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Research Article

Adapting Hydralazine as a Reliable Low-Toxicity Alternative to Cobalt Chloride for Hypoxia Modeling in Breast Cancer Cells

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Abstract

Background: Hypoxia represents a critical characteristic of the breast cancer microenvironment, responsible for tumor progression and therapeutic resistance. Although cobalt chloride ($CoCl_2$), which is used to induce cellular hypoxia, its application is restricted by severe cytotoxicity and off-target side effects. ***Objective***: To evaluate hydralazine as an alternative for hypoxia induction in human MCF-7 breast cancer cells. ***Methods***: MCF-7 cells were treated with increasing concentrations of hydralazine and $CoCl_2$ to compare the efficacy and cytotoxicity. Cell viability and stabilization of hypoxia-inducing factor-1 alpha (HIF-1α) were assessed using MTT and Western blotting assays, respectively. GAPDH protein was used as a loading control. ***Results***: Hydralazine maintained near 98% cell viability across all tested doses up to 1600 µM at 24 hours, whereas $CoCl_2$ treatment resulted in dose-dependent cytotoxicity with a significant viability reduction to approximately 50% at the same maximum concentration. Furthermore, prolonged exposure (72 hours) to 1600 µM $CoCl_2$ caused severe cytotoxicity (<20% viability), while hydralazine-treated cells maintained significantly higher viability, enabling safer conditions for chronic hypoxia during long-term studies. Interestingly, Western blot results showed that hydralazine induced and stabilized HIF-1α protein, with a higher expression level than observed in $CoCl_2$-treated cells across the 50 to 150 µM concentration range. ***Conclusions***: Hydralazine efficiently induces hypoxia in MCF-7 cells with favorable biocompatibility compared to $CoCl_2$ and can be a simple, safer, and more reliable chemical model for investigating signals mediated by HIF-1α in luminal breast cancer research, avoiding the variability of confounding heavy metal toxicity.

***Keywords*:** Breast cancer; Cobalt chloride; Hydralazine; Hypoxia; HIF-1α; MCF-7 cells.

تكييف الهيدرالازين كبديل موثوق منخفض السمية لكلوريد الكوبالت لنمذجة نقص الأكسجين في خلايا سرطان الثدي

الخلاصة

الخلفية: نقص الأكسجين يمثل خاصية حاسمة في بيئة سرطان الثدي الدقيقة، مسؤولة عن تقدم الورم والمقاومة العلاجية. على الرغم من استخدام كلوريد الكوبالت ($CoCl_2$) لتحفيز نقص الأكسجين الخلوي، إلا أن تطبيقه محدود بسبب السمية الخلوية الشديدة وآثار جانبية غير مستهدفة. **الهدف**: تقييم الهيدرالازين كبديل لتحفيز نقص الأكسجين في خلايا سرطان الثدي MCF-7 البشرية. **الطرائق**: تم معاملة خلايا MCF-7 بتركيزات متزايدة من الهيدرالازين و$CoCl_2$ لمقارنة الفعالية والسمية الخلوية. تم تقييم بقاء الخلايا واستقرار عامل ألفا المحفز لنقص الأكسجين (HIF-1α) باستخدام اختبارات MTT وWestern Blutting على التوالي. تم استخدام بروتين GAPDH كمادة تحكم في التحميل. **النتائج**: حافظ الهيدرالازين على فعالية خلوية تقارب 98٪ عبر جميع الجرعات المختبرة حتى 1600 ميكرومولار خلال 24 ساعة، بينما أدى علاج $CoCl_2$ إلى سمية خلوية تعتمد على الجرعة مع انخفاض كبير في قابلية العيش إلى حوالي 50٪ عند نفس التركيز الأقصى. علاوة على ذلك، تسبب التعرض المطول (72 ساعة) ل 1600 ميكرومولار في $CoCl_2$ في تسمم خلوي شديد (<20٪ من البقاء)، بينما حافظت الخلايا المعالجة بالهيدرازين على قدرة أكبر بشكل ملحوظ، مما أتاح ظروف أكثر أمانا لنقص الأكسجين المزمن خلال الدراسات طويلة الأمد. ومن المثير للاهتمام أن نتائج Western Blot أظهرت أن الهيدرالازين استثب وثبت بروتين HIF-1α، بمستوى تعبير أعلى مما لوحظ في الخلايا المعالجة ب $CoCl_2$ عبر نطاق تركيز 50 إلى 150 ميكرومول. **الاستنتاجات**: يسبب الهيدرالازين نقص الأكسجين بكفاءة في خلايا MCF-7 ذات التوافق الحيوي الإيجابي مقارنة ب $CoCl_2$، ويمكن أن يكون نموذجا كيميائيا بسيطا وأكثر أمانا وموثوقية للتحقيق في الإشارات التي يتوسط HIF-1α في أبحاث سرطان الثدي المضيئة، متجنبا تفاوتات سمية المعادن الثقيلة المربكة.

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INTRODUCTION

Solid tumors, including breast cancer, represent one of the most frequent newly diagnosed cancers with a high mortality rate worldwide [1,2]. The disease is considered highly heterogeneous and comprises several molecular subtypes, including luminal A (MCF7 cells), which express positive estrogen receptors [3]. A key feature shared by the most aggressive solid tumors is containing insufficient and abnormal vasculature, which creates an aggressive microenvironment with regions of low oxygen levels [4]. Hypoxia can develop in the core of the tumor, resulting in the activation and stabilization of the hypoxia-inducible factor 1 alpha (HIF-1α) protein. This transcription factor is constitutive under normal levels of oxygen and rapidly degraded by prolyl hydroxylase domain (PHD) enzymes [5]. However, under hypoxic conditions, this enzyme is inhibited, causing HIF-1α stabilization and translocation to the nucleus and subsequent transcription of various genes (such as VEGF, BNIP3, and genes involved in glycolysis) that

improve cellular adaptation, angiogenesis, and metastasis and develop resistance to chemotherapy and radiotherapy [6–8]. For investigating new therapies in preclinical studies, it is essential to model this crucial environment by using a hypoxia-mimicking in vitro model. The usual method is using special hypoxic chambers, which control the environment of the cell culture with an oxygen level around 1-5% [9]. However, these methods are more expensive, require a special gas mixture, and present difficulties in providing a constant level of highly degradable HIF-1α protein (especially after moving cells out of these chambers), which creates a challenge for high-throughput screening [10,11]. For this reason, a simple model using hypoxia mimetic agents has been developed. Cobalt chloride ($CoCl_2$) represents one of the most commonly used chemicals to mimic hypoxia by substituting Fe^{2+} in the active site of the PHD enzyme, resulting in the inhibition of the enzyme's activity and increasing the stability of HIF-1α [12–14]. However, studies showed that $CoCl_2$ produces higher cellular toxicity, oxidative stress, and iron chelation independent of the actual oxygen-sensing pathway, which confounds the experimental results [12,13]. Therefore, it is essential to identify a less toxic, more suitable, and clinically relevant agent that can be used as an alternative to produce a hypoxic response. This study was established to investigate the effect of hydralazine, the well-known vasodilator used as an antihypertensive agent, as a less toxic agent for the induction of hypoxia in human breast cancer cell lines, specifically MCF-7 cells. Hydralazine has been shown to induce hypoxia in vitro by directly activating the HIF-1 pathway through inhibiting PHD enzyme activity, which prevents the hydroxylation and subsequent degradation of HIF-1α, but with a different or lower off-target toxicity profile than $CoCl_2$ [15,16]. Therefore, the objective of this study was to compare the efficacy and cytotoxicity of hydralazine against $CoCl_2$ in the MCF-7 breast cancer cell line. It is hypothesized that hydralazine would induce robust HIF-1α stabilization with substantially lower cytotoxicity than $CoCl_2$, thereby establishing a more reliable and biocompatible *in vitro* model for cellular and molecular hypoxia research in breast cancer.

