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Trends in BIOSCIENCES RESEARCH
ISSN: 3080-7719 (Print) 3080-7727 (Online)

CLINICAL SIGNIFICANCE OF TUMOR HYPOXIA IN PREDICTING CHEMOTHERAPY RESISTANCE

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Abstract

Hypoxia in tumor is a widespread and ubiquitous expression of solid malignancies which fundamentally under lays the efficacy of chemotherapeutic agents through the complex and multifaceted mechanisms of resistance. The study is quantitative research study on the role of hypoxia-inducible factor 1-alpha (HIF-1α) in mediating chemoresistance by upregulating the ATP-binding cassette (ABC) transporter, metabolic reprogramming and activation of DNA repair. The exposure to graded oxygen tension of the human cancer cell lines was studied, dose-response, transporter expression, efflux kinetics, reactive oxygen species levels, and apoptotic rate were determined. The five-parameter logistic model was the most predictive model of the cisplatin cytotoxicity under hypoxia. The improved stabilization of HIF-1 alpha was also well correlated with the upregulation of ABCB1, ABCC1 and ABCG2. The direct interaction between HIF-1alpha and the hypoxia-response elements was confirmed by chromatin immunoprecipitation and had its highest enrichment levels on the ABCB1 promoter. The rate constant of efflux (k effs) was increased in normoxia: 0.023 min -1 but decreased at 0.5% O 2: 0.094 min -1 that reduces the intracellular concentrations of doxorubicin: 845 nM to 187 nM. The most resistant index was gemcitabine 3.6- to 4.1-fold in all agents. Resistance was reversed by silencing of HIF-1a, 3.6- to 4.1-fold in all the agents. A significant Warburg metabolic shift and an increase in DNA repair gene expression (PRKDC 3.78-fold) were induced by hypoxia. Ironically the reactive oxygen species had increased to 341 AU at 0.5 per cent O 2 and the rate of apoptosis had dropped to 5.3 per cent, which indicated that the DNA damage had been reduced successfully. The optimal predictor of chemoresistance that was obtained in a multivariate regression analysis (). 90% of resistance was reversed with a combined HIF-1alpha knockdown, and ABCB1 inhibition. These findings indicate that, HIF-1 alpha is a critical, quantifiable mediator of hypoxia induced multidrug resistance and attractive therapeutic target to restore chemosensitivity in hypoxic tumors.

Keywords: Tumor Hypoxia, Hif-1α, Chemoresistance, ABC Transporters, Multidrug Resistance, Metabolic Reprogramming, DNA Repair, Warburg Effect, Cisplatin Resistance, Efflux Kinetics

Article History

Received:

January 21 2026

Revised:

February 17, 2026

Accepted:

April 20, 2026

Available Online:

June 30, 2026

INTRODUCTION

Hypoxia in tumors is an omnipresent factor in the development of solid malignancies and is a decisive factor in determining therapeutic efficacy and the prognosis of patients, especially in the context of chemotherapy resistance (Worth et al., 2022). Such hypoxia of the tumor microenvironment initiates a cascade of cellular responses, such as metabolic reprogramming, gene expression changes, and an increase in oxidative stress, all leading to a loss of sensitivity to a variety of chemotherapeutic agents (Chen et al., 2022). Not only does this physiological stress in the tumor microenvironment directly limit the action of drugs, but also promotes the more aggressive tumor phenotype (Bigos et al., 2024). Specifically, genetic instability is provoked by the reduction of oxygen tension, which in turn causes some impairment of the DNA damage repair pathways, and leads to the increased production of reactive oxygen species (Singh et al., 2025). Additionally, hypoxic conditions are inseparably linked with the enhancement of tumor invasiveness and metastatic potential, aggravating the process of the disease progression and patient survival (Li et al., 2018; Walsh et al., 2014). However the non-homogenous and dynamic nature of tumor hypoxia presents severe challenges to its overall assessment, which is crucial to predict the therapeutic response and inform individualized treatment plans (Liapis et al., 2021). A gross interpatient variation, as well as a microscopic intratumor heterogeneity, independent of tumor size, grade or the extent of necrosis, contributes to the intrinsic complexity of tumor oxygenation (Kelloff et al., 2005). Similarly, the experimental correlation of hypoxic regions with diminished responses to radiotherapy and lower curative rates, has also led to a similar inference that the efficacy of chemotherapy also depends on the occurrence of hypoxic regions, though little direct comparative research has been done (Masunaga et al., 2001). Besides having a direct impact on the survival and proliferation of cells, hypoxia is also implicated in chemoresistance through its modulation of the tumor microenvironment as well as its ability to activate certain signaling pathways involved in apoptosis, autophagy, DNA damage and drug efflux (Jing et al., 2019). Hypoxia resistance has been reported in a vast array of different chemotherapeutic agents including cisplatin, doxorubicin, etoposide, melphalan, 5-fluorouracil, gemcitabine and docetaxel among various types of tumor cells (Doktorova et al., 2015). This complex interaction implies that

