ compared between *H + C and saline alone, #H + C and CONPs alone, and $H + C and Herceptin alone.

is worth of further investigating exactly how the levels of HIF-1α and as well its responsiveness to hypoxia is upregulated during the Herceptin-resistance acquiring process.

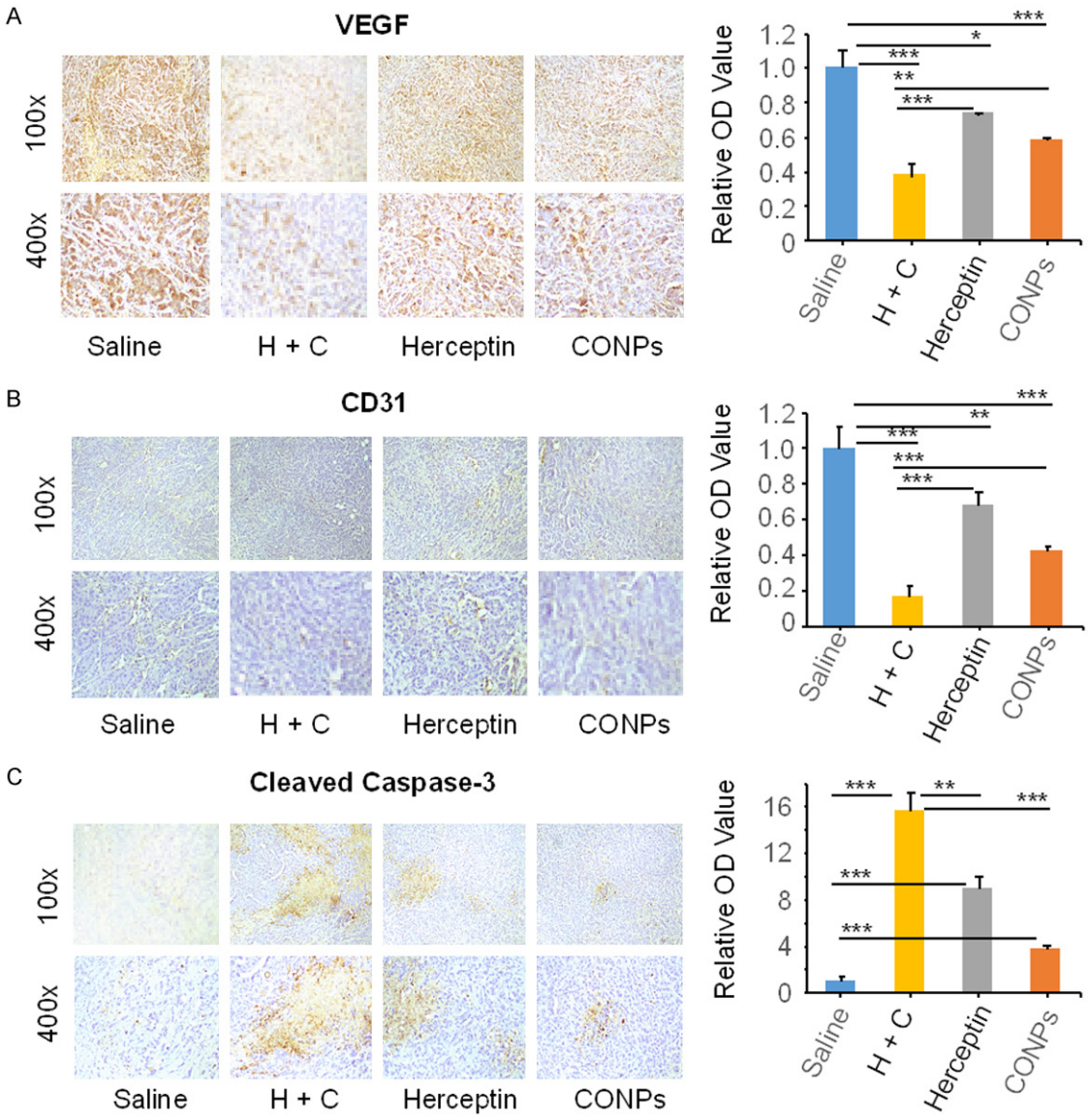

Figure 7. CONPs sensitize Herceptin-resistant cells to the inhibitory effect of Herceptin on tumor angiogenesis and survival. The tumor tissues collected from the mice described in **Figure 6** were processed for IHC staining for expression of the angiogenic factor protein VEGF (A), the vascular endothelial marker protein CD31 (B), and the apoptotic marker protein cleaved caspase-3 (C). Optical density (OD) for expression quantification was obtained by ImageJ Fiji color deconvolution analysis and normalized to the Saline group value. *P<0.05; **P<0.01; ***P<0.001.

How HIF-1α contributes to the Herceptin resistance is unclear. HIF-1α is well known to work as a transcription activator. Once induced by hypoxia, HIF-1α activates the transcription of target genes such as VEGF among many other genes [30, 31] that favor cell proliferation, survival and angiogenesis, suggesting that the overexpressed HIF-1α signaling activity could be responsible for the Herceptin resistance through enhanced proliferation, survival and angiogenesis. Indeed, as shown in the results of immunohistochemical staining of the tumor tissue for expression of the cellular marker proteins for proliferation, angiogenesis and apoptosis (**Figures 6**, **7**), treatment with CONPs plus Herceptin causes significant reduction in the cell capability of survival and proliferation as well as the tumor angiogenesis, via likely blocking the HIF-1α signaling, resulting in suppressed tumor growth and metastasis (**Figures 6**, **8**).

