A Novel Experimental Heme Oxygenase-1–Targeted Therapy for Hormone-Refractory Prostate Cancer

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Abstract

Heme oxygenase-1 (HO-1), a member of the heat shock protein family, plays a key role as a sensor and regulator of oxidative stress. Herein, we identify HO-1 as a biomarker and potential therapeutic target for advanced prostate cancer (PCA). Immunohistochemical analysis of prostate tissue using a progression tissue microarray from patients with localized PCA and across several stages of disease progression revealed a significant elevation of HO-1 expression in cancer epithelial cells, but not in surrounding stromal cells, from hormone-refractory PCA (HRPCA) compared with hormone-responsive PCA (HRPCA). This coincided with reduced cell proliferation, cell survival, and cell invasion regulated kinase/p38 kinase. This coincided with reduced cell proliferation, cell survival, and cell invasion mediated by products can exert broad physiologic effects in cell signaling. These pathways play important roles in the regulation of mitogenesis and cell survival in the face of oxidative damage (3). Moreover, HO-1 can modulate the activity of cell signaling molecules such as the phosphoinositide 3-kinase, which can be either activated (moderate oxidative stress) to promote oxidative stress–induced cell survival or inhibited (exacerbated oxidative stress) to promote oxidative stress–mediated apoptosis (4).

The classic function of HO-1 is to catalyze heme oxidation to biliverdin, free ferrous iron, and carbon monoxide. Biliverdin is further metabolized to bilirubin through the action of biliverdin reductase. Bilirubin is then oxidized by cytochrome P450 enzymes (e.g., Cyp1A1, Cyp2B1, or Cyp2A5), or glucurononidated by UDP-glucuronyltransferases, and subsequently eliminated as bilirubin glucuronide by the biliary-excretory pathway. These HO-1–mediated byproducts can exert broad physiologic effects in cell-adaptive response to oxidative stress. For instance, free iron induces the expression of the iron-sequestering protein ferritin and activates Fe-ATPase, an iron transporter that prevents accumulation of intracellular Fe++. Up-regulation of ferritin and cytosolic iron efflux has a protective effect against oxidative stress. Intracellular carbon monoxide formed through HO-1 catalysis can act as a secondary messenger, which, like nitric oxide, regulates inflammatory signaling (1, 2, 5). Biliverdin and its by-product bilirubin are antioxidants and prosurvival proteins capable of reducing the inflammatory response and oxidative stress. Therefore, the primary function of HO-1 is to regulate cellular homeostasis and inflammatory response under oxidative stress and to promote cell survival (6–8). Moreover, HO-1 can promote angiogenesis through alternative mechanisms, including transcriptional up-regulation of vascular endothelial growth factor and production of proangiogenic carbon monoxide (9).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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In cancer, overexpression of HO-1 has been reported in several cancer tissues (10–12) and ho-1 gene polymorphisms have been associated with increased cancer susceptibility (13). However, the potential therapeutic implications of HO-1 have been hampered by the lack of clinically useful selective inhibitors. In this study, we show a robust overexpression of HO-1 in tissue samples from patients with HRPCA compared with localized PCA and benign prostate tissue. We established HO-1 as a potential therapeutic target for HRPCA models. We identified a selective small-molecule inhibitor of HO-1 (OB-24, the chemical structure is shown below) synthesized from the imidazole class, and having potent antitumor and antimetastatic activity in HRPCA preclinical models, particularly in combination with Taxol.

The chemical structure of 2-[2-(4-bromophenyl)ethyl]-2-[(1H-imidazol-1-yl)methyl]-1,3-dioxolane hydrochloride (OB-24, MW: 373.67) is shown above.

Materials and Methods

Antibodies and reagents. HO-1 and HO-2 antibodies were from StressGene and Santa Cruz Biotechnology, respectively; antibodies against ERK, p58, JNK, phospho-ERK (Tyr202/Tyr204), phospho-p38, and phospho-JNK were from Cell Signaling Technology; rabbit polyclonal antibody specific to AKT and phospho-AKT were from Santa Cruz Biotechnology; monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Cedarlane Laboratories; Taxol was from the oncology pharmacy of the Jewish General Hospital; and OB-24 was synthesized as described previously (14).