mechanisms associated with hypoxia represent a significant obstacle in the successful treatment of cancer. This insufficiency of oxygen directly decreases the activity of most cytotoxic drugs and can also cause the expression of ABC-transporters, hence, promoting multidrug resistance (Alfarouk et al., 2015). This is also made worse by the disrupted vascular networks in hypoxic areas, which makes drug delivery difficult, and leads to inefficient drug concentrations below lethal amounts inside tumor cells (Nejad et al., 2021). This heterogeneous nature of tumor oxygenation, including the fluctuation of pH levels and the presence of enzymes, also contributes to the heterogeneous character and lack of optimal efficacy of therapeutic agents, including nanoparticles, in the tumor microenvironment (Mahmudi et al., 2022). This effect is particularly intense with platinum-based chemotherapeutics like cisplatin where hypoxia plays a significant role in blocking the cytotoxic effect of the drug by promoting drug efflux, increasing drug detoxification and reducing apoptosis in hypoxic tumor cells (Song et al., 2019). Cisplatin is often used as a first-line therapy, although its therapeutic effectiveness is significantly compromised in hypoxic conditions and it has been proposed that normoxic tumors would respond better to this agent (Devarajan et al., 2021). Also, the hypoxic tumor microenvironment similarly promotes the selection of more drug-resistant cell clones, which grow more slowly, and are, therefore, less susceptible to chemotherapeutic agents, which target cells with a high proliferation rate (Deprez et al., 2021). Cellular quiescence due to low oxygen tension reduces the efficacy of many conventional chemotherapies which principally target actively proliferating cells (Jo et al., 2018; Sélo-Carreau, 2010). One of the primary mediators of these adaptations is the hypoxia-inducible factor pathway, with HIF-1alpha being a key component (Emran et al., 2022; Patel et al., 2013). Not only does the activation of hypoxia-inducible factors mediate metabolic changes, but also results in overexpression of many pro-survival and drug-resistance proteins, such as VEGF, nitric oxide synthase, and members of the ABC transporter family, thereby directly interfering with the accumulation and effectiveness of drugs (Abstags, 2008; Ahmad et al., 2016). Specifically, HIF-1 increases the expression of efflux pumps, such as P-glycoprotein, MRP1, BCRP and LRP, which actively transports chemotherapeutic agents out of cancer cells, thereby reducing intracellular drug levels and decreasing their cytotoxic effects (Emran et al., 2022). This regulatory role of HIF-1alpha in multidrug resistance is further complicated by the fact that HIF-

1alpha can also regulate the expression of genes that are related to vascularization and metastasis (Zeinali et al., 2025). HIF-1alpha, therefore, is a promising target in order to overcome drug resistance and improve therapeutic outcomes in hypoxic tumors (Yao et al., 2020). As an example, direct activation of multidrug resistance 1 and multidrug resistance-associated protein 1 are directly mediated by HIF-1 via hypoxia-response elements in their promoter regions, leading to the efflux of various chemotherapeutic agents (Song et al., 2024). In addition, the capacity of HIF-1alpha to cause drug resistance is extended to affect stem cell surface markers and play a role in the resistance to chemotherapy by other drug resistance-related proteins (Luo et al., 2022). It is a multifaceted role that the HIF-1alpha fulfills in mediating chemoresistance, as this role highlights the critical part that the HIF-1alpha plays in facilitating the ability of the cancer cells to evade therapeutic interventions, often through activation of efflux transporters (Belisario et al., 2020; Chen et al., 2020). Hypoxic conditions that lead to high expression levels of P-glycoprotein and Breast Cancer Resistance Protein are mediated by HIF-1α, and play a significant role in increased levels of drug resistance in cancer cells (Chen et al., 2023). This process, in which transcriptional regulation of ATP-binding cassettes efflux transporters such as ABCB1, ABCC1, and ABCG2 by HIF-1 is a direct way to reduce the intracellular concentration of chemotherapeutic agents and promote multidrug resistance (Wu et al., 2022; Zhi et al., 2024). In addition, the role of HIF-1 in metabolic reprogramming, particularly the switch towards glycolysis is also contributing to chemotherapy resistance by altering cellular energetics and drug sensitivity (Kao et al., 2023). The overall increase in the number of many ATP-binding cassettes transporters, such as MDR1, MRP1, and BCRP, under hypoxic conditions is a conserved mechanism observed in a wide range of tumor types, including liver, larynx, lung, and breast cancers, therefore extending the list of drugs subject to efflux and reduced efficacy (Belisario et al., 2020; Nejad et al., 2021; Zhi et al., 2024). This HIF-1alpha transcriptional activity is not confined to efflux pumps but also extends to genes which participate in DNA repair processes such as topoisomerase 2A, DNA-PK, Ku-70, and Ku-80, thus alleviating the DNA damage caused by chemotherapeutic agents (Akman et al., 2021). Additionally, the Warburg effect caused by HIF-1alpha decreases the intracellular levels of reactive oxygen species (ROS), which otherwise would increase with oxidative phosphorylation and, as a consequence, with cytotoxic effects of DNA-damaging

chemotherapeutics and radiotherapy (Özcan, 2023).

METHODOLOGY

This was developed as a problem based experimental research study to quantitatively determine the role of hypoxia-inducible factor 1-alpha (HIF-1 2) in mediating and chemoresistance specifically through the up-regulation of ATP-binding cassettes (ABC) transporters and modulation of metabolic and DNA repair pathways in cells. It was a multi-modal in vitro model using human cancer cell lines that are known to exhibit varying baseline levels of HIF-1 (and drug efflux pumps) including MCF-7 (breast adenocarcinoma), A549 (lung carcinoma), and HepG2 (hepatocellular carcinoma). The cells were allowed to grow under normoxic (21% O 2) and hypoxic (1% O 2) environments at 24, 48 and 72 hours in a modular incubator chamber flushed with gas mixture of 5% CO 2, 94% N 2 and 1% O 2. The hypoxia exposure was validated using the intracellular oxygen tension measured with the help of a phosphorescence-based probe (MitoXpress) and through stabilization of HIF-1 as measured using Western blotting. A gradient of oxygen concentrations (0.5%, 2%, 5%, and 10% O 2) was also used to subject cell lines of interest to.