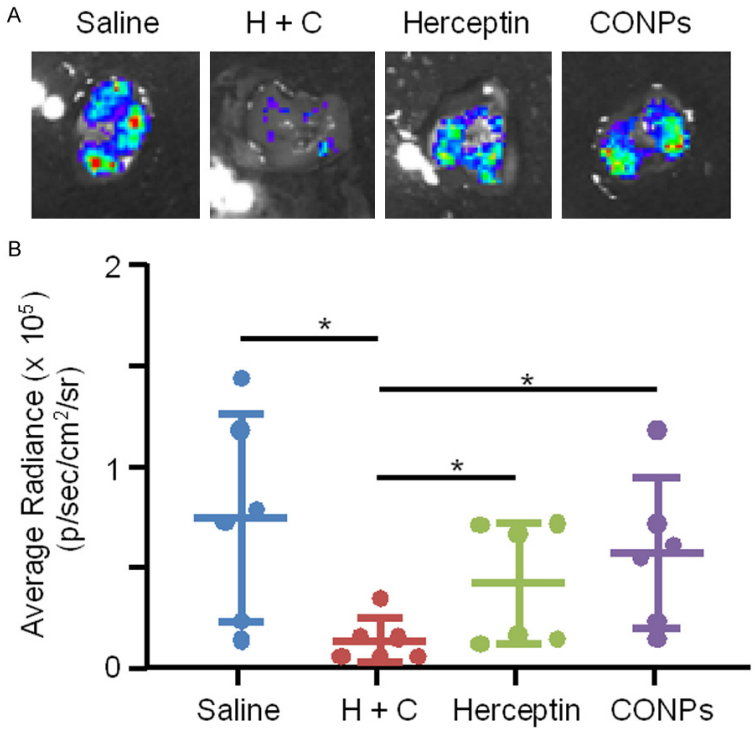

Figure 8. CONPs sensitize the Herceptin-resistant breast cancer cells to the inhibitory effect of Herceptin on the lung metastasis. During tissue collection from the mice described in **Figure 6**, the lung specific BLI was performed (A) and bioluminescent intensity for the lung metastasis quantified (B). *P<0.05.

We noticed that under the hypoxic culture conditions, treatment with CONPs in combination with Herceptin killed the Herceptin-sensitive and Herceptin-resistant cells to a similarly significant extent (**Figures 1**, **2**) regardless of the marked difference in the expression of HIF-1α and VEGF between the cells (**Figures 3**-**5**). For these reasons, HIF-1α may be targeted by the combination therapy in the Herceptin-resistant cells only. In other words, the combination treatment may kill the Herceptin-sensitive cells independently of HIF-1α signaling. In addition, there is no angiogenic activity under the tissue culture conditions. Thus, the angiogenic signaling may not play a major role under the cell culture conditions but is critically targeted *in vivo* by the combination treatment in the tumor-bearing mice and patients. Likewise, CONPs could also possibly target cancer stem cells that are predominantly homed in the hypoxic and acidic tumor core *in vivo*.

Among several candidate target genes tested, we found that VEGF, like HIF-1α, has a higher basal level under the normoxic culture conditions and even more upregulated upon hypoxia in the Herceptin-resistant cells compared to that in the Herceptin-sensitive cells. Our results indicate that treatment with CONPs, particularly in combination with Herceptin, leads to rapid inhibition of VEGF secretion followed by a relatively slow decrease in intracellular VEGF levels (**Figures 4**, **5**). This suggests that the treatment acts first on the pre-existing VEGF protein by presumably targeting the VEGF protein for degradation and/or inhibiting its secretion independent of HIF-1α while blocking HIF-1α induction and subsequent VEGF transcription. It is likely that CONPs block VEGF expression by preventing HIF-1α upregulation. The mechanism by which CONPs cause the reduction of HIF-1α could be at any level of expression regulation starting from gene promoter activity through to protein stability which deserves further investigation. It likely has something to do with the pro-oxidant activity of CONPs given the concurrence of hypoxia and acidity in the cancer cell microenvironment [30, 31].

Herceptin remains the most widely used drug to treat HER2$^+$ breast cancer. However, resistance to the drug, even if given in combination with chemotherapy [5, 6], is a significant clinical problem that severely limits the efficacy of the drug [1, 3, 4]. Our results collectively show that the non-toxic CONPs can sensitize the HER2$^+$ human breast cancer cells to the cytotoxicity of Herceptin to multiple cellular aspects including survival, proliferation, angiogenesis and potentially cancer stem cells associated with HIF-1α signaling induction and activation. Therefore, this study provides novel insights into the mechanisms underlying the Herceptin resistance and opens a new avenue on developing effective therapeutics for the patients to overcome the drug resistance and maximize relapse-free survival.

Acknowledgements

This work was supported by an NIH grant (CA132977), a Florida Breast Cancer Foundation grant, the LIFE@UCF Richard Tucker Grant and an institutional grant to J.Z.

Disclosure of conflict of interest

None.

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