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Original Research

Inhibiting autophagy does not decrease drug resistance in breast cancer stem-like cells under hypoxic conditions

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ABSTRACT

Introduction: Breast cancer has been one of the most frequently diagnosed cancers in women over the past decade. The presence of a breast cancer stem cell (BCSC) population within breast tumors significantly contributes to malignancy and drug resistance. Under the hypoxic conditions of breast tumors, BCSCs exhibit increased resistance to chemotherapy, complicating treatment and reducing its efficacy. Consequently, targeting BCSCs is considered a crucial strategy for breast cancer treatment. We hypothesize that the chemotherapy resistance of BCSCs is dependent on the upregulation of autophagy triggered by hypoxia. This study aims to investigate the relationship between autophagy and drug resistance of BCSCs in hypoxic conditions. **Methods**: BCSCs were isolated from Vietnamese breast cancer cell line 1 (VNBRCA1) based on expression of CD44 and CD24. Cobalt (II) chloride ($CoCl_2$) was used to simulate a hypoxic environment in monolayer cell cultures. The hypoxic status of BCSCs was confirmed by the upregulation of the *HIF-1α* gene. Autophagy activation was assessed through the expression of autophagy-related genes (*LC3B, Beclin-1, AMPK, PI3K, AKT*). Drug resistance was evaluated by measuring the expression of *ABCC10* and assessing cell proliferation under cisplatin treatment. **Results**: The study revealed that $CoCl_2$ treatment led to high expression of *HIF-1α* and significant upregulation of *LC3B* in BCSCs, together with enhanced expression of *ABCC10*. Consequently, BCSCs exhibited marked resistance to cisplatin in the hypoxic environment. However, inhibition of autophagy using the autophagy inhibitor 3-methyladenine (3-MA) did not reduce cisplatin resistance in hypoxic conditions. **Conclusion**: The findings indicate that in hypoxic conditions, BCSCs enhance both the autophagy process and drug resistance. However, the drug resistance of BCSCs in hypoxic conditions is not directly associated with the upregulation of autophagy. Therefore, targeting autophagy inhibition may not be an effective strategy to reduce drug resistance in hypoxia.

Key words: breast cancer stem-like cells, hypoxia, autophagy, drug resistance, cobalt chloride

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INTRODUCTION

According to the 2022 GLOBOCAN statistics, breast cancer is the second most common cancer, making up 11.5% of the 19,976,499 new cases, and ranks fourth in cancer-related deaths, accounting for 6.8% of the 9,743,832 fatalities globally. Furthermore, this data indicates that in Viet Nam, breast cancer represented the highest proportion of new cancer diagnoses in 2022, with the majority of patients being women. Primary prevention measures like reducing excess body weight, lowering alcohol consumption, increasing physical activity, and promoting breastfeeding can help reduce breast cancer incidence. In 2021, WHO launched the Global Breast Cancer Initiative to reduce mortality by 2.5% annually, aiming to save 2.5 million lives in 20 years through health promotion, timely diagnosis, and comprehensive management[1].

Cancer stem cells (CSCs) play an important role in contributing to the heterogeneity of many kinds of cancer[2]. About 20 years ago, Al-Hajj *et al.* determined that a small breast cancer cell population strongly expressed CD44 and weakly expressed CD24, which was able to develop tumors in NOD/SCID mouse models[3]. Later, ALDH1 was also detected as a novel marker for breast cancer stem cell isolation, which is correlated with poor clinical outcomes in breast cancer patients[4]. Breast cancer stem cells are significant due to their role in metastasis and therapy resistance. Efforts are focused on characterizing them, understanding their pathways, and developing targeted therapies to eradicate them. New therapeutic approaches, including drug delivery, gene targeting, and immunotherapy, hold promise for effectively targeting breast cancer stem cells in cancer treatment[5].

Cancer stem cells are usually located at the core of solid tumors, distant from the blood vessels, creating a hypoxic microenvironment (hypoxia). In comparison, while the level of oxygen pressure in normal tissue reaches over 40 mmHg, concentration in cancer tumors is frequently maintained between 0 and

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20 mmHg. Transcription factors, which are responsible for adapting to hypoxic conditions, are known as hypoxia-induced factors (HIFs)[6]. We have reviewed the important effects of hypoxia as well as the roles of HIFs during tumor progression[7]. Particularly, *HIF-1α* and *HIF-2α* regulate cancer stem cells' adaptation to hypoxia and resistance to therapies[8].

The term "autophagy" comes from a Greek word meaning "eating itself" and was first introduced in 1963 by a Belgian physiologist Christian de Duve. Mechanisms related to this biological process were studied and published by Yoshinori Ohsumi and his colleagues from the 1960s to the 1990s[9,10]. This multi-step process includes autophagy induction, recognition, autophagosome formation, fusion with lysosome, and autophagolysosome destruction[11]. Each step involves the regulation of many genes called autophagy-related genes (ATGs). At least 40 ATGs have been discovered and 20 of them play key roles in the autophagy of eukaryotic cells[12]. In cancer cells, autophagy acts not only as a "friend" since it stimulates cancer cells to proliferate, but also as a "foe" due to its inhibitory impacts on cancer cell survival[13]. In the case of tumor suppression, autophagy protects cancer cells by preventing the accumulation of DNA-damaging reactive oxygen species (ROS) and by removing damaged proteins and organelles to maintain homeostasis[14,15]. Conversely, autophagy activation also aids tumor cell survival by regulating various mediators[16,17]. Overall, the process of autophagy in cancer cells is highly complex and not yet fully understood.

Hypoxia has been shown to induce autophagy in some types of cancer stem cells[18,19], which has led to studies on signaling pathways related to autophagy in hypoxic cancer stem cells. In this study, we use cobalt (II) chloride ($CoCl_2$) to mimic hypoxic conditions in breast cancer stem-like cell populations to investigate changes in the expression of genes related to autophagy induction and regulation. Additionally, we examine changes in drug resistance gene expression *ABCC10* and cisplatin resistance in breast cancer stem-like cells in a hypoxia-mimic model. Based on these results, we draw preliminary conclusions about the molecular relationship between autophagy and drug resistance in hypoxic breast cancer stem-like cells.

METHODS

Cell Culture

VNBRCA1 stands for the Vietnamese breast cancer cell line no. 1, which was isolated and characterized from Vietnamese breast cancer patients and was described and used by our laboratory in previous studies[20]. Cancer cells were cultured in DMEM (Lonza, Switzerland) supplemented with 10% FBS (Thermo Fisher Scientific, USA) and 1% antimycotic-antibiotic (HyClone, USA) for enrichment in a T-25 flask (SPL Life Science, Korea Republic). All flasks were incubated at 37°C and 5% CO_2, and the medium was freshly changed after 48 to 72 hours. After reaching 80-90% confluency, cells were harvested by Deattachment reagent (Regenmedlab, Viet Nam) to perform subculture.

Flow cytometry assay for $CD44^{high}CD24^{low}$ population

Cells were harvested by Deattachment reagent as described above. Approximately 1×10^7 VNBRCA1 cells were stained with 10 μL of anti-CD44 (BD Biosciences, USA) and 10 μL of anti-CD24 (Sigma-Aldrich, USA) in phosphate-buffered saline (supplemented with 2% BSA) at 4°C in the dark for 20 minutes. Then, all cells were sorted based on fluorescent-bound antigen signals using the BD FACSMelody Cell Sorter (BD Biosciences, USA). The cell population that is strongly positive for CD44 and negatively or dimly expressed CD24 is considered breast cancer stem cells (BCSCs). They are marked as BCSCs hereafter in this article. BCSCs were cultured in M171 medium (Thermo Fisher Scientific, USA) supplemented with 1% MEGS (Thermo Fisher Scientific, USA) at 37°C and 5% CO_2.

Cobalt (II) Chloride Cytotoxicity Assay

Cytotoxicity was determined using the AlamarBlue assay[21]. BCSCs were seeded at a density of 3,000 cells per well in a 96-well plate (SPL Life Science, Korea Republic). Each well was filled with 100 μL of M171. After 24 hours of incubation, the medium was withdrawn and replaced with new medium containing $CoCl_2$ at different concentrations. Medium without $CoCl_2$ was used as a negative control. The percentage of cell viability was calculated based on the following formula:

$$Rate\ of\ viable\ cells\ (\%) = \frac{OD\ value\ of\ treated\ group - OD\ value\ of\ blank}{OD\ value\ of\ untreated\ group - OD\ value\ of\ blank} x\ 100\%$$

Cisplatin Cytotoxicity Assay

Breast cancer stem-like cells were seeded at a density of 3,000 cells per well in a 96-well plate. They were then pretreated with different concentrations of $CoCl_2$ for various periods of time. Subsequently, the

M171 medium was withdrawn and replaced with new medium containing cisplatin (Sigma-Aldrich, USA) at concentrations of 50, 100, 150, 200, and 250 μM, respectively. BCSCs were incubated at 37°C for the next 24 hours, and cell viability was assessed via the AlamarBlue assay as described above.

Gene Expression Analysis

RNA isolation was performed using the easy-BLUE^{TM} Total RNA Extraction Kit (iNtRON Biotechnology, South Korea). The expression levels of target genes were determined by real-time quantitative RT-PCR (reverse transcriptase polymerase chain reaction) with specific primers (listed in **Table 1**). All primers were purchased from PHUSA Biochem Co., Viet Nam. RNA samples were divided into qPCR tubes (Thermo Fisher Scientific, USA) with appropriate amounts and supplemented with the necessary components for a standard PCR reaction, following the instructions in the Luna Universal One-Step RT-qPCR Kit (New England Biolabs, USA). Thermal cycling was performed using the Replex system (Eppendorf, USA). Gene expression levels were semi-quantitated using the Livak formula, with β*-actin* used for normalization.

Statistical Analysis

All assays were performed in triplicate. Raw data were collected using Microsoft Excel 365 and analyzed with GraphPad Prism 9.0 software. All data are displayed as mean ± standard error of the mean (SEM). Differences between treatments were examined using the one-way ANOVA method, with a p-value < 0.05 considered statistically significant.

Table 1: Sequences of primer pairs used for qRT-PCR

Genes	Primer sequence (5'-3')	Referrence
β*-actin F*	CTGGAACGGTGAAGGTGACA	[23]
β*-actin R*	AAGGGACTTCCTGTAACAATGCA	
HIF-1α F	TGGTGACATGATTTACATTTCTGA	[24]
HIF-1α R	AAGGCCATTTCTGTGTGTAAGC	
LC3B F	TGTCCGACTTATTCGAGAGCAGCA	[25]
LC3B R	TTCACCAACAGGAAGAAGGCCTGA	
Beclin-1 F	ATGCAGGTGAGCTTCGTGTG	[26]
Beclin-1 R	CTGGGCTGTGGTAAGTAATGGA	
ABCC10 F	CGGGTTAAGCTTGTGACAGAGC	[27]
ABCC10 R	AACACCTTGGTGGCAGTGAGCT	
AMPK F	CAGGCATATGGTGGTCCATAGAG	[28]
AMPK R	TCATGGGATCCACCTGCAGC	
PI3K F	TGTGTGGGACTTATTGAGGTG	[22]
PI3K R	ACACAGTATCCAGCACATGAAC	
AKT F	TCTATGGCGCTGAGATTGTG	[29]
AKT R	CTTAATGTGCCCGTCCTTGT	

Abbreviations: **F**: Forward, **R**: Reverse

RESULTS

Establishing model of hypoxic BCSCs using $CoCl_2$

After sorting, the BCSC population predominantly exhibits a $CD44^{high}CD24^{low}$ phenotype (over 96%) and shows an approximately 1.5-fold increase in ALDH1 gene expression compared to the pre-sorting population (**Figure 1A**). However, this increase in ALDH1 expression is not statistically significant (p-value = 0.20) (**Figure 1B**). This population was used for further analysis.

In the first assay to evaluate the cytotoxicity of $CoCl_2$, $CoCl_2$ displayed high toxicity in BCSCs when used in high concentrations. Indeed, when compared to cells not treated with $CoCl_2$, the survival rate of BCSCs markedly decreases in a concentration- and time-dependent manner when exposed to $CoCl_2$ concentrations ranging from 100 to 800 μM for 24 to 72 hours (**Figure 1C**). In contrast, BCSCs exposed to $CoCl_2$ concentrations below 100 μM for 24 hours show a generally reduced survival rate, but it is not significantly different from the negative control (**Figure 1D**).

At low concentrations of $CoCl_2$ (25 to 100 μM), BCSCs demonstrated a significant rise in *HIF-1α* gene expression. Notably, exposure to 100 μM $CoCl_2$ results in the most significant increase, with a 4.54-fold elevation in *HIF-1α* gene expression compared to cells not treated with $CoCl_2$ (**Figure 1E**).

Autophagy is stimulated under hypoxic condition

The hypoxic environment caused by adding $CoCl_2$ strongly induced autophagy in BCSCs. In fact, in the presence of $CoCl_2$, *LC3B*, a central marker of autophagy induction, exhibits increased gene expression in a concentration-dependent manner. As $CoCl_2$ concentration rises, *LC3B* gene expression increases from a 1.45 to a 4.09-fold change compared to untreated cells. The expression of the *Beclin-1* gene, another autophagy marker, also increases at $CoCl_2$ concentrations of 50, 75, and 100 μM (1.49, 2.28,

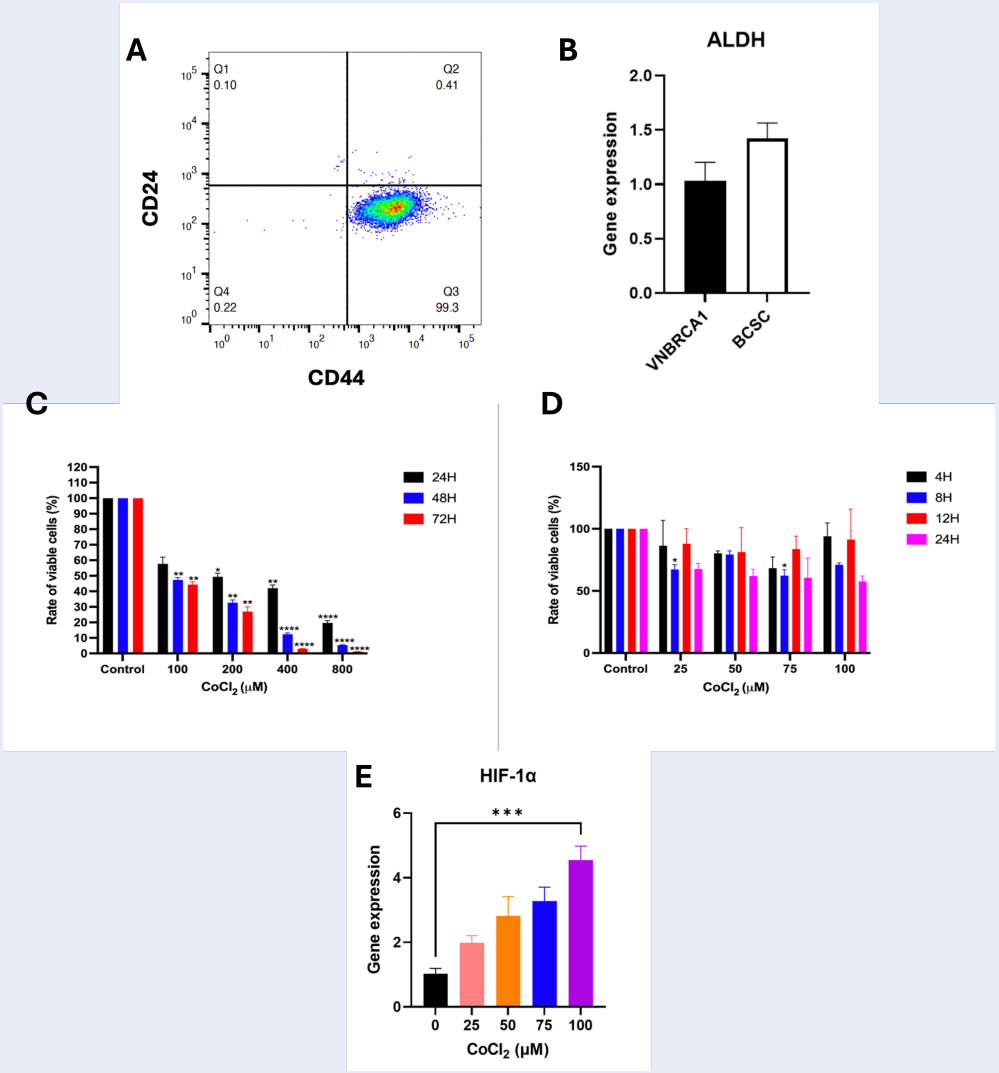

Figure 1: **Model of hypoxic BCSCs using $CoCl_2$**. The expression of CD24, CD44, and ALDH1 gene on breast cancer stem-like cell populations. (**A**) Flow cytometry is applied to evaluate expression of marker CD44 and CD24 on the surface of breast cancer stem-like cell populations. (**B**) The expression of *ALDH1* gene in both group VNBRCA1 and BSCS were semiquantitavely analyzed by Livak method (normalized by *β-actin*). Cytotoxicity effects of cobalt (II) chloride ($CoCl_2$) on survival rate of BCSCs accessed via AlamarBlue method (compared to negative control). Under the presence of (**C**) high concentrations of $CoCl_2$ for a long period of time, the survival rate of BCSCs tends to decrease sharply, while in BCSCs treated with (**D**) lower concentrations of $CoCl_2$ for a short period of time, their survival rate is slightly reduced (*p-value < 0.05; **p-value < 0.01; ****p-value < 0.0001). (**E**) The expression of *HIF-1α* increases in a $CoCl_2$ concentration-dependent manner (compared to BCSCs not treated with $CoCl_2$, ***p-value < 0.001). **Abbreviations**: **BCSCs**: Breast cancer stem cells, VNBCRA1: Vietnamese breast cancer cell line No. 1

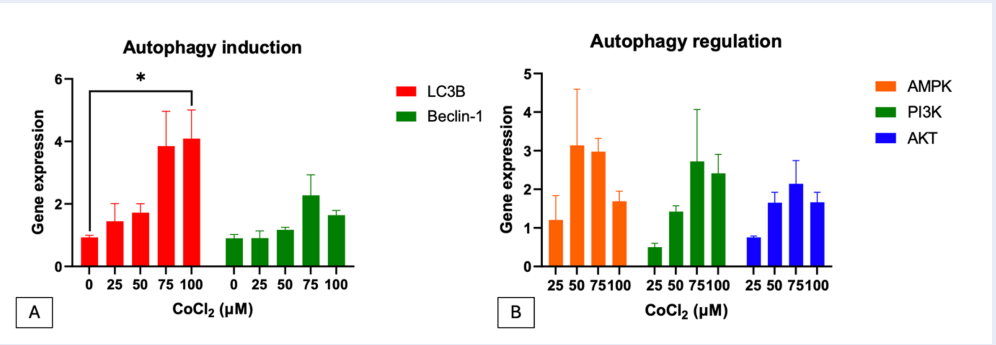

Figure 2: **Expressions of autophagy-related genes are changed upon $CoCl_2$ treatment** (compared to BCSCs not treated with $CoCl_2$). Hypoxic condition induces increased expression of gene involved in (**A**) autophagy induction such as *LC3B, Beclin-1* and (**B**) autophagy regulation such as *AMPK, PI3K, AKT*. Specifically, certain concentrations of $CoCl_2$ cause a significant increase in the expression of the aforementioned genes. **Abbreviations**: **3-MA**: 3-Methyladenine, **BCSCs**: Breast cancer stem cells, *p-value < 0.05