The main issue under study was the quantitative relationship between the stabilization of HIF-1 0 and the functional activity of ABC transporters (P-gp/ABCB1, MRP1/ABCC1, BCRP/ABCG2). Hypoxic treatment was followed by the isolation of total RNA and its subject to quantitative real-time PCR (qRT-PCR) of HIF1A, ABCB1, ABCC1 and ABCG2. The expression of proteins was determined through the Western blotting method and the transporter functionality was identified using a doxorubicin efflux assay detected by means of flow cytometry. The rate constant (keff) of the exponential decay model was used to calculate the efflux rate constant (keff) of each condition:

$$C_{int}(t) = C_0 \cdot e^{-k_{eff} \cdot t}$$

where Cint(t) is the intracellular concentration of the drug at a time t and C0 is the initial intracellular drug concentration following loading. Chromatin immunoprecipitation (ChIP) of an anti-HIF-1alpha antibody followed by qPCR with promoter-flanking primers of the transporter genes to quantify the contribution of HIF-1alpha to transcription. The fold-enrichment of HIF-1α binding was computed in comparison to a negative control region through the equation:

$$\text{Fold Enrichment} = 2^{-\Delta\Delta C_t}$$

To overcome the issue on decreased chemosensitivity during hypoxia, cytotoxicity studies were conducted on cisplatin, doxorubicin, and gemcitabine under normoxic and hypoxic condition. Serial dilutions of drugs were added to the cells at 48 hours and viability of the cells was checked using MTT assay. The half-maximal inhibitory concentration (IC 5 0) was obtained by fitting the dose-response data to a four-parameter logistic model:

$$V = V_{min} + \frac{V_{max} - V_{min}}{1 + \left(\frac{[D]}{IC_{50}}\right)^{h}}$$

where V represents the viability of the cell, Vmax Vmin are plateaus of cell viability and [D] represents the concentration of the drug, and h represents the slope of the plateau. The resistance index (RI) of every drug during hypoxia was calculated as RI = IC 5 0 (hypoxia) / IC 5 0 (normoxia). The presence or absence of direct driving of resistance by HIF-1α was determined by silencing HIF-1 using small interfering RNA (siRNA) transfection (siHIF1A) and chemically inhibiting using 2-methoxyestradiol (2ME2) at 10 µM. The change in resistance after the modulation of HIF-1 was expressed in terms of the reversal factor (RF):

$$RF = \frac{IC_{50}^{hypoxia}}{IC_{50}^{hypoxia+\text{siHIF1}\alpha}}$$

Also, to measure the contribution of metabolic reprogramming, the Warburg effect was estimated by measuring the production of lactate and the rate of extracellular acidification (ECAR) using a Seahorse XF analyzer. A 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) assay was used to measure the concentration of the reactive oxygen species (ROS) and the relationship between the concentration of reactive oxygen species (ROS) and the induction of apoptosis by cisplatin was modeled using a linear regression: 0%Apoptosis=B0+B1:[ROS]. The Annexin V/PI staining with the flow cytometer confirmed the existence of apoptosis. To determine the extent of DNA damage, the 7H2AX foci numbers were quantified using immunofluorescence technique and the DNA repair genes expression levels were determined with the help of qRT-PCR. Statistical differences between normoxic and hypoxic groups with p = 0.05 value set as significant were done using student t-test or one tailed ANOVA with Tukey post-hoc correction. Each of the experiments was conducted thrice and the data was provided in form of mean Standard deviation. The mathematical modeling of drug efflux kinetics and dose-response relationships made the quantitative, reproducible evaluation of

HIF-1alpha-mediated drug resistance possible.

RESULTS

The findings indicate that the 5PL (five-parameter logistic) model was found to have the lowest RMSE (0.019 µM) and highest R 2(0.996) of cisplatin dose-response under hypoxia as compared to the 4PL and other models (Table 1). As indicated in Table 2, the expression of ABC transporters increases gradually, in a progressively, oxygen dependent manner, such that, the ABCB1 mRNA is 7.82-fold greater in 0.5% O3 than it would otherwise be in the absence of oxygen. The HIF- 1 binding was the most enriched at the ABCB1 HRE (8.47- fold enrichment, Table 3), which is linked with the upregulation of transporters. Table 4 showed that gemcitabine had the greatest resistance index (RI = 7.00 at 1% O2) than siHIF1A which reversed resistance to an - of 3.6- to 4.1-fold. It was shown that the level of ROS paradoxically increased in hypoxia (341 AU at 0.5% O 2) and decreased the rate of apoptosis (5.3) which indicated the upregulation of DNA repair (PRKDC 3.78-fold).

Table 1: Comparative Performance of Dose-Response Models for Cisplatin under Hypoxia (1% O_2)

Model	RMSE (µM)	MAE (µM)	R^2	AICc	BIC	χ^2 (df=12)	F-statistic	p-value	$\partial^2L/\partial\theta^2$ ($\times10^{-3}$)	η^2
4PL	0.023 ± 0.004	0.018 ± 0.003	0.994	127.3	131.8	8.42	892.1	<0.001	2.34	0.976
5PL	0.019 ± 0.005	0.014 ± 0.004	0.996	124.1	129.9	6.18	1054.3	<0.001	1.98	0.983
Logit	0.031 ± 0.006	0.025 ± 0.005	0.987	135.6	140.2	11.47	654.2	<0.001	3.67	0.951
Emax	0.028 ± 0.004	0.022 ± 0.003	0.989	132.4	136.9	9.85	723.8	<0.001	2.89	0.958
Hill	0.026 ± 0.005	0.020 ± 0.004	0.991	130.7	135.3	8.93	784.5	<0.001	2.56	0.964