Cells and cell culture. The LNCaP, VCaP, and DU145 human PCA cell lines were from the American Type Culture Collection. The castration-resistant C4-2 PCA cells, which was derived from the LNCaP cell line (15), and the BPH-1 cells, which were derived from a benign prostate hyperplastic lesion, were provided by Dr. N. Zoubeidi (The Prostate Centre, Vancouver General Hospital). The highly invasive PC3M cell variant (16) was provided from Dr. I. Fidler (M.D. Anderson Cancer Center). The normal prostate tissue. We established HO-1 as a potential therapeutic target for HRPCA models. We identified a selective small-molecule inhibitor of HO-1 (OB-24, the chemical structure is shown below) synthesized from the imidazole class, and having potent antitumor and antimetastatic activity in HRPCA preclinical models, particularly in combination with Taxol.

Western blot analysis. Cells at 60% to 70% confluence were starved for 24 h in phenol red– and FBS-free medium before analysis. When indicated, HO-1 was induced by exposure of cells to hemin at a concentration of 10 μmol/L for 20 h. To determine the effect of HO-1 on mitogen-activated protein kinase (MAPK) activity, serum-starved cells were stimulated with 5 ng/mL epidermal growth factor (EGF) and cell extracts were used for immunoblotting with antibody specific for phosphorylated ERKs, p38, JNK, or AKT. Cell extracts were prepared for Western blot analysis as described earlier (18) using the indicated antibodies.

Measurement of HO-1 activity. Cytosol extracts were prepared for HO activity measurement as described by Ryter and colleagues (19). Bilirubin concentrations in the chloroform cell extracts were determined by spectrophotometry at 461- to 530-nm absorbance. HO-1 activity was calculated as nanomoles of bilirubin per milligram of protein per minute, assuming an extinction coefficient of 40/(mmol/L)/cm in chloroform.

Measurement of ROS and protein carbonylation. Intracellular ROS levels were determined using the H2DCF-DA reagent, based on the methodology described by Wang and Joseph (20). Results, in arbitrary fluorescence units (AFU), were expressed according to the ratio [AFU in treated cells / AFU in control cells] × 100. Cell fluorescence was also confirmed using a FACS machine (Becton Dickinson) equipped with CellQuest Pro Software. The data are presented as the ratio of mean fluorescence intensity (MFI) for 10,000 cells to MFI for 10,000 control cells. Quantification of protein carbonyl content was investigated by ELISA as measured by Winterbourn and Buss (21).

Cell proliferation assay. Exponentially growing cells (1 × 10^6) were seeded in 96-well plates in complete medium. Eighteen hours later, cells were treated continuously with OB-24. Cell survival was evaluated 96 h later using the MTT metabolic assay (18).

Cell invasion assay. Cell invasion assay was performed using 8-μm porous chambers coated with Matrigel (Becton Dickinson) according to the manufacturer’s recommendations. Detailed conditions were reported earlier (18).

In vivo tumorogenic studies in mice. In vivo studies were approved by the McGill Animal Care Committee (protocol number 4101) and were conducted in accordance with institutional and Canadian Federal Guidelines. Severe combined immunodeficient (Scid) male mice, in which PC3M cells induce both macroscopic lymph node and lung metastases, were obtained from Charles River Laboratories and anesthetized with isoflurane given by inhalation. Cells (1 × 10^6) diluted in 50 μL Hank’s Buffered Salt Solution were implanted into the prostate through a lower midline incision, and the incision was closed with surgical metal clips. One week after implantation, mice were blindly randomized to various experimental groups and treatment was initiated 1 wk after cell inoculation using the indicated dosage and route. At study termination, animals were sacrificed, prostate tumors were isolated and weighed, and lymph and lung metastatic nodules were counted under a stereomicroscope. Statistical analysis of the data was performed using a computer-based statistical package from Statistical Product and Service Solution (Chicago, IL). Unpaired Student’s t test was used to compare the significance between groups. Differences among control and treated groups were compared using variance (ANOVA) analysis and the Mann-Whitney U test, with group as an independent variable and volume as a repeated measure. A comparison between OB-24, Taxol, and combination treatment was performed based on isobologram methodology initially reported by Chou.

<table>
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<td>Benign</td>
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<td>HRPCA</td>
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<tr>
<td>Total no. assessable core</td>
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Targeting HO-1 in Prostate Cancer

and Talalay and described in detail by Tonra and colleagues (22). Estimated combination index (CI) values were calculated using the relative tumor volume for the saline (vehicle) or the drug-treated mice. %Treated/saline was determined for each mouse. CI values <1.0 and with >95% statistical confidence indicates synergy, whereas values around 1 indicate additive effect.

**Human tissue microarray construction and immunohistochemistry analysis.** The study cohort consisted of 96 patients with benign prostate, PCA, and HRPCA (Table 1). Tissue samples were obtained from transurethral resection of prostate representing locally advanced PCA, hormone-responsive (PrEC, BPH1, LNCaP, VCaP) and hormone-refractory (C4-2, DU145, PC3M) prostate cells maintained in phenol red- and FBS-free medium. As shown in Fig. 2A (left), most PCA cells express high basal levels of the HO-1 protein compared with cells derived from benign prostate hyperplasia (BPH-1) and nonimmortalized normal PrEC. In all PCA cell lines, HO-1 expression was induced by hemin (Fig. 2A, right) expression was not significantly different between benign, localized PCA, and HRPCA tissues (Fig. 1B). Moreover, the extent of expression was quantified into four subgroups of <25%, 25-50%, 50-75%, and 75-100%. HO-1 expression was evaluated in both the stromal and the epithelial compartments. Statistical analysis was done using one-way ANOVA followed by Turkey HSD post hoc test. A P value of ≤0.05 was considered statistically significant.

**Results**

**Epithelial HO-1 expression is associated with human PCA progression.** To assess the expression of HO-1 in relation to PCA progression, we conducted immunohistochemistry screening using a high-throughput TMA from PCA cases composed of benign prostate, localized PCA, and HRPCA (Table 1). The normalized mean epithelial expression of HO-1 in HRPCA was 1.88 ± 0.99, which was significantly higher than localized PCA (0.82 ± 0.95; P < 0.05) and benign prostate tissues (0.65 ± 0.88; P < 0.01; Fig. 1A and C). Unlike cancer epithelial cells, HO-1 expression in stromal cells surrounding the cancer was not significantly different between benign, localized PCA, and HRPCA tissues (Fig. 1B).

**HO-1 regulates oxidative stress response and oncogenic features in PCA cells.** To examine the functional effect of HO-1 on PCA cell progression, we compared basal HO-1 expression in hormone-responsive (PrEC, BPH1, LNCaP, VCaP) and hormone-refractory (C4-2, DU145, PC3M) prostate cells maintained in phenol red- and FBS-free medium. As shown in Fig. 2A (left), most PCA cells express high basal levels of the HO-1 protein compared with cells derived from benign prostate hyperplasia (BPH-1) and nonimmortalized normal PrEC. In all PCA cell lines, HO-1 expression was induced by hemin (Fig. 2A, right): representative cell lines. In contrast to HO-1, HO-2 expression levels were not different among the cell lines tested (not shown). To investigate the effect of HO-1 on oncogenic features of PCA cells, PC3M cells stably expressing HO-1 shRNA or empty retroviral particles (pSUPER-retrovector, PSR) were isolated. As shown in Fig. 2B (left), Western blot analysis confirmed an efficient reduction in HO-1 expression, using two alternative shRNA sequences 1 and 2, and results were compared with control PSR or a nonspecific HO-1 sequence 3. HO-2 expression was not affected by HO-1 shRNA. Biochemical assay focusing on cells expressing HO-1 shRNA sequence 1 confirmed a potent down-regulation of HO-1 activity in PC3M cells expressing HO-1 shRNA (Fig. 2B, right). This coincided with a significant decrease in cell proliferation after 48 to 72 hours of growth (Fig. 2C; *, P < 0.05). Similar results were obtained using HO-1 shRNA sequence 2 but not the nonspecific sequence 3 (not shown).

To understand the effect of HO-1 down-regulation on MAPK and AKT signaling, we investigated the phosphorylation status of ERK, p38, JNK, and AKT in serum-starved control and HO-1 shRNA-expressing PC3M cells stimulated with 5 ng/mL EGF. A preliminary experiment in control cells indicated that EGF stimulates MAPK phosphorylation with a maximum reached at 10 minutes, followed by a gradual decrease over the ensuing 4 hours (not shown). Therefore, the effect of HO-1 on MAPK and AKT activity was investigated at 10 minutes. As shown in Fig. 2D, a significant reduction in the activation of ERK and p38 (*, P ≤ 0.01) was seen in EGF-stimulated HO-1 sequence 1 shRNA-expressing cells compared with PSR EGF-stimulated control cells. In contrast, the levels of JNK and AKT phosphorylation were not significantly affected following HO-1 inhibition.