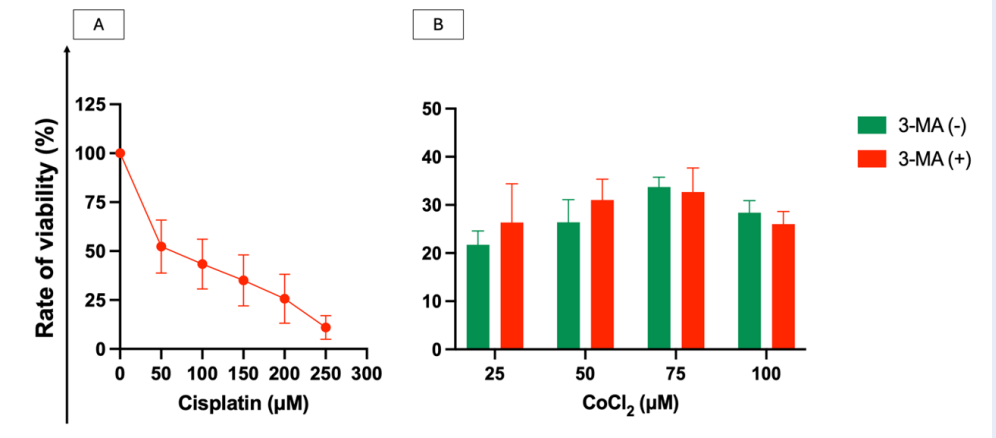

Figure 3: **Evaluation of cisplatin resistance in hypoxic BCSCs**. (**A**) The viability rate of BCSCs decreases as the concentration of cisplatin increases, ranging from 0 to 250 μM. The AlamarBlue assay determines the IC_{50} value of cisplatin for BCSCs to be approximately 60 μM. (**B**) Pretreatment with $CoCl_2$ enhances the resistance of breast cancer stem-like cells to the cytotoxic effects of cisplatin, regardless of pretreatment with the autophagy inhibitor 3-MA, compared to the negative control.
Abbreviations: **3-MA**: 3-Methyladenine, **BCSCs**: Breast cancer stem cells, *p-value < 0.05

and 1.64-fold change, respectively), but it shows no significant change at 25 μM ($p > 0.05$). Overall, both genes display increased expression with higher $CoCl_2$ concentrations, although these changes are not statistically significant ($p > 0.05$) (**Figure 2A**). In general, genes involved in autophagy regulation exhibit elevated expression levels when exposed to various $CoCl_2$ concentrations (**Figure 2B**). The AMPK gene shows the greatest expression increase at 50 μM $CoCl_2$ (3.14-fold change), while PI3K and AKT genes reach their peak expression at 75 μM $CoCl_2$ (2.69 and 2.14-fold change, respectively). After reaching peak expression, the levels of these genes gradually decline with further increases in $CoCl_2$ concentration.

The inhibition of autophagy did not reduce the drug resistance of BCSCs

The potential for drug resistance in BCSCs was assessed using a cytotoxicity assay with the anti-tumor drug cisplatin. The drug resistance of hypoxic BCSCs to cisplatin was evaluated either in the presence or absence of the autophagy inhibitor 3-MA in the culture medium. In this assay, BCSCs were first induced to become hypoxic by culturing them in a medium containing $CoCl_2$ (ranging from 25 to 100 μM) for 24 hours. Subsequently, these hypoxic BCSCs were cul-

tured in a fresh medium containing 60 μM cisplatin for the next 24 hours.

The AlamarBlue assay results indicated that the viability of hypoxic BCSCs increased with the concentration of cobalt (II) chloride, although the increase was not significant. Concurrent treatment with 60 μM cisplatin and 5 mM autophagy inhibitor 3-MA also enhanced the viability of the BCSCs population in relation to the concentration of $CoCl_2$.

The results showed that, whether treated with or without 3-MA, the cell viability of BCSCs remained similar and peaked at 75 μM $CoCl_2$ (**Figure 3**).

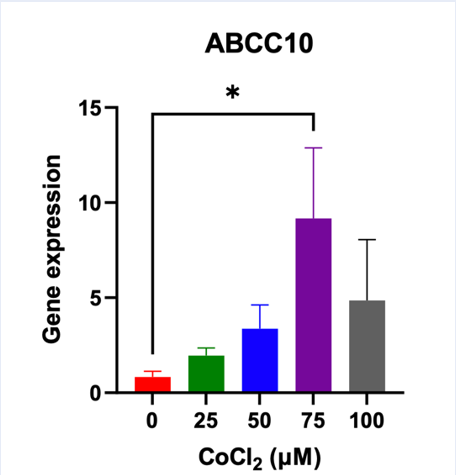

Figure 4: **The expression of the drug resistance gene *ABCC10* increased when breast cancer cell populations were treated with gradually increasing concentrations of $CoCl_2$ compared to BCSCs not treated with $CoCl_2$.** *p-value < 0.05

Hypoxia enhanced the expression of *ABCC10*

Treatment with $CoCl_2$ results in an upregulation of the drug resistance gene *ABCC10*. The most significant increase in expression, observed as a 9.17-fold change, occurs at a concentration of 75 μM $CoCl_2$ compared to breast cancer cells not exposed to $CoCl_2$ (see **Figure 4**).