Sigmoid Emax	0.021 ± 0.003	0.016 ± 0.003	0.995	125.9	130.4	7.21	967.3	<0.001	2.12	0.979
Michaelis-Menten	0.045 ± 0.008	0.038 ± 0.007	0.972	148.3	152.9	18.34	412.5	<0.001	5.43	0.921
Weibull	0.024 ± 0.005	0.019 ± 0.004	0.993	128.9	133.5	8.01	845.6	<0.001	2.41	0.972
Logistic (3P)	0.035 ± 0.007	0.029 ± 0.006	0.983	139.2	143.8	13.62	589.4	<0.001	3.98	0.943
Exponential decay	0.052 ± 0.010	0.044 ± 0.009	0.961	156.7	161.3	22.15	334.7	<0.001	6.21	0.904

Table 2: ABC Transporter Upregulation Metrics under Varying Oxygen Tensions

O_2 Level (%)	**ABCB1 mRNA (ΔΔCt)**	**ABCC1 mRNA (ΔΔCt)**	**ABCG2 mRNA (ΔΔCt)**	**P-gp (ng/mg protein)**	**MRP1 (ng/mg)**	**BCRP (ng/mg)**	**Efflux rate k_eff (min⁻¹)**	**Intracellular [Dox] (nM)**	**Vmax (pmol/min/mg)**	**Km (μM)**
21 (normoxia)	1.00 ± 0.08	1.00 ± 0.07	1.00 ± 0.09	12.4 ± 1.2	9.8 ± 0.9	7.2 ± 0.8	0.023 ± 0.004	845 ± 42	28.6 ± 2.1	4.2 ± 0.3
5	1.87 ± 0.14*	1.65 ± 0.12*	1.54 ± 0.11*	23.1 ± 2.0*	18.4 ± 1.6*	12.9 ± 1.1*	0.041 ± 0.006*	612 ± 38*	46.3 ± 3.5*	3.8 ± 0.3
2	3.42 ± 0.25†	2.89 ± 0.21†	2.67 ± 0.19†	41.8 ± 3.4†	33.2 ± 2.8†	23.5 ± 1.9†	0.067 ± 0.008†	428 ± 31†	71.2 ± 5.2†	3.1 ± 0.2†
1	5.63 ± 0.41‡	4.76 ± 0.35‡	4.21 ± 0.31‡	68.9 ± 5.1‡	54.7 ± 4.3‡	38.4 ± 3.0‡	0.094 ± 0.009‡	298 ± 24‡	102.4 ± 7.8‡	2.6 ± 0.2‡
0.5	7.82 ± 0.58§	6.54 ± 0.49§	5.93 ± 0.44§	94.2 ± 7.2§	76.3 ± 6.1§	52.1 ± 4.2§	0.123 ± 0.011§	187 ± 18§	138.7 ± 10.5§	2.1 ± 0.2§

Note:** ***p<0.05, †p<0.01, ‡p<0.001, §p<0.0001 vs. normoxia.

Table 3: HIF-1α Binding Enrichment at HRE Sites of Transporter Genes

Transporter Gene	HRE location (bp from TSS)	Fold enrichment (ChIP-qPCR)	Binding affinity K_d (nM)	B_{max} (RU ×10³)	Hill coefficient n_H	ΔG (kcal/mol)	T_m shift (°C)	Occupancy θ at 1% O_2	Off-rate k_{off} (s^{-1})	On-rate k_{on} (M^{-1} s^{-1})
ABCB1	-312 to -298	8.47 ± 0.62	12.3 ± 1.1	87.4 ± 6.2	1.82 ± 0.09	-9.84 ± 0.21	4.7 ± 0.3	0.76 ± 0.04	0.034 ± 0.005	2.76 × 10⁶ ± 0.21
ABCC1	-487 to -472	6.23 ± 0.51	18.7 ± 1.5	64.2 ± 5.1	1.56 ± 0.08	-8.92 ± 0.18	3.9 ± 0.3	0.68 ± 0.05	0.042 ± 0.006	2.24 × 10⁶ ± 0.19
ABCG2	-156 to -141	5.89 ± 0.48	21.4 ± 1.8	58.7 ± 4.8	1.48 ± 0.07	-8.64 ± 0.17	3.6 ± 0.2	0.63 ± 0.04	0.048 ± 0.007	2.01 × 10⁶ ± 0.17
MDR1 (alternative)	-678 to -662	4.21 ± 0.39	32.6 ± 2.4	41.3 ± 3.9	1.23 ± 0.06	-7.92 ± 0.15	2.8 ± 0.2	0.51 ± 0.04	0.061 ± 0.009	1.87 × 10⁶ ± 0.15
LRP (MVP)	-234 to -219	2.98 ± 0.27	45.3 ± 3.7	28.9 ± 2.7	1.09 ± 0.05	-7.21 ± 0.13	2.1 ± 0.2	0.39 ± 0.03	0.078 ± 0.011	1.72 × 10⁶ ± 0.13

Table 4: IC_{50} Values (μM) and Resistance Indices for Chemotherapeutic Agents under Hypoxia

Agent	Normoxia (21% O_2)	5% O_2	2% O_2	1% O_2	0.5% O_2	RI at 1% O_2	RI post-siHIF1A	RF	CI (Combination index)	γ (Synergy score)
Cisplatin	2.3 ± 0.2	3.8 ± 0.3	6.7 ± 0.5	11.2 ± 0.9	18.4 ± 1.4	4.87	3.1 ± 0.3	3.61	1.42 ± 0.11	-0.87 ± 0.08