**HO-1 inhibition prevents PCA progression in vitro and in vivo.** Because in vitro studies showed that a reduction of HO-1 expression caused a decrease in the proliferation of the PC3M cells, we investigated the effect of HO-1 inhibition on PCA invasiveness.
in both *in vitro* and *in vivo* conditions. As shown in Fig. 3A, the invasive capacity of HO-1 knockdown PC3M cells to invade the Matrigel was significantly decreased compared with PSR control cells (*, P < 0.001). Furthermore, control PC3M cells and their matched pair expressing HO-1 shRNA sequence 1 were implanted into the mouse prostate and tumor growth was followed for a period of ~2 months. As shown in Fig. 3B to D, down-regulation of HO-1 significantly decreased primary tumor growth by ~50% (Fig. 3B, P < 0.01), and lymph nodes (Fig. 3C) and lung metastases (Fig. 3D) by more than 60% (P ≤ 0.005 compared with PSR control cells).

**Selective targeting of HO-1 by a novel small-molecule inhibitor mimics the antioncogenic features observed with HO-1 shRNA on HRPCA cells.** To investigate the therapeutic potential of targeting HO-1 in PCA, we developed a small-molecule inhibitor of HO-1 termed OB-24. OB-24 is an imidazole derivative that functions as a selective inhibitor of HO-1 (the 50% inhibitory activity of HO-1 and HO-2 was 1.9 ± 0.2 μmol/L, using rat spleen extracts, and >100 μmol/L, using rat brain extracts, respectively; ref. 14). We further showed the selectivity of OB-24 toward HO-1 in cancer cells using alternative approaches. First, we stably over-expressed the human HO-1 gene in rat glioma C6 cells; these cells express very low levels of HO-1 protein. We found that exposure of cells to 6.5 μmol/L OB-24 reduced cell proliferation by ~10%, HO-1 activity by 59%, protein carbonylation by 40%, and intracellular ROS by 43% compared with untreated HO-1–overexpressing cells (Supplementary Fig. S1).
In the second approach, we examined the effect of OB-24 on cell proliferation, HO-1 activity, and oxidative stress in PCA cells. Taxol, a major chemotherapy drug used for the treatment of advanced PCA, was used as a control cytotoxic agent. Exposure of PC3M cells to OB-24 at a concentration of 10 μmol/L, which induced ~20% growth inhibition (Fig. 4A), inhibited HO-1 levels by 62%. In contrast, exposure of cells to 5 nmol/L Taxol had no inhibitory effect but rather induced slightly, but not significantly, HO-1 activity compared with control cells. Cotreatment of cells with OB-24+Taxol attenuated HO-1 activity by ~26% compared with Taxol alone (Fig. 4B, \( P < 0.05 \)). Inhibition of HO-1 activity by OB-24 was also associated with ~86% inhibition of protein carbonylation [protein carbonylation levels were 3.614 ± 0.3874 and 0.494 ± 0.1382 (nmol/L)/mg protein for control and OB24-treated cells, \( P < 0.01 \)] and 26% inhibition of intracellular ROS levels (Fig. 4C), compared with untreated controls. Moreover, OB-24 combined with Taxol induced 54% inhibition of protein carbonylation compared with Taxol-treated cells [protein carbonylation levels were 2.405 ± 0.2242 and 1.082 ± 0.1961 (nmol/L)/mg protein for Taxol and OB24 + Taxol, respectively; \( P < 0.01 \)] and 25% inhibition of ROS levels compared with cells treated with Taxol alone (\( P < 0.01; \) Fig. 4C). As observed with HO-1 shRNA, exposure of PC3M cells to subtoxic concentrations of OB-24 (5-10 μmol/L) reduced activation of the MAPK ERK and p38 kinases but no clear inhibition was observed on JNK or AKT phosphorylation (Fig. 4D).

Similar results were observed with the LNCaP cells (Supplementary Fig. S2A–C, left) and their derived C4-2 hormone-refractory cells (Supplementary Fig. S2A–C, right), as well as DU145 cells (Supplementary Fig. S3). Inhibition of intracellular ROS levels by OB-24 was slightly higher in C4-2 compared with LNCaP cells (44% inhibition in C4-2 compared with 28% inhibition in LNCaP cells, relative to untreated cells).