DISCUSSION

The Cluster of Differentiation 44 (CD44) is a complex transmembrane adhesion glycoprotein involved in numerous signaling pathways significant in carcinogenesis and tumor progression[30]. Meanwhile, Cluster of Differentiation 24 (CD24) serves as a crucial biomarker, indicating poor prognoses like increased invasion, metastasis, and reduced survival in cancer patients[31]. The enzyme Aldehyde dehydrogenase (ALDH1) catalyzes the conversion of intracellular aldehydes to carboxylic acids and is expressed highly in a small subset of breast tumor cells capable of tumor generation in implanted mice[32]. Breast cancer cells displaying the $CD44^{+}CD24^{-/low}ALDH1^{high}$ phenotype are considered cancer stem cells due to their association with poorer outcomes in breast cancer patients[33]. In this study, breast cancer cells isolated from Vietnamese patients were cultured in DMEM with 10% FBS to expand their population. Flow cytometry sorting was then used to purify cells with high CD44 expression and low CD24 expression. As such, $CD44^{high}CD24^{low}$ breast cancer cells exhibiting elevated ALDH1 and EpCAM can be classified as breast cancer stem-like cells (BCSCs).

Cobalt (II) chloride ($CoCl_2$) has been used previously to modulate HIF expressions in various cancer[34–36] and non-cancer cell lines[37,38]. Past studies commonly used $CoCl_2$ concentrations over 100 μM; however, our research demonstrated that BCSC proliferation was significantly inhibited at concentrations above 100 μM for extended durations (24 to 72 hours). Consequently, we opted for lower concentrations of $CoCl_2$, under 100 μM, administered for shorter times (4 to 24 hours). The AlamarBlue assay revealed reduced BCSC proliferation, with no significant difference compared to cells cultured without $CoCl_2$. Similarly, a Thai study reported comparable outcomes using $CoCl_2$ concentrations of 50 or 100 μM[39]. To prevent extensive cell death affecting other evaluations, we employed $CoCl_2$ concentrations ranging from 25 to 100 μM in subsequent experiments.

Autophagy under controlled hypoxia promotes cancer cell survival; however, under severe hypoxic stress, it can lead to cell death[40]. In normoxic conditions, hypoxia-inducing factors (HIFs) are deactivated as prolyl hydroxylase enzymes hydroxylate them using oxygen. In hypoxia, HIFs stabilize and translocate to the nucleus, activating oncogenes[41]. Among HIF isoforms, HIF-1α is the most studied due to its mRNA levels increasing with the severity of hypoxia in many cancers[42–44]. Thus, HIF-1α can serve as a biomarker for evaluating tumor hypoxia levels. Using RT-qPCR, we found that $CD44^{high}CD24^{low}$ breast cancer cells exhibited increased HIF-1α expression correlating with rising $CoCl_2$ concentrations. Various mechanisms involving cobalt ions (Co^{2+}) enhancing HIF-1α expression have been suggested, generally affecting cofactors, inhibiting PHDs, and stabilizing HIF-1α[45]. Higher $CoCl_2$ concentrations in experimental models reflect a more pronounced hypoxic state in cancer tumors.

Beclin-1 is essential for signaling pathways activating autophagy and autophagosome formation, requiring interaction with hVsp34, while LC3B, a lipidated form of microtubule-associated protein light-chain 3, is a reliable autophagy marker, increasing in response to stressors[46]. Our study found that *Beclin-1* and *LC3B* gene expressions increased with rising $CoCl_2$ concentrations, indicating strong autophagy induction by this agent, similar to results in other cell lines[47]. The observed increase in HIF-1α expression suggests a link between autophagy and hypoxic response in BCSCs. According to Chen et al., $CoCl_2$ exposure increased HIF-1α and its downstream protein BNIP3, involved in autophagy induction through interaction with *Beclin-1* and *LC3*[47]. The AMPK/PI3K/AKT pathway is implicated in autophagy across mammalian cell lines, typically with AMPK activation and increased expression, while *PI3K* and *AKT* expressions decrease under autophagy-inducing agents[48–50]. However, our results showed all three genes increased expression in BCSCs exposed to 25-75 μM $CoCl_2$, decreasing at 100 μM. Prior research also indicated mTOR, downstream of PI3K and AKT, plays a key role in autophagy regulation, with reduced PI3K and AKT expressions potentially inhibiting mTOR and initiating autophagy[51]. AMPK activation relates to numerous autophagy process steps, given its involvement in several autophagy factors[52]. Variances in gene expressions within BCSCs suggest autophagy regulation through alternate signaling pathways, necessitating further investigation into AMPK/PI3K/AKT and autophagy relationships.

Autophagy links to drug resistance in different cancer cell lines. Experiments with cisplatin on $CD44^{high}CD24^{low}$ BCSCs, treated with $CoCl_2$ to simulate hypoxia and stimulate autophagy, showed increased cell survival correlating with $CoCl_2$ concentrations. This survival increase aligned with changes in autophagy-related gene expressions and *ABCC10*, a gene associated with drug resistance in breast cancer stem cells[27]. We earlier posited that autophagy acts as a "double-edged sword" in drug resistance among cancer stem cells, potentially enhancing sensitivity or decreasing responsiveness to chemotherapy drugs based on the signaling pathway involved[53]. Thus, targeting autophagy may offer a strategy to combat drug resistance in cancer cells. Numerous studies revealed that inhibiting autophagy through chemicals or genetic alterations enhances apoptosis in chemotherapy-resistant cell lines[54–57]. We hypothesize that autophagy suppression increases sensitivity to cisplatin in $CD44^{high}CD24^{low}$ BCSCs. Using 5 mM 3-MA, an autophagy inhibitor, we observed significant *LC3B* expression reduction compared to untreated cells (data not shown). Nevertheless, the response to cisplatin remained unchanged under 3-MA influence, suggesting a weaker linkage between autophagy and cisplatin response in our cell line compared to others. Hence, more specific methods are required to evaluate signaling pathways linking these properties in Vietnamese breast cancer stem-like cells.

CONCLUSIONS

BCSCs exhibit increased expression of the *HIF-1α* gene when exposed to higher concentrations of cobalt (II) chloride ($CoCl_2$), which also leads to the upregulation of genes involved in the induction (*Beclin-1, LC3B*) and regulation (*AMPK, PI3K, AKT*) of autophagy. Under hypoxia-mimicking conditions, BCSCs show increased resistance to cisplatin. However, inhibiting autophagy with the inhibitor 3-MA does not reduce the drug resistance of hypoxic BCSCs. Furthermore, under these conditions, an increase in the expression of the drug resistance gene *ABCC10* is observed in response to $CoCl_2$ levels. These findings suggest that the drug resistance of BCSCs under hypoxia is related to both autophagy upregulation and ABCC10 expression. Therefore, addressing drug resistance in BCSCs should consider both autophagy and ABCC10 pathways.