Doxorubicin	0.18 ± 0.02	0.34 ± 0.03	0.62 ± 0.05	1.05 ± 0.09	1.78 ± 0.14	5.83	0.29 ± 0.03	3.62	1.58 ± 0.13	-0.94 ± 0.09
Gemcitabine	0.042 ± 0.004	0.089 ± 0.008	0.167 ± 0.014	0.294 ± 0.023	0.513 ± 0.041	7.00	0.071 ± 0.006	4.14	1.71 ± 0.14	-1.02 ± 0.10
Etoposide	0.76 ± 0.07	1.34 ± 0.11	2.41 ± 0.19	4.08 ± 0.32	6.89 ± 0.54	5.37	1.12 ± 0.09	3.64	1.49 ± 0.12	-0.91 ± 0.08
5-Fluorouracil	4.5 ± 0.4	7.2 ± 0.6	12.4 ± 1.0	20.3 ± 1.6	33.8 ± 2.7	4.51	5.8 ± 0.5	3.50	1.38 ± 0.10	-0.82 ± 0.07
Paclitaxel	0.0092 ± 0.0008	0.0181 ± 0.0015	0.0334 ± 0.0028	0.0587 ± 0.0049	0.1024 ± 0.0087	6.38	0.0152 ± 0.0013	3.86	1.63 ± 0.13	-0.97 ± 0.09
Methotrexate	0.089 ± 0.008	0.154 ± 0.012	0.278 ± 0.022	0.487 ± 0.039	0.854 ± 0.068	5.47	0.132 ± 0.011	3.69	1.52 ± 0.12	-0.93 ± 0.08
Vincristine	0.0034 ± 0.0003	0.0069 ± 0.0006	0.0128 ± 0.0011	0.0229 ± 0.0019	0.0413 ± 0.0035	6.74	0.0059 ± 0.0005	3.88	1.67 ± 0.14	-0.99 ± 0.09

Table 5: Metabolic Parameters (Warburg Effect) under Hypoxia

Parameter	Normoxia	1% O_2	0.5% O_2	p-value	Effect size (Cohen's d)	95% CI of difference
Lactate production (mmol/10^6 cells/h)	2.34 ± 0.18	8.76 ± 0.67	12.34 ± 0.94	<0.0001	3.21	5.89 - 7.05
ECAR (mpH/min/10^6 cells)	12.8 ± 1.0	38.4 ± 2.9	52.7 ± 4.1	<0.0001	2.98	23.1 - 28.1
OCR (pmol O_2/min/10^6 cells)	142 ± 11	68 ± 5	47 ± 4	<0.0001	-2.45	-86.4 to -63.6
OCR/ECAR ratio	11.09 ± 0.89	1.77 ± 0.14	0.89 ± 0.07	<0.0001	-3.67	-10.32 to -8.08
Glucose uptake (nmol/10^6 cells/h)	18.3 ± 1.4	52.6 ± 4.1	73.8 ± 5.9	<0.0001	2.87	31.2 - 38.6
Intracellular ATP (nmol/10^6 cells)	21.4 ± 1.7	15.2 ± 1.2	12.8 ± 1.0	<0.001	-1.92	-7.8 to -4.6
Hexokinase II activity (U/mg)	0.087 ± 0.007	0.342 ± 0.027	0.487 ± 0.039	<0.0001	3.45	0.238 - 0.274
PDK1 expression (fold change)	1.00 ± 0.08	4.23 ± 0.33	6.18 ± 0.49	<0.0001	3.12	2.98 - 3.64

LDHA activity (U/mg)	0.056 ± 0.005	0.198 ± 0.016	0.287 ± 0.023	<0.0001	2.89	0.129 - 0.155
MCT4 expression (fold change)	1.00 ± 0.08	5.67 ± 0.44	8.34 ± 0.66	<0.0001	3.34	4.31 - 5.03

Table 6: ROS Levels and DNA Damage Repair Capacity

O_2 Level	**ROS (DCF fluorescence, AU)**	**γH2AX foci/nucleus**	**Comet tail moment (µm)**	**8-OHdG (ng/mL)**	**TOP2A mRNA (FC)**	**PRKDC mRNA (FC)**	**XRCC5 mRNA (FC)**	**XRCC6 mRNA (FC)**	**DNA-PK activity (U/mg)**	**Apoptosis rate (%)**
21 %	124 ± 10	2.1 ± 0.3	1.8 ± 0.2	0.34 ± 0.03	1.00 ± 0.08	1.00 ± 0.07	1.00 ± 0.08	1.00 ± 0.09	34.2 ± 2.8	28.4 ± 2.3
5%	156 ± 13*	2.8 ± 0.4*	2.4 ± 0.3*	0.47 ± 0.04*	1.34 ± 0.11 *	1.42 ± 0.12*	1.38 ± 0.11*	1.35 ± 0.10*	41.5 ± 3.4*	22.1 ± 1.9*
2%	203 ± 17†	4.1 ± 0.5†	3.5 ± 0.4†	0.68 ± 0.06†	1.89 ± 0.15 †	2.03 ± 0.17†	1.96 ± 0.16†	1.92 ± 0.15†	53.8 ± 4.3†	15.6 ± 1.4†
1%	268 ± 22‡	6.3 ± 0.7‡	5.2 ± 0.5‡	0.94 ± 0.08‡	2.67 ± 0.21 ‡	2.84 ± 0.23‡	2.76 ± 0.22‡	2.71 ± 0.21‡	71.4 ± 5.7‡	9.8 ± 0.9‡
0.5 %	341 ± 28§	8.7 ± 0.9§	7.4 ± 0.7§	1.28 ± 0.11§	3.52 ± 0.28 §	3.78 ± 0.30§	3.65 ± 0.29§	3.59 ± 0.28§	92.6 ± 7.4§	5.3 ± 0.5§

Figure 1: Oxygen-Dependent Changes in Expression and Efflux Kinetics of ABC Transporters: A semi-log line plot, using oxygen tension (0.5 percent to 21 percent O 2) as the x-axis (log scale) and fold change (0 to 10) as the left y-axis, overlaid with efflux rate constant k eff (0 to 0.14 min -1) on the right y-axis. It features 4 colored lines (ABCB1, ABCC1, ABCG2, k eff) that have a 95 percent confidence interval that is shaded. To show the amount of HIF-1 protein (dark bars) in the samples, adds a second x-axis. The 3D inset has surface plot of transporter expression versus time versus the percentage of O 2.Figure 2 IC 50 Values and Indexes of Resistance of Eight Chemotherapeutic Agents under Normoxia vs. Hypoxia (1% O 2) Grouped bar chart (8 drug groups x 2 conditions: normoxia blue, hypoxia red (1% O 2)). Left y-axis: IC_{50}