METHODS

Chemicals and biological materials

Penicillin, streptomycin, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and trypsin were purchased from Capricorn Scientific, Germany. Dimethyl sulfoxide (DMSO) was obtained from Euroclone, Italy. Cell culture flasks and plates used in this study were provided by Nest-Biotechnology, China. Hydralazine and $CoCl_2$ were provided by MedChemExpress (MCE), NJ, USA. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) compound was purchased from Promega, USA. HIF-1α polyclonal antibody was obtained from FineTest, China. GAPDH polyclonal antibody was purchased from Elabscience (Houston, USA). All compounds were dissolved in water and sterilized through filtration with a 0.22 µm Millipore syringe filter.

Cell culture and maintenance

The human breast cancer cell line, MCF-7 (Luminal A, Estrogen Receptor-Positive), was obtained from the American Type Culture Collection (ATCC). Cells were routinely cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin antibiotic solution. The incubation environment is maintained at 37°C in 98% humidity with a 5% CO_2 atmosphere. Regular passages were done using 0.05% Trypsin when cells reached 70-80% confluence. Cells underwent routine testing for bacteria and mycoplasma infections.

In vitro cell cytotoxicity assay

The cytotoxicity effects of compounds were assessed using the MTT assay [17,18]. In 96-well plates, MCF-7 cells were seeded at a density of 5 x 103 cells/well. The next day, cells were treated with hydralazine or $CoCl_2$ in a series of concentrations (1600, 800, 400, 200, 100, 50, 25, 12.5, and 6.25 µM). In the control group, only DMEM supplemented with an equal percentage of solvent was added to the cells. After 24, 48, or 72 hours of incubation, the medium was removed, and fresh media containing MTT solution (0.5 mg/ml) were added to each well. Plates were then foiled and incubated for 4 hours at 37°C and 5% CO_2 to form formazan crystals by metabolically active cells. After the incubation period was completed, the culture media were discarded, and 100 µl of DMSO was added with shaking for 10 min to dissolve the crystals formed. Optical density (OD) for each well was measured using a GloMax Microplate Reader (Promega, USA) at 560 and 600 nm. Cell viability was calculated using the following equation: Cell viability % = [OD sample/OD control] x 100

Western blotting assay

To verify activation and stabilization of the hypoxic pathway, the Western blotting method was used [19]. Concentration ranges (50-200 µM) for comparison were selected depending on the pre-study optimizing procedure to identify the highest dose that induced visible HIF-1α stabilization (via Western blot) while maintaining >90% cell viability. Cells were seeded at a 2 x 10^5 cell/well density using 6-well plates. After 6 hours of treatment with different doses of hydralazine or $CoCl_2$, cells were lysed, and total proteins were extracted using a cold RIPA lysis buffer containing protease and phosphatase inhibitors. Protein concentrations were measured using the Bicinchoninic Acid (BCA) assay. By using 30 µg of total protein for each sample, proteins were then loaded and separated through the Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

(SDS-PAGE) method using a concentration of separating gel equal to 6% (for HIF-1α) or 10% (for GAPDH). Separated proteins were transferred into a polyvinylidene fluoride (PVDF) membrane. After the transfer process, PVDF membranes were blocked with skimmed milk and then incubated with the primary antibody at a dilution ratio equal to 1:1000 for HIF-1α and 1:5000 for GAPDH at 4°C overnight. The next day, PVDF membranes were incubated with a suitable horse radish peroxidase (HRP)-conjugated secondary antibody (dilution ratio 1:5000), and protein bands were detected using a chemiluminescence detection assay using the ChemiDoc imaging system, Bio-Rad (USA). Band density was quantified using ImageJ analysis software and normalized against the loading control [19].

Ethical considerations

The study protocol was approved by the Research Ethics Committee of the College of Pharmacy, Mustansiriyah University.

Statistical Analysis

Experiments for cell viability were performed in biological triplicate, while a biological duplicate was used in the Western blot assay. Mean and standard error of the mean (SEM) were used to express the results data. GraphPad Prism (version 8.0.2, GraphPad Software, San Diego, CA, USA) was used to analyze data. Statistical comparisons among groups were performed using two-way analysis of variance (ANOVA) for multiple comparisons in MTT cell viability assays, whereas one-way ANOVA was used for Western blot densitometry analysis. Differences with a *p*-value less than 0.05 were considered statistically significant.

RESULTS

An MTT cell viability assay was conducted on MCF-7 breast cancer cells, comparing the cytotoxicity of hydralazine against $CoCl_2$ across varying concentrations (6.25-1600 µM) and three distinct exposure times (24 h, 48 h, and 72 h). Cell viability showed a dose and time-dependent decrease for both treatments. During the 24-hour exposure time, lower concentrations of both compounds are generally well tolerated by the MCF-7 cells. Cell survival varies at the maximum concentration of 1600 µM. At this dose, cells treated with hydralazine maintain approximately 98% viability, whereas $CoCl_2$ significantly ($p< 0.0001$) reduces cell viability to nearly 50% (Figure 1A). This indicates that $CoCl_2$ induces rapid, acute cytotoxicity at high doses while hydralazine remains exceptionally well-tolerated. However, exposure to $CoCl_2$ for 48 and 72 hours increases the accumulation of heavy metals, leading to hazardous consequences (Figure 1B, C). After 72-hour exposure at a mid- to high concentration of 800 µM, cells treated with $CoCl_2$ exhibit significant cytotoxicity, decreasing to around 40% viability and further reduction to approximately 20% at 1600 µM (Figure 1C). In contrast, hydralazine-treated cells maintain a substantial survival advantage over chronic exposure, with viability remaining constant at around 60% viability at the same high concentrations.

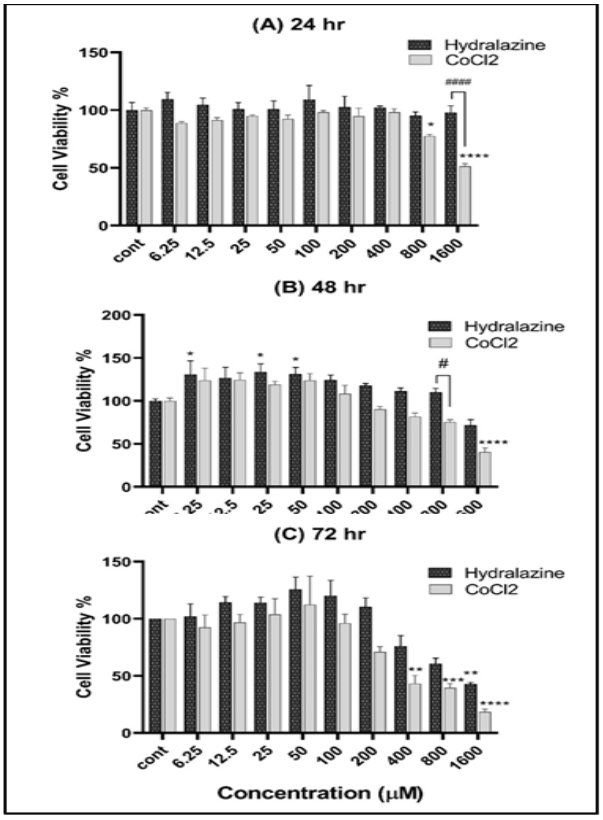

Figure 1: Effect of Hydralazine and $CoCl_2$ on the viability of MCF-7 cells. Confluent cultures of MCF-7 cells were treated with constant increasing concentrations of Hydralazine and $CoCl_2$ (6.25-1600 µM). Cell viability was measured using the MTT assay after (**A**) 24 hours, (**B**) 48 hours, and (**C**) 72 hours of incubation. Data are presented as mean +/- standard error of the mean (SEM). *p<0.05 and ****p<0.0001 *vs*. control, and $^{\#}p$<0.05 and $^{\#\#\#\#}p$<0.0001 *vs*. Hydralazine. Data analyzed using two-way ANOVA followed by Sidak's post-test, n=3.

Due to the sustained viability of the cells across the majority of the tested doses, determining the IC50 was particularly limited. GraphPad Prism could only calculate exact IC50 values at certain time points when cell viability decreased considerably. For $CoCl_2$, the IC_{50} values were found to be 665 µM at 48 hours and 534 µM at 72 hours. For hydralazine, an IC50 value was only calculated at 1447 µM after 72 hours of exposure. At all other time intervals for both treatments, cell viability remained over the 50% threshold, preventing the software from determining precise IC50 values. To validate the dose-dependent effect of hypoxia induction through Hydralazine and $CoCl_2$, HIF-1α protein was analyzed using a Western Blotting assay (Figure 2). Under normal conditions, the HIF-1α protein band was rapidly degraded and barely detectable (Figure 2A, Lane 1). Treatment with hydralazine (Figure 2A, Lanes 2-5) and $CoCl_2$ (Figure 2A, Lanes 6-9) results in accumulation of HIF-1α with intense bands in lower doses (50 and 100

µM) compared to less intense bands in the higher doses (150 and 200 µM).

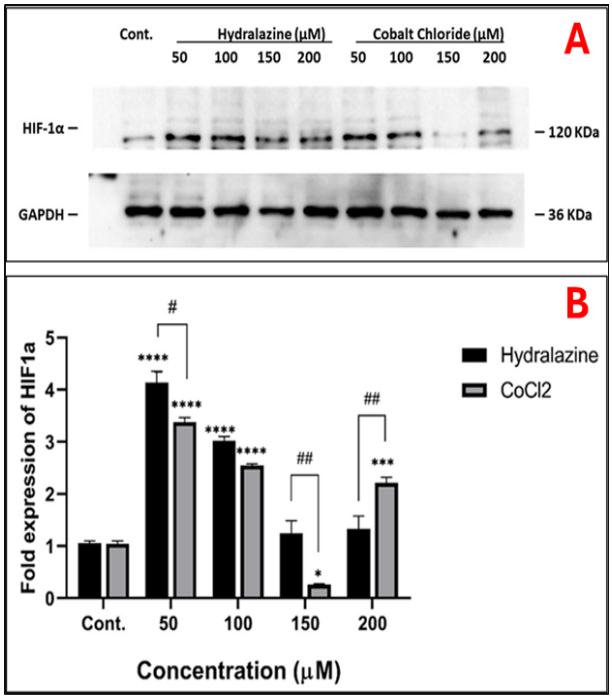

Figure 2: Representative western blots for differentiation proteins and corresponding densitograms. MCF-7 cells were incubated under normoxic conditions (Cont.) or treated with increasing concentrations (50, 100, 150, and 200 µM) of Hydralazine and $CoCl_2$ for 6 hours. GAPDH was used as a loading control to ensure equal protein loading. (**A**) The bands demonstrate a stabilization of HIF-1α in treated groups compared to the control. (**B**) The relative fold expression of HIF-1α protein was measured compared to the untreated control (Cont.). Data are presented as mean +/- standard error of the mean (SEM) and analyzed using one-way ANOVA followed by Tukey's post-test. The highest expression was observed at 50 µM for both treatments, followed by a decline at higher doses (100 and 150 µM) with a favorable increase in the Hydralazine group. A notable rebound in HIF-1α expression for $CoCl_2$ was observed at 200 µM. *p<0.05, ***p<0.001, and ****p<0.0001 vs. control;# p<0.05 and ## p<0.01 of hydralazine vs. CoCl2 group; n=2.