ABBREVIATIONS

3-MA: 3-Methyladenine, ***ABCC10***: ATP-Binding Cassette Subfamily C Member 10, **AKT**: Protein Kinase B, **AMPK**: AMP-Activated Protein Kinase, **ANOVA**: Analysis of Variance, **BCSC**: Breast Cancer Stem Cell, **$CoCl_2$**: Cobalt (II) Chloride, **CSC**: Cancer Stem Cell, **DMEM**: Dulbecco's Modified Eagle Medium, **FBS**: Fetal Bovine Serum, **HIF**: Hypoxia-Induced Factor, **HIF-1α**: Hypoxia-Induced Factor 1 alpha, ***LC3B***: Microtubule-Associated Proteins 1A/1B Light Chain 3B, **mRNA**: Messenger Ribonucleic Acid, **PI3K**: Phosphoinositide 3-Kinase, **RT-qPCR**: Real-Time Quantitative Polymerase Chain Reaction, **SEM**: Standard Error of the Mean, **VNBRCA1**: Vietnamese Breast Cancer Cell Line No. 1

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AUTHOR'S CONTRIBUTIONS

Bui Dinh Khan and Tran Ngo The Nhan equally contributed to developing the research methodology, conducting the experimental work, and collecting the data. Bui Dinh Khan took primary responsibility for data analysis and manuscript content. Pham Van Phuc suggested the layout, discussed the results, revised entire manuscript, and supervised the study. All authors contributed significantly to this work and have read and approved the final version of the manuscript.

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AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a Cancer Journal for Clinicians. 2024;74(3):229–63. PMID: 38572751. Available from: https://doi.org/10.3322/caac.21834.
2. Beck B, Blanpain C. Unravelling cancer stem cell potential. Nature Reviews Cancer. 2013;13(10):727–38. PMID: 24060864. Available from: https://doi.org/10.1038/nrc3597.
3. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(7):3983–8. PMID: 12629218. Available from: https://doi.org/10.1073/pnas.0530291100.
4. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell. 2007;1(5):555–67. PMID: 18371393. Available from: https://doi.org/10.1016/j.stem.2007.08.014.
5. Scioli MG, Storti G, D'Amico F, Gentile P, Fabbri G, Cervelli V. The role of breast cancer stem cells as a prognostic marker and a target to improve the efficacy of breast cancer therapy. Cancers (Basel). 2019;11(7):1021. PMID: 31330794. Available from: https://doi.org/10.3390/cancers11071021.
6. Barth DA, Prinz F, Teppan J, Jonas K, Klec C, Pichler M. Long-Noncoding RNA (lncRNA) in the regulation of hypoxia-inducible factor (HIF) in Cancer. Non-Coding RNA. 2020;6(3):27. PMID: 32640630. Available from: https://doi.org/10.3390/ncrna6030027.
7. Khan BD, Chau HN, Nhi NT. Roles of hypoxia in tumor progression and novel strategies for cancer treatment. Biomedical Research and Therapy. 2022;9(10):5361–74. Available from: https://doi.org/10.15419/bmrat.v9i10.774.
8. Tong WW, Tong GH, Liu Y. Cancer stem cells and hypoxia-inducible factors (Review). International Journal of Oncology. 2018;53(2):469–76. PMID: 29845228. Available from: https://doi.org/10.3892/ijo.2018.4417.
9. Ashrafizadeh M, Ahmadi Z, Mohammadinejad R, Kaviyani N, Tavakol S. Monoterpenes modulating autophagy: A review study. Basic & Clinical Pharmacology & Toxicology. 2020;126(1):9–20. PMID: 31237736. Available from: https://doi.org/10.1111/bcpt.13282.
10. Mizushima N. A brief history of autophagy from cell biology to physiology and disease. Nature Cell Biology. 2018;20(5):521–7. PMID: 29686264. Available from: https://doi.org/10.1038/s41556-018-0092-5.
11. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annual Review of Genetics. 2009;43(1):67–93. PMID: 19653858. Available from: https://doi.org/10.1146/annurev-genet-102808-114910.
12. Sakamoto H, Nakada-Tsukui K, Besteiro S. The Autophagy Machinery in Human-Parasitic Protists; Diverse Functions for Universally Conserved Proteins. Cells. 2021;10(5):1258. PMID: 34069694. Available from: https://doi.org/10.3390/cells10051258.
13. Yun CW, Jeon J, Go G, Lee JH, Lee SH. The dual role of autophagy in cancer development and a therapeutic strategy for cancer by targeting autophagy. International Journal of Molecular Sciences. 2020;22(1):179. PMID: 33375363. Available from: https://doi.org/10.3390/ijms22010179.
14. Guo JY, Xia B, White E. Autophagy-mediated tumor promotion. Cell. 2013;155(6):1216–9. PMID: 24315093. Available from: https://doi.org/10.1016/j.cell.2013.11.019.
15. Bischof J, Westhoff MA, Wagner JE, Halatsch ME, Trentmann S, Knippschild U, et al. Cancer stem cells: the potential role of autophagy, proteolysis, and cathepsins in glioblastoma stem cells. Tumour Biology. 2017;39(3):1010428317692227. PMID: 28347245. Available from: https://doi.org/10.1177/1010428317692227.
16. Hu YL, DeLay M, Jahangiri A, Molinaro AM, Rose SD, Carbonell WS. Hypoxia-induced autophagy promotes tumor cell survival and adaptation to antiangiogenic treatment in glioblastoma. Cancer Research. 2012;72(7):1773–83. PMID: 22447568. Available from: https://doi.org/10.1158/0008-5472.CAN-11-3831.
17. Gao L, Dou ZC, Ren WH, Li SM, Liang X, Zhi KQ. CircCDR1as upregulates autophagy under hypoxia to promote tumor cell survival via AKT/ERK/mTOR signaling pathways in oral squamous cell carcinomas. Cell Death & Disease. 2019;10(10):745. PMID: 31582727. Available from: https://doi.org/10.1038/s41419-019-1971-9.
18. Samanta D, Gilkes DM, Chaturvedi P, Xiang L, Semenza GL. Hypoxia-inducible factors are required for chemotherapy resistance of breast cancer stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(50):5429–38. PMID: 25453096. Available from: https://doi.org/10.1073/pnas.1421438111.
19. Qureshi-Baig K, Kuhn D, Viry E, Pozdeev VI, Schmitz M, Rodriguez F. Hypoxia-induced autophagy drives colorectal cancer initiation and progression by activating the PRKC/PKC-EZR (ezrin) pathway. Autophagy. 2020;16(8):1436–52. PMID: 31775562. Available from: https://doi.org/10.1080/15548627.2019.1687213.
20. Van Phuc P, et al. Isolation and characterization of breast cancer stem cells from malignant tumours in Vietnamese women.