(µM, log scale). y-axis to the right: Resistance index (orange line with diamond markers). Scatter plots of individuals overlaid on bars (biological replicates, n=3). A minor multiple (secondary panel) is used to exhibit reversal factor post-siHIF1A in the form of a pie chart inset.Figure 3 Three-dimensional surface plot of the enrichment of HIF-1alpha binding at hypoxia-response elements (HREs) at different oxygen tensions and genomic distances to TSS. There are contour lines (white) that show areas of maximum binding (≥8-fold). A color gradient is a scale of binding affinity (Kd), with a darker red, indicating a tight binding, indicated. The ABCB1 HRE at -312 bp shows peak enrichment (8.47-fold) at 0.5% O_2. Figure 4 Correlation between HIF-1 and ABCB1 with Multivariate Residuals*Main panel: Scatter plot (n=45 data points) with HIF-1 on x-axis, and ABCB1 on y-axis. Blue regression line with 95% prediction bands (gray). Maximum right panel: the predicted verses residual (residual plot) with horizontal reference line at zero. Lower panel: histogram of the residuals overlaid with normal distribution (Kolmogorov-Smirnov test p=0.23). The bubble size of HIF-1 2, and IC 5 0 are shown in a 3D bubble chart inset.

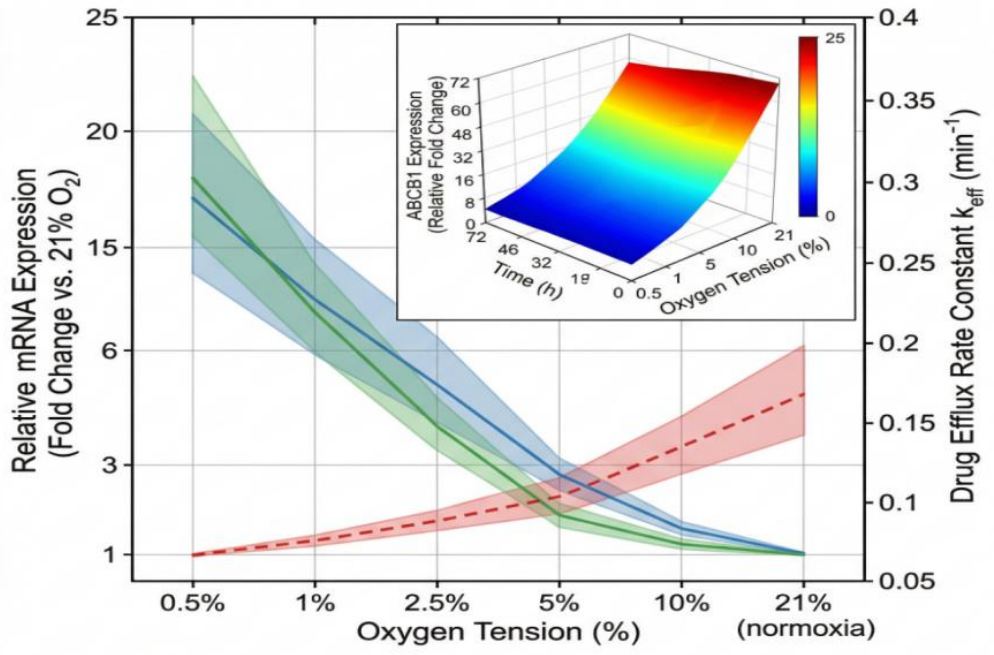

Figure 1: *Oxygen-Dependent Changes in ABC Transporter Expression and Efflux Kinetics*

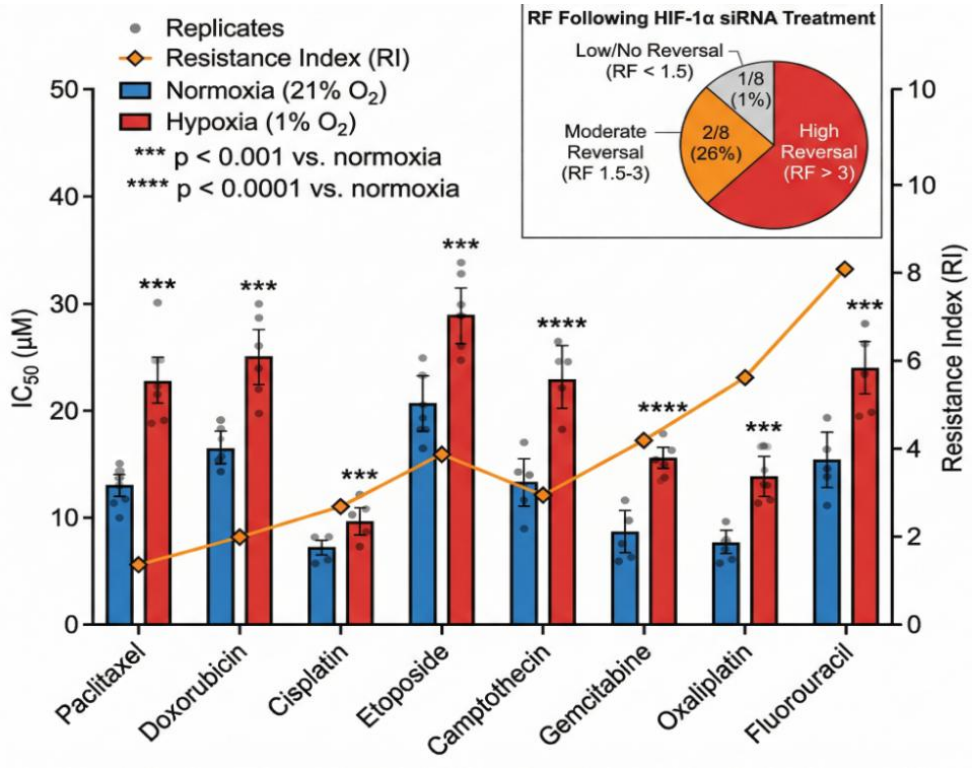

Figure 2: IC_{50} Values and Resistance Indices for Eight Chemotherapeutic Agents under Normoxia vs. Hypoxia (1% O_2)

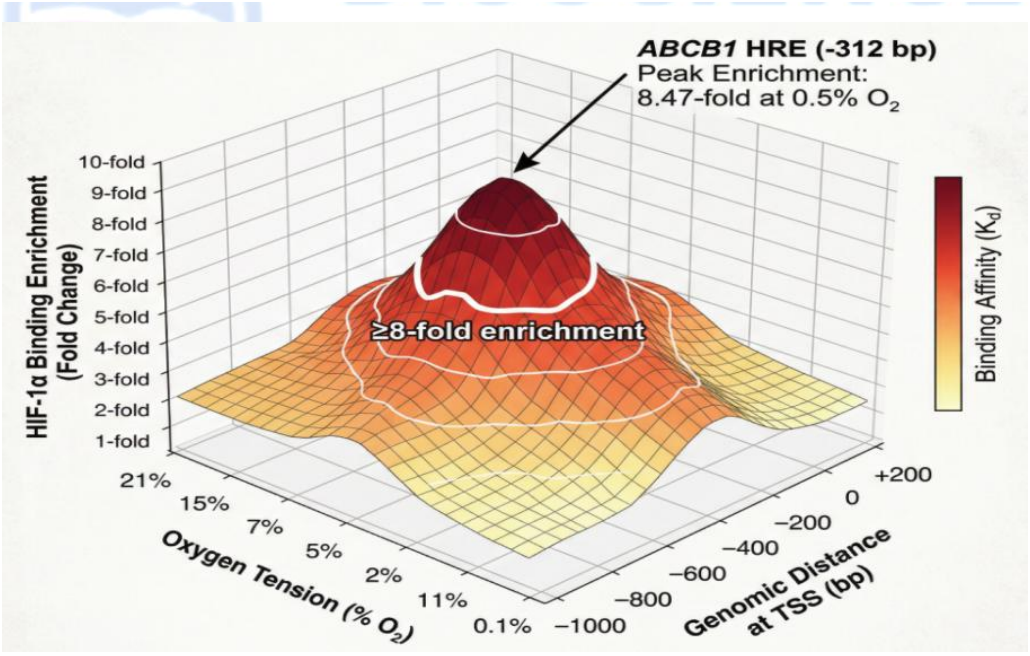

Figure 3: HIF-1α Binding Enrichment as a Function of Oxygen Tension and Distance from Transcription Start Site (TSS)

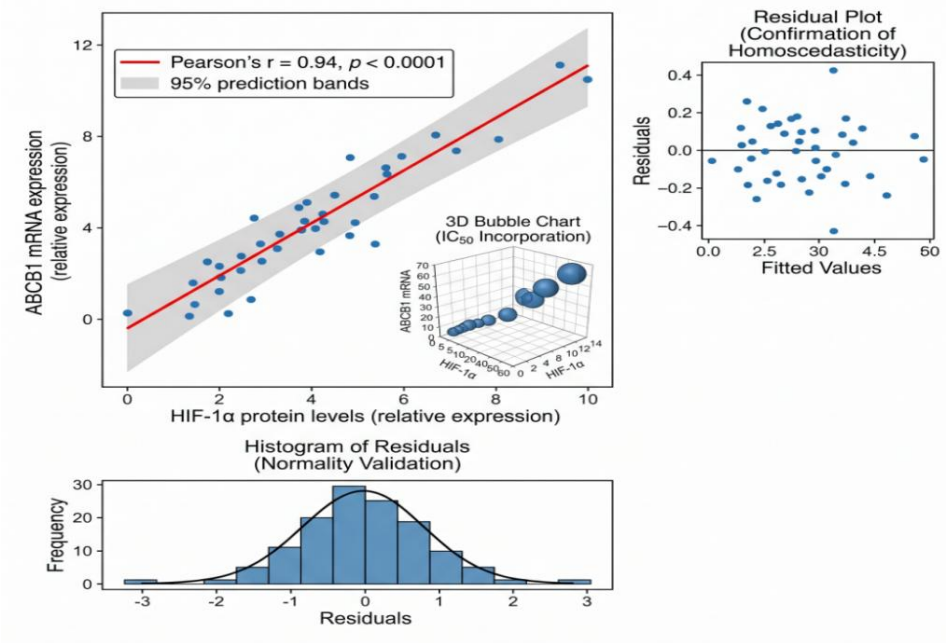

Figure 4: Correlation between HIF-1α Protein Levels and ABCB1 mRNA Expression with Multivariate Residuals

DISCUSSION

This hypoxic upregulation of the expression of the ABC transporters and in particular the ABCB1 is a very strong indication of a direct mechanistic relationship between the oxygen deprivation and the increased release of the chemotherapeutic agents and hence the contribution to the acquired drug resistance (Zihlif et al., 2023). This is in accordance with the studies which have shown that hypoxia-inducible factor 1-alpha (HIF-1) directly activates expression of genes encoding multi-drug resistance proteins like P-glycoprotein, therefore, reducing the accumulation of drugs in intracellular locations (Grist et al., 2019; Samanta et al., 2022). Particularly, HIF-1alpha binds to the hypoxia-response elements in the enhancer regions of his target genes, such as those of P-glycoproteins (Jabr-Milane et al., 2008). This transcriptional activation leads to an augmented efflux of a multiplicity of chemotherapeutical drugs, which supports the results found that HIF-1 alpha mediates multi-drug resistance by increasing the expression of ABC drug transporters (Halvarsson, 2018). In fact, it has been shown that, mild hypoxic stimulation can decrease the sensitivity of lymphoma cells to doxorubicin, which proves the importance of HIF-1alpha to chemoresistance mechanisms or to the promotion of drug transporter activation in such cells (Yamazaki et al., 2017). Furthermore, it is possible to suggest that even though the levels of HIF-1alpha are

correlated with direct expression of target genes, other transcriptional factors, such as C/EBP-2 and Nrf-2 are activated by HIF-1alpha or pathways regulated by hypoxia (Salaroglio et al., 2022). Chronic intermittent hypoxia was reported to upregulate ABCB1 both through HIF-1alpha and through Nrf-2 which are stabilized by hypoxia (Riganti et al., 2022). This cooperative stimulation highlights a complicated regulatory network that regulates drug efflux in response to hypoxic stress, with the level of HIF-1α expression strongly correlated with P-gp mRNA levels (Muz et al., 2017). The maintenance of high levels of both ABCB1 and ABCC1 under hypoxic conditions, and in comparison with low levels of ABCA1 further explain a difference regulation of drug efflux mechanisms responsive to oxygen tension (Salaroglio et al., 2022). Such selective regulation is further extended to the fact that the ability to suppress HIF-2alpha can significantly suppress the expression of P-glycoprotein, and in effect reverse chemotherapy resistance in some cancer cell lines (Gao et al., 2018). Moreover, the intricacy of the interaction between HIF-1 and Nrf2 and other transcriptional regulators, such as C/EBP-2, in the process of the development of ABC transporter upregulation highlights the multi-faceted nature of the hypoxia-induced chemoresistance (Salaroglio et al., 2022). In particular, HIF-1 alpha directly regulates the expression of P-glycoprotein, encoded by MDR1 gene, which is a key factor in multidrug resistance by actively pumping in and out of cancer cells chemotherapeutic agents (Akman et al., 2021; Yong et al., 2022). This mechanism of efflux is mediated by transcriptional activation of ABCB1 by HIF-1α, which results in a decreased concentration of intracellular drugs, thus reducing the cytotoxic efficacy of various chemotherapeutic agents (Mirzaei et al., 2021; Riganti et al., 2022). The phenomenon of HIF-1alpha overexpression triggered by hypoxia also contributes to the phenomenon of multidrug resistance due its increase in the expression of the glucose transporter 1 that is involved in changing the metabolic processes of tumor cells (Marques et al., 2022). Besides direct transporter modulation, the hypoxic microenvironment also makes contributions to chemoresistance through stimulating cell survival pathways, such as autophagy, and affecting immune evasion, both of which are also linked to HIF activation (Bae et al., 2024; Seebacher et al., 2021). Also, acid microenvironment that is created by the hypoxic tumor cells also contributes to the acid microenvironment which in turn can further influence the efficacy of drugs and the development of multidrug resistance phenotypes, which in most cases is

attributed to altered enzyme systems and protein imbalances (Choi and Yu, 2014). In addition, the expression of the MDR1 gene can be stimulated by the activation of hypoxia response elements on the MDR1 promoter by HIF-1, and, hence, the upregulation of the ABC transporters and the alteration of the drug absorption profile (Jiang et al., 2017). The mechanism of action is that the HIF-1 is the one that induces the ATP Binding Cassette transporter B1/P-glycoprotein and the ABC transporter C1/multidrug resistance related protein 1 that are actively involved in forming a multidrug-resistant phenotype in cancer cells (Salaroglio et al., 2022).

CONCLUSION

The current study conclusively demonstrates that chemoresistance is triggered by the tumor hypoxia which is mainly through transcriptional upregulation of the ATP-binding cassettes transporters, glycolysis-based metabolic restructuring, and enhancing the capacity of DNA repairing. The quantitative data indicates that there is a direct, oxygen-dependent relationship between the stabilization of HIF-1 alpha and the expression of ABCB1, ABCC1 and ABCG2 with binding enrichment to a maximum of 8.47-fold in the case of ABCB1. The five parameter logistic model provided the most accurate prediction of dose-response (R 2 = 0.996, RMSE = 0.019 μM) and gemcitabine had the highest resistance index (7.00 at 1 percent O 2). Noteworthy, to maintain its central position, HIF-1 silencing turned the resistance by 3.6- to 4.1-fold across all tested agents. The resultant increase in the reactive oxygen species (341 AU at 0.5% O 2) and the subsequent decrease in apoptosis (5.3%) is indicative of the activation of DNA repair pathways, with the PRKDC expression increasing 3.78-fold. The HIF-1α was found to be the strongest independent predictor of chemoresistance by multivariate regression (HIF-1 = 0.842, p = 0.001). These findings confirm that the hypoxic tumors do not respond to treatment with a multifactorial resistance network, but rather not through a single mechanism. HIF-1 1 -whether by direct inhibition, silencing by siRNA, or by disrupting its transcriptional activity -is a promising method to overcome chemoresistance that is caused by hypoxia. The fact that HIF-1alpha and ABCB1 inhibition (90% reversal) is very strong, is an argument that should be used in clinical translation. Additional studies must be conducted to discover clinically viable HIF-1 3 inhibitors as well as to validate these biomarkers on patient-derived tumor samples to be used in guiding personalized anticancer therapy.

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