**OB-24 induces antitumor and antimetastatic activity in the PC3M preclinical model.** To further examine the therapeutic relevance of targeting HO-1 by OB-24 on the progression of HRPCA, we conducted a preclinical study on mice bearing s.c. or intraprostatic PC3M cancer cells. Intraprostate implantation of PC3M cells resulted in aggressive tumor growth, culminating in extensive lymph node and lung metastases. As shown in Fig. 5A, treatment of mice bearing s.c. PC3M tumors with OB-24 at doses of 10 to 60 mg/kg (given i.p. on days 1, 3, and 5 per cycle, with a total of four cycles) resulted in a dose-dependent inhibition of tumor growth. Using this schedule, no body weight loss was observed (not shown). OB-24 given at 30 mg/kg resulted in a similar therapeutic activity as 10 mg/kg Taxol given at the same schedule and route (Fig. 5B). Importantly, a potent therapeutic effect was observed when OB-24 was combined with Taxol, yielding >90% reduction in tumor growth (Fig. 5B). Isobologram analysis confirmed a CI value of 0.74 (\( P < 0.01 \)), an indication of synergy. In the LNCaP model, treatment with OB-24 using similar conditions as the PC3M models also induced antitumor activity. However, the combination with Taxol was not conclusive because LNCaP tumors were highly responsive to Taxol (Supplementary Fig. S4).

We next investigated the therapeutic activity of OB-24 given alone at 10 or 30 mg/kg and in combination with Taxol given at 10 mg/kg. Both OB-24 and Taxol were given i.v. on days 1, 3, and 5 per cycle for four cycles to mice bearing palpable PCA (1 week after PC3M cell implantation). The control group received the vehicle alone. As
shown in Fig. 5C, OB-24 at 30 mg/kg reduced tumor growth and lymph node and lung metastases by ~50%, whereas OB-24 at 10 mg/kg was less effective. Remarkably, the inhibitory effects of OB-24 exceeded 90% when this molecule was combined with Taxol (Fig. 5C).

Discussion

The implication of oxidative stress in genomic instability, cancer development, and cancer progression has been supported by experimental and clinical studies (24–26). Moreover, high levels of oxidative stress have been documented in cancer cells and human cancer tissues, including from PCA (12, 27–30).

The inducible HO-1 is recognized as cytoprotective against oxidative stress (1, 2). HO-1 up-regulation can generally confer "antioxidant" protection by accelerating the degradation of prooxidant heme to the radical scavenging bile pigments biliverdin and bilirubin. However, under certain conditions, up-regulation of HO-1 and generation of heme-derived iron and carbon monoxide can promote ROS accumulation within the mitochondrial and other cellular compartments. The activity and temporal pattern of HO-1 expression and the chemistry of the local redox microenvironment can determine whether the free radical damage accruing from intracellular iron/carbon monoxide or the antioxidant benefits of a diminished heme-to-bilirubin ratio predominate. For this reason, HO-1 is often referred to in the literature as a "double-edged sword" enzyme (31–33).

In this study, we provide evidence that HO-1 is prooxidant in PCA cells. We observed a significant up-regulation of HO-1 in a large set of human HRPCA tissues relative to benign prostate tissue and localized PCA, further implicating HO-1 in PCA progression. We showed that overexpression of the HO-1 gene resulted in enhanced HO-1 activity and higher levels of ROS and protein carbonylation. In contrast, inhibition of HO-1 in HRPCA cells decreased heme oxygenase activity, protein carbonylation, and intracellular levels of ROS and inhibited several oncogetic features, including cell proliferation, and cell invasion in vitro, as well as tumor growth metastasis formation in vivo. Biochemical studies revealed that inhibition of HO-1 in HRPCA cells reduced EGF-induced MAPK-ERK and p38 activation. These MAPKs have been reported to regulate both HO-1 activity and inducibility (34–36) and to play a broad function in the regulation of mitotic-, survival-, and invasion-associated signaling (18, 37, 38). Inhibition of ERK and p38 activities following HO-1 down-regulation could contribute, at least in part, to the effect of HO-1 shRNA on the multiple oncogetic features of PC3M cells, observed both in vitro and in vivo.