However, the overall signal intensity of the hydralazine-treated group was markedly stronger than that observed in the $CoCl_2$ groups at comparable concentrations (Figure 2A). The fold expression of HIF-1α protein in a range of concentrations (50, 100, 150, and 200 µM) of relatively safe doses of both compounds was calculated relative to untreated controls (Figure 2B). After a 6-hour incubation period, both treatments exhibited a significant peak expression (p< 0.0001) at the lowest concentration examined (50 µM) compared to the control. At this dose, hydralazine produced the highest response with an approximately 4-fold increase, which is a significant difference from $CoCl_2$ (p< 0.05) with a response of nearly a 3-fold increase (Figure 2B). Subsequent increases in doses exhibited a progressive decline in HIF-1α expression for both groups. At a concentration of 100 µM, the levels of protein expression were decreased but remained significantly elevated compared to the control (p< 0.0001 for hydralazine, p< 0.001 for $CoCl_2$). However, at a 150 µM dose, a noticeable suppression was observed in the hydralazine group, with protein expression returning to a near control level, while $CoCl_2$ showed a significantly (p< 0.05) stronger inhibition, with folding expression less than 0.25. Interestingly, at the highest dose (200 µM), the two treatments displayed a significant difference in the level of HIF-1α (p< 0.05); while hydralazine remained at the same level, $CoCl_2$ demonstrated a resurgence in expression (p< 0.001), reaching approximately 2-fold (Figure 2B).

DISCUSSION

To understand breast cancer progression and therapy resistance, it is important to model an accurate in vitro replication of the hypoxic tumor microenvironment. In this study, hydralazine has been investigated as a novel, non-toxic alternative to the conventional chemical mimetic $CoCl_2$ for inducing hypoxia in MCF-7 breast cancer cells. Although $CoCl_2$ is widely used as a consequence of its lower cost and accessibility, excessive concern has emerged about inducing cytotoxicity before achieving a desirable stable level of HIF-1α protein [12]. On the other hand, Hydralazine exhibited a good safety profile with a promising effect on inducing the hypoxia response in normal cells [10], making it a more favorable model for examining the microenvironment of luminal A breast cancer. In this study, the MTT results of $CoCl_2$ treatment showed a significant decline in the viability of MCF-7 cells at high concentrations (≥ 400 µM), especially after incubation for 48 and 72 hours (Figure 1). This is consistent with the findings produced by Li et al., who showed that although $CoCl_2$ can increase hypoxic markers, it can concurrently stimulate significant apoptosis and oxidative stress in MCF-7 cells, possibly through the production of reactive oxygen species and mitochondrial fission [12]. This toxic effect makes it difficult to discriminate the reason for cell death, whether it is from the true hypoxic signaling or heavy-metal-induced cytotoxicity. In contrast, the current study results demonstrated that hydralazine-treated cells maintain a distinct survival advantage over $CoCl_2$.. This sustained gap across the chronic exposure boards demonstrates that hydralazine's lower toxicity is more than just a temporary delay in cell death but rather could represent a fundamental physiological difference in how cells respond to the drug compared to the severe oxidative stress caused by heavy metals. As hydralazine is an FDA-approved antihypertensive drug, its mechanism of action is different from heavy metal toxicity, which makes hydralazine favorable over $CoCl_2$ in that any change in MCF-7 behavior might be related to the activation of hypoxia signaling rather than apoptosis or non-precise cellular stress. Ultimately, this viability data supports the main hypothesis of the study by demonstrating that hydralazine has a larger therapeutic window and a superior safety profile compared to $CoCl_2$.. In addition, hydralazine is suggested to be a more reliable reagent for

long-term, chronic in vitro hypoxia studies (such as studying metabolic shift phenomena, the Warburg effect, and the development of drug resistance) in MCF-7 cells than $CoCl_2$, as it avoids severe and rapid toxicity at elevated doses and extended time points. This is important for examining the chronic adaptation of luminal breast cancer cells to hypoxia, which is more representative of the clinical tumor mass in patients with breast cancer. To investigate the expression level of the HIF-1α protein, the results of the western blotting assay in this study showed that hydralazine significantly induced hypoxia with a higher fold increase in HIF-1α protein expression than $CoCl_2$ at effective concentrations (50 and 100 µM). However, the drop in HIF-1α expression at 150 µM at the 6-hour mark may be attributed to shifted temporal dynamics, wherein a more rapid initial accumulation stimulates robust negative feedback loops, such as PHD upregulation, to remove the protein before extraction. Conversely, the rebound in stabilization at 200 µM is probably due to acute cellular stress that temporarily overwhelms or saturates the proteasomal degradation mechanism, resulting in protein accumulation despite the high metabolic viability maintained at 24 hours. The favorable higher expression of HIF-1α protein in hydralazine compared to $CoCl_2$ at effective doses is probably due to their separate mechanisms of action on the inhibition of PHD enzymes. The function of $CoCl_2$ is primarily by the substitution of ferrous ions from the active site of PHD, which prevents the hydroxylation and consequent degradation of HIF-1α [12,13]. However, Knowles et al. demonstrated that hydralazine can act as a direct inhibitor of PHDs without altering the hemostasis of cellular ions or producing metal toxicity [15]. Validating the non-toxic hypoxia model of hydralazine for MCF-7 cells is particularly relevant for clinical applications, as this cell line represents estrogen receptor-positive (ER+) breast cancer. Evidence has emerged about a complex relationship between hypoxia and ER signaling [20,21]. Studies introduced by Capatina et al. and Jehanno *et al*. have revealed that hypoxia can reduce the expression of ERα, resulting in a reduction in the response of MCF-7 cells to hormonal treatment such as tamoxifen [8,22]. Hence, hydralazine can be used to investigate the delicate signaling pathways by evaluating the effect of HIF-1α inhibitors, such as PX-478, to increase the efficacy of hormonal therapy. Moreover, recent studies by Lafi *et al*. and Yahyapour *et al*. demonstrate the potential repurposing of hydralazine in combination with other drugs, such as ATRA or doxorubicin, for cellular modification at the epigenetic level in breast cancer [23,24]. This proposes that hydralazine is not only a laboratory tool but also could be a bioactive agent with relevance to the cancer microenvironment. Consequently, these findings suggest the valuable use of hydralazine over $CoCl_2$ due to potential activation and stabilization of HIF-1α in MCF-7 cells without significant cell death. Although this research supports the biocompatibility and efficacy of hydralazine as a hypoxia mimetic, several limitations must be acknowledged. First of all, the experimental model is completely based on the MCF-7 cell line. Although this is relevant for modeling luminal A breast cancer, the findings need to be confirmed against other molecular subtypes, such as triple-negative breast cancer (e.g., MDA-MB-231), to ensure broad application. Secondly, due to resource restrictions during the extended optimization process, the western blot analysis was limited to two biological replicates. Finally, while this study confirms the primary accumulation and stabilization of the HIF-1α protein, it did not assess the subsequent transcriptional activation of the pathway. Future investigations should emphasize validating the functional transcriptional output of HIF-1α by measuring mRNA levels of downstream hypoxia effector genes, such as VEGF, BNIP3, and GLUT1 via qPCR analysis, thereby fully establishing the mechanistic equivalence of hydralazine to both $CoCl_2$ and true physical hypoxia. Furthermore, investigating whether hydralazine alters the sensitivity of MCF-7 cells to endocrine therapy would be a potential next step.

Conclusion

Hydralazine potentially produces a simple, safer, more effective, and mechanistically distinct alternative to $CoCl_2$ for inducing hypoxia in MCF-7 cells. By possibly reducing off-target cytotoxicity, hydralazine exhibits a favorable model for investigating the molecular relationship between hypoxia and estrogen signaling in luminal breast cancer.

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Conflict of interests

The authors declared no conflict of interest.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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