Journal of Cell and Animal Biology. 2010;4(12):163–9. Available from: https://doi.org/10.5897/JCAB.9000104.
21. Kumar P, Nagarajan A, Uchil PD. Analysis of cell viability by the alamarBlue assay. Cold Spring Harbor Protocols, 2018. 2018(6): p. pdb. prot095489. 2018;Available from: https://doi.org/10.1101/pdb.prot095489.
22. Akrami H, Mahmoodi F, Havasi S, Sharifi A. PlGF knockdown inhibited tumor survival and migration in gastric cancer cell via PI3K/Akt and p38MAPK pathways. Cell Biochemistry and Function. 2016;34(3):173–80. PMID: 26968576. Available from: https://doi.org/10.1002/cbf.3176.
23. Wang J, Ni Z, Duan Z, Wang G, Li F. Altered expression of hypoxia-inducible factor-1α (HIF-1α) and its regulatory genes in gastric cancer tissues. PLoS One. 2014;9(6):e99835. PMID: 24927122. Available from: https://doi.org/10.1371/journal.pone.0099835.
24. Li G, Shan C, Liu L, Zhou T, Zhou J, Hu X, et al. Tanshinone IIA inhibits HIF-1α and VEGF expression in breast cancer cells via mTOR/p70S6K/RPS6/4E-BP1 signaling pathway. PLoS One. 2015;10(2):e0117440. PMID: 25659153. Available from: https://doi.org/10.1371/journal.pone.0117440.
25. Liu Q, Fang Q, Ji S, Han Z, Cheng W, Zhang H. Resveratrol-mediated apoptosis in renal cell carcinoma via the p53/AMP-activated protein kinase/mammalian target of rapamycin autophagy signaling pathway. Molecular Medicine Reports. 2018;17(1):502–8. PMID: 29115429.
26. Wu S, Sun C, Tian D, Li Y, Gao X, He S. Expression and clinical significances of Beclin1, LC3 and mTOR in colorectal cancer. International Journal of Clinical and Experimental Pathology. 2015;8(4):3882–91. PMID: 26097572.
27. Sobczak M, Strachowska M, Gronkowska K, Robaszkiewicz A. Activation of ABCC Genes by Cisplatin Depends on the CoREST Occurrence at Their Promoters in A549 and MDA-MB-231 Cell Lines. Cancers (Basel). 2022;14(4):894. PMID: 35205642. Available from: https://doi.org/10.3390/cancers14040894.
28. Kohjima M, Higuchi N, Kato M, Kotoh K, Yoshimoto T, Fujino T. SREBP-1c, regulated by the insulin and AMPK signaling pathways, plays a role in nonalcoholic fatty liver disease. International Journal of Molecular Medicine. 2008;21(4):507–11. PMID: 18360697. Available from: https://doi.org/10.3892/ijmm.21.4.507.
29. Li JZ, Wang ZL, Xu WH, Li Q, Gao L, Wang ZM. MicroRNA-495 regulates migration and invasion in prostate cancer cells via targeting Akt and mTOR signaling. Cancer Investigation. 2016;34(4):181–8. PMID: 27031291. Available from: https://doi.org/10.3109/07357907.2016.1156690.
30. Xu H, Niu M, Yuan X, Wu K, Liu A. CD44 as a tumor biomarker and therapeutic target. Experimental Hematology & Oncology. 2020;9(1):36. PMID: 33303029. Available from: https://doi.org/10.1186/s40164-020-00192-0.
31. Fang X, Zheng P, Tang J, Liu Y. CD24: from A to Z. Cellular & Molecular Immunology. 2010;7(2):100–3. PMID: 20154703. Available from: https://doi.org/10.1038/cmi.2009.119.
32. Douville J, Beaulieu R, Balicki D. ALDH1 as a functional marker of cancer stem and progenitor cells. Stem Cells and Development. 2009;18(1):17–25. PMID: 18573038. Available from: https://doi.org/10.1089/scd.2008.0055.
33. Ricardo S, Vieira AF, Gerhard R, Leitão D, Pinto R, Cameselle-Teijeiro JF. Breast cancer stem cell markers CD44, CD24 and ALDH1: expression distribution within intrinsic molecular subtype. Journal of Clinical Pathology. 2011;64(11):937–46. PMID: 21680574. Available from: https://doi.org/10.1136/jcp.2011.090456.
34. Mikeladze MA, Dutysheva EA, Kartsev VG, Margulis BA, Guzhova IV, Lazarev VF. Disruption of the complex between GAPDH and Hsp70 sensitizes C6 glioblastoma cells to hypoxic stress. International Journal of Molecular Sciences. 2021;22(4):1520. PMID: 33546324. Available from: https://doi.org/10.3390/ijms22041520.
35. Tang Y, Jin YH, Li HL, Xin H, Chen JD, Li XY. PBRM1 deficiency oncogenic addiction is associated with activated AKT-mTOR signalling and aerobic glycolysis in clear cell renal cell carcinoma cells. Journal of Cellular and Molecular Medicine. 2022;26(14):3837–49. PMID: 35672925. Available from: https://doi.org/10.1111/jcmm.17418.
36. Yang L, Zeng L, Wang Z, Hu X, Xiong H, Zhang T. Differentiated embryo chondrocyte 1, induced by hypoxia-inducible factor 1α, promotes cell migration in oral squamous cell carcinoma cell lines. Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology. 2022;133(2):199–206. PMID: 34758939. Available from: https://doi.org/10.1016/j.oooo.2021.08.022.
37. Nakuluri K, Mukhi D, Mungamuri SK, Pasupulati AK. Stabilization of hypoxia-inducible factor 1α by cobalt chloride impairs podocyte morphology and slit-diaphragm function. Journal of Cellular Biochemistry. 2019;120(5):7667–78. PMID: 30387200. Available from: https://doi.org/10.1002/jcb.28041.
38. Zhang C, Chen M, Tao Q, Chi Z. Cobalt chloride-stimulated hypoxia promotes the proliferation of cholesteatoma keratinocytes via the PI3K/Akt signaling pathway. International Journal of Medical Sciences. 2021;18(15):3403–11. PMID: 34522167. Available from: https://doi.org/10.7150/ijms.60617.
39. Jampasri S, Reabroi S, Tungmunnithum D, Parichatikanond W, Pinthong D. Plumbagin Suppresses Breast Cancer Progression by Downregulating HIF-1α Expression via a PI3K/Akt/mTOR Independent Pathway under Hypoxic Condition. Molecules (Basel, Switzerland). 2022;27(17):5716. PMID: 36080483. Available from: https://doi.org/10.3390/molecules27175716.
40. Mazure NM, Pouysségur J. Hypoxia-induced autophagy: cell death or cell survival? Current Opinion in Cell Biology. 2010;22(2):177–80. PMID: 20022734. Available from: https://doi.org/10.1016/j.ceb.2009.11.015.
41. Pezzuto A, Carico E. Role of HIF-1 in cancer progression: novel insights. A review. Current Molecular Medicine. 2018;18(6):343–51. PMID: 30411685. Available from: https://doi.org/10.2174/1566524018666181109121849.
42. Yoshikawa T, Tsuburaya A, Miyagi Y, Sekiguchi H, Kimura M, Cho H. Up-regulation of hypoxia-inducible factor-1 alpha and VEGF mRNAs in peritoneal dissemination of patients with gastric cancer. Anticancer Research. 2006;26:3849–53. PMID: 17094412.
43. Qin J, Liu Y, Lu Y, Liu M, Li M, Li J. Hypoxia-inducible factor 1 alpha promotes cancer stem cells-like properties in human ovarian cancer cells by upregulating SIRT1 expression. Scientific Reports. 2017;7(1):10592. PMID: 28878214. Available from: https://doi.org/10.1038/s41598-017-09244-8.
44. Maxwell PJ, Gallagher R, Seaton A, Wilson C, Scullin P, Pettigrew J. HIF-1 and NF-kappaB-mediated upregulation of CXCR1 and CXCR2 expression promotes cell survival in hypoxic prostate cancer cells. Oncogene. 2007;26(52):7333–45. PMID: 17533374. Available from: https://doi.org/10.1038/sj.onc.1210536.
45. Muñoz-Sánchez J, Chánez-Cárdenas ME. The use of cobalt chloride as a chemical hypoxia model. Journal of Applied Toxicology. 2019;39(4):556–70. PMID: 30484873. Available from: https://doi.org/10.1002/jat.3749.
46. Miracco C, Cevenini G, Franchi A, Luzi P, Cosci E, Mourmouras V. Beclin 1 and LC3 autophagic gene expression in cutaneous melanocytic lesions. Human Pathology. 2010;41(4):503–12. PMID: 20004946. Available from: https://doi.org/10.1016/j.humpath.2009.09.004.
47. Chen R, et al. Effects of cobalt chloride, a hypoxia-mimetic agent, on autophagy and atrophy in skeletal C2C12 myotubes. ioMed research international. 2017;2017:7097580. PMID: 28706950. Available from: https://doi.org/10.1155/2017/7097580.
48. Lin Y, Kuang W, Wu B, Xie C, Liu C, Tu Z. IL-12 induces autophagy in human breast cancer cells through AMPK and the PI3K/Akt pathway. Molecular Medicine Reports. 2017;16(4):4113–8. PMID: 28765958. Available from: https://doi.org/10.3892/mmr.2017.7114.
49. Yin H, Zuo Z, Yang Z, Guo H, Fang J, Cui H, et al. Nickel induces autophagy via PI3K/AKT/mTOR and AMPK pathways in mouse kidney. Ecotoxicology and Environmental Safety.

2021;223:112583. PMID: 34352574. Available from: https://doi.org/10.1016/j.ecoenv.2021.112583.

50. Hu S, Yin J, Yan S, Hu P, Huang J, Zhang G, et al. Chaetocochin J, an epipolythiodioxopiperazine alkaloid, induces apoptosis and autophagy in colorectal cancer via AMPK and PI3K/AKT/mTOR pathways. Bioorganic Chemistry. 2021;109:104693. PMID: 33609914. Available from: https://doi.org/10.1016/j.bioorg.2021.104693.
51. Deleyto-Seldas N, Efeyan A. The mTOR - autophagy axis and the control of metabolism. Frontiers in Cell and Developmental Biology. 2021;9:655731. PMID: 34277603. Available from: https://doi.org/10.3389/fcell.2021.655731.
52. Wang S, Li H, Yuan M, Fan H, Cai Z. Role of AMPK in autophagy. Frontiers in Physiology. 2022;13:1015500. PMID: 36505072. Available from: https://doi.org/10.3389/fphys.2022.1015500.
53. Bui KD, Van Pham P. The relationship between autophagy and multidrug resistance in cancer stem cells. Science and Technology Development Journal. 2022;25(4):2625–36. Available from: https://doi.org/10.32508/stdj.v25i4.4036.
54. Lefort S, Joffre C, Kieffer Y, Givel AM, Bourachot B, Zago G. Inhibition of autophagy as a new means of improving chemotherapy efficiency in high-LC3B triple-negative breast cancers. Autophagy. 2014;10(12):2122–42. PMID: 25427136. Available from: https://doi.org/10.4161/15548627.2014.981788.
55. Guo XL, Li D, Hu F, Song JR, Zhang SS, Deng WJ. Targeting autophagy potentiates chemotherapy-induced apoptosis and proliferation inhibition in hepatocarcinoma cells. Cancer Letters. 2012;320(2):171–9. PMID: 22406827. Available from: https://doi.org/10.1016/j.canlet.2012.03.002.
56. Liu D, Yang Y, Liu Q, Wang J. Inhibition of autophagy by 3-MA potentiates cisplatin-induced apoptosis in esophageal squamous cell carcinoma cells. Medical Oncology (Northwood, London, England). 2011;28(1):105–11. PMID: 20041317. Available from: https://doi.org/10.1007/s12032-009-9397-3.
57. Li J, Hou N, Faried A, Tsutsumi S, Kuwano H. Inhibition of autophagy augments 5-fluorouracil chemotherapy in human colon cancer in vitro and in vivo model. European Journal of Cancer (Oxford, England). 2010;46(10):1900–9. PMID: 20231086. Available from: https://doi.org/10.1016/j.ejca.2010.02.021.