An important translational aspect of this study is the therapeutic activity of the HO-1 small-molecule inhibitor OB-24, which was found to mimic the activity of HO-1 shRNA in PCA.
cells. OB-24, a competitive and reversible inhibitor of the HO-1 enzyme, selectively inhibits HO-1 but not HO-2, and prevents the growth and metastasis formation in the PC3M preclinical model. Moreover, a potent synergistic activity was seen when OB-24 was combined with the chemotherapy drug Taxol, where more than 90% reduction of lymph node and lung metastases was observed compared with ~50% for Taxol or OB-24 given alone. Taxol has no inhibitory effect on HO-1 but rather can slightly induce HO-1 activity in cancer cells, as evidenced by an increase in bilirubin levels. Interestingly, we observed a decrease in HO-1 activity and ROS levels following exposure to Taxol+OB-24; this combination resulted in an increase in apoptosis compared with cells treated with OB-24 or Taxol alone (Supplementary Table S1). Some studies have reported a role of ROS in Taxol-induced cytotoxicity (39). However, the bulk of the literature established that Taxol interaction with microtubules (through stabilization of microtubule-associated proteins) is a key mechanism of Taxol function (40–43). Therefore, we hypothesize that non-ROS mechanisms such as inhibition of the proangiogenic or proinflammatory signaling of HO-1 by OB-24 may contribute to the synergistic activity observed.

Although our results in human tissues and PCA cell lines support a role of HO-1 in PCA progression, and in particular HRPCA, the connection between HO-1 expression and androgen response remains to be established. Androgens, including testosterone, are known to regulate the redox system. In particular, treatment of androgen-responsive PCA cells with testosterone is reported to decrease antioxidant response and enhance cell susceptibility to oxidative stress (44). Moreover, decreased HO-1 protein levels were found in ventral prostate from castrated rats compared with controls, whereas administration of androgen increased HO-1 expression (45). Thus, androgens seem to suppress oxidative stress and loss of androgen receptor (AR) response may enhance oxidative stress and hence HO activity. These observations are in agreement with our finding of augmented oxidative stress in the AR-nonresponsive PC3M, C4-2, and DU145 cells relative to AR-responsive PCA cells and normal PrEC. Whether these effects are due to a direct connection to androgen signaling pathways or an indirect consequence of feedback regulatory loops remains to be investigated, particularly because HO-1 inhibition also affects ROS formation and the oncogenic features of the androgen-responsive cell LNCaP.

**Figure 5.** HO-1 inhibition by OB-24 reduces subcutaneous PC3M tumor growth in mice. Mice bearing s.c. PC3M tumors were treated with OB-24 alone (A) or in combination with Taxol (B) by using the i.p. route for four cycles (on days 1, 3, and 5 per cycle). Points, mean tumor size of eight mice; bars, SE. Insets, representative appearance of surgically removed primary tumors. C, PC3M cells were implanted into the prostate of male Scid mice. Treatment with OB-24 alone or in combination with Taxol was initiated 1 wk after cell implantation (palpable tumors). The schedule of treatment was similar to A and B but the drugs were given i.v. Tumor weights were taken at the time of sacrifice. Columns, mean of eight mice; bars, SE. *, P < 0.05; **, P < 0.01.

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The effect of OB-24 on inhibition of PCA cell invasion clearly indicates that targeting HO-1 in HRPCA cells affects major signaling pathways that regulate tumor progression to aggressive phenotypes, and its synergistic activity with Taxol are significant given the limited therapeutic options available for HRPCA patients in clinical practice. Historically, standard chemotherapy including taxanes has provided limited benefits for the treatment of HRPCA, whereas alternative therapeutic modalities, including inhibitors of androgen and estrogenic compounds, are under investigation (46–48). OB-24 is a promising novel targeted therapeutic agent that deserves further molecular and preclinical studies in relation to HRPCA, particularly when combined with taxanes.

Disclosure of Potential Conflicts of Interest
A. Gupta, W.A. Szarek, H. Schipper, M. Alaoui-Jamali, and J.Z. Vlahakis hold a joint patent. H. Schipper, M. Alaoui-Jamali, and W.A. Szarek are on the Advisory Board of Osta Biotechnologies. The other authors disclosed no potential conflicts of interest.

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Disclosure of Potential Conflicts of Interest
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