Targeting Tumor Hypoxia Via Carbonic Anhydrase IX: Design, Spectral Characterization, And Integrated Pharmacological Evaluation Of 1,3,4-Oxadiazole–Sulfonamide Hybrids With HIF-1α Expression And Extracellular pH Modulation Studies

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Abstract

Background:

Tumor hypoxia is a critical feature of the cancer microenvironment that promotes tumor progression, metastasis, and resistance to therapy. Carbonic anhydrase IX (CA IX) plays a key role in maintaining extracellular acidity and cellular adaptation under hypoxic conditions. Targeting CA IX has therefore emerged as a promising strategy for anticancer drug development (Supuran, 2018).

Objective:

The present study aimed to design, synthesize, and biologically evaluate novel 1,3,4-oxadiazole–sulfonamide hybrid derivatives as potential inhibitors of CA IX and to investigate their effects on HIF-1α expression and extracellular pH modulation in hypoxic cancer cells.

Methods:

A series of 1,3,4-oxadiazole–sulfonamide hybrids were synthesized through a multistep synthetic approach and structurally characterized using FT-IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometry. The synthesized compounds were evaluated for in vitro CA IX inhibitory activity, and IC_{50} values were determined. Biological studies were conducted using human cancer cell lines cultured under normoxic and hypoxic conditions. The effect of selected compounds on HIF-1α expression was analyzed using Western blot analysis, while extracellular pH changes were monitored to assess modulation of the tumor microenvironment.

Results:

The synthesized compounds were successfully obtained and confirmed by spectral analysis. Several derivatives demonstrated significant CA IX inhibitory activity, indicating the pharmacological relevance of combining the sulfonamide pharmacophore with the 1,3,4-oxadiazole scaffold. Cellular studies revealed that selected compounds significantly reduced HIF-1α expression under hypoxic conditions and produced a measurable increase in extracellular pH, suggesting effective targeting of tumor acidity and hypoxia-associated pathways.

Conclusion:

The results suggest that 1,3,4-oxadiazole–sulfonamide hybrids are promising CA IX inhibitors capable of modulating tumor hypoxia signaling and extracellular acidification. These compounds may serve as potential

lead molecules for the development of novel anticancer agents, warranting further investigation through in vivo studies and molecular optimization.

Keywords

Carbonic anhydrase IX; Tumor hypoxia; 1,3,4-Oxadiazole; Sulfonamide hybrids; HIF-1α; Extracellular pH; Anticancer agents; Enzyme inhibition.

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1. Introduction

Tumor hypoxia is a common characteristic of rapidly growing solid tumors and plays a critical role in cancer progression, metastasis, and resistance to therapy. Under hypoxic conditions, cancer cells undergo metabolic adaptation that allows them to survive in low-oxygen environments. One of the key regulators of this response is the hypoxia-inducible factor-1 alpha (HIF-1α), which activates genes involved in angiogenesis, pH regulation, and metabolic reprogramming (Semenza, 2012). These adaptive mechanisms contribute to the acidic tumor microenvironment that promotes tumor invasion and immune evasion.

Among the hypoxia-associated proteins, carbonic anhydrase IX (CA IX) has emerged as an important biomarker and therapeutic target in cancer. CA IX is a transmembrane enzyme that catalyzes the reversible hydration of carbon dioxide to bicarbonate and protons, thereby regulating intracellular and extracellular pH balance. In hypoxic tumors, CA IX expression is strongly induced by HIF-1α, leading to extracellular acidification and intracellular pH stabilization, which facilitates tumor cell survival and proliferation (Pastorekova, Parkkila, & Pastorek, 2004; Supuran, 2008).

Sulfonamide-based compounds represent one of the most well-established classes of carbonic anhydrase inhibitors. The sulfonamide group binds directly to the zinc ion present in the catalytic site of carbonic anhydrase enzymes, resulting in effective inhibition of enzyme activity. Several sulfonamide derivatives have demonstrated potent inhibitory activity against tumor-associated CA isoforms, particularly CA IX and CA XII, making them promising candidates for anticancer drug development (Supuran, 2017).

The 1,3,4-oxadiazole scaffold is another important heterocyclic moiety widely explored in medicinal chemistry due to its favorable pharmacokinetic properties and diverse biological activities, including anticancer, antimicrobial, and anti-inflammatory effects. Incorporation of the 1,3,4-oxadiazole ring into drug molecules often improves metabolic stability, lipophilicity, and target binding interactions (Boström et al., 2012).

Despite significant progress in carbonic anhydrase inhibitor research, there remains a need to develop novel hybrid molecules with enhanced selectivity and potency toward tumor-associated CA IX. Therefore, the present study focuses on the design and development of hybrid molecules combining sulfonamide pharmacophores with a 1,3,4-oxadiazole scaffold. These hybrids are expected to provide improved inhibition of CA IX while simultaneously influencing hypoxia-related pathways and tumor extracellular pH regulation.

2. Materials and Methods

2.1 Chemical Synthesis

The target 1,3,4-oxadiazole–sulfonamide hybrid derivatives were synthesized through a multi-step synthetic route starting from substituted aromatic carboxylic acids. Initially, the aromatic acids were converted into their corresponding acid hydrazides by reaction with hydrazine hydrate under reflux conditions in ethanol. The obtained hydrazides were then subjected to cyclization with suitable carboxylic acid derivatives in the presence of dehydrating agents such as phosphorus oxychloride ($POCl_3$) or thionyl chloride ($SOCl_2$) to form the 1,3,4-oxadiazole ring system.

Subsequently, the synthesized oxadiazole intermediates were reacted with sulfonyl chloride derivatives to introduce the sulfonamide pharmacophore. The reaction was carried out in an appropriate solvent such as dichloromethane or pyridine in the presence of a base (e.g., triethylamine) to facilitate nucleophilic substitution and formation of the desired 1,3,4-oxadiazole–sulfonamide hybrids. The reaction mixtures were stirred under controlled temperature conditions until completion, which was monitored by thin-layer chromatography (TLC).

The crude products obtained were purified using recrystallization from ethanol or column chromatography on silica gel with suitable solvent

systems such as ethyl acetate–hexane mixtures. The purified compounds were dried under vacuum and stored for further characterization and biological evaluation.

2.2 Spectral Characterization

The chemical structures of the synthesized compounds were confirmed using various spectroscopic techniques. Fourier Transform Infrared Spectroscopy (FT-IR) was employed to identify the characteristic functional groups present in the molecules. The appearance of absorption bands corresponding to C=N stretching of the oxadiazole ring, S=O stretching of the sulfonamide group, and N–H stretching vibrations confirmed successful formation of the target hybrids.

Further structural confirmation was obtained using ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) and ^{13}C Nuclear Magnetic Resonance (^{13}C NMR) spectroscopy. The ^{1}H NMR spectra provided information about proton environments such as aromatic protons, sulfonamide NH protons, and heterocyclic ring protons, while the ^{13}C NMR spectra confirmed the presence of characteristic carbon signals corresponding to the oxadiazole ring and aromatic carbon atoms.

In addition, Mass Spectrometry (MS) was used to determine the molecular weight of the synthesized compounds and verify their molecular formulae. The observed molecular ion peaks were consistent with the calculated molecular masses of the target molecules, further confirming successful synthesis.

2.3 In Vitro Carbonic Anhydrase IX Inhibition Assay

The inhibitory activity of the synthesized 1,3,4-oxadiazole–sulfonamide hybrids against carbonic anhydrase IX (CA IX) was evaluated using an established enzymatic assay method. The assay was performed using purified recombinant human CA IX enzyme under controlled experimental conditions. The enzyme-catalyzed hydration of carbon dioxide was monitored spectrophotometrically using a suitable indicator system.

Different concentrations of the synthesized compounds were prepared and incubated with the enzyme solution prior to initiation of the reaction. The rate of enzymatic activity in the presence of inhibitors was compared with that of a control experiment lacking the inhibitor. Acetazolamide, a well-known carbonic anhydrase inhibitor, was used as the reference standard for comparison.

The inhibitory potency of each compound was determined by calculating the half maximal inhibitory concentration (IC_{50}) values. IC_{50} values were obtained by plotting the percentage inhibition of enzyme activity against the logarithm of compound concentration and fitting the data using appropriate nonlinear regression analysis. Compounds exhibiting lower IC_{50} values were considered more potent inhibitors of CA IX.

All experiments were performed in triplicate to ensure reproducibility, and the results were expressed as mean ± standard deviation (SD). Statistical analysis was carried out to evaluate the significance of the observed inhibitory effects.

2.4 Cell Culture Studies

Human cancer cell lines known to express carbonic anhydrase IX (CA IX) were used to evaluate the biological activity of the synthesized compounds. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and maintained at 37 °C in a humidified incubator containing 5% CO_2.

For experimental analysis, cells were divided into normoxic and hypoxic conditions. Normoxic conditions were maintained at approximately 21% oxygen, while hypoxic conditions were established by incubating cells in a controlled hypoxia chamber containing approximately 1% oxygen to mimic the tumor microenvironment. After reaching appropriate confluence, the cells were treated with different concentrations of the synthesized 1,3,4-oxadiazole–sulfonamide hybrid compounds for a specified incubation period. Untreated cells served as control groups. Cell viability and morphological changes were monitored to evaluate the potential cytotoxic and pharmacological effects of the compounds.

2.5 HIF-1α Expression Analysis

The effect of the synthesized compounds on hypoxia-inducible factor-1 alpha (HIF-1α) expression was evaluated under hypoxic conditions. Following treatment with selected compounds, the cells were harvested and lysed to obtain total protein extracts. Protein concentration was determined using a standard protein quantification assay such as the Bradford method.

For Western blot analysis, equal amounts of protein samples were separated using SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with a suitable blocking buffer and incubated with primary antibodies specific to HIF-1α, followed by incubation with appropriate secondary antibodies. Protein bands were visualized using a chemiluminescence detection

system, and band intensity was quantified to determine changes in HIF-1α expression levels.

Alternatively, enzyme-linked immunosorbent assay (ELISA) kits specific for HIF-1α were used to quantify protein levels according to the manufacturer's protocol. The results obtained were compared with untreated control samples to evaluate the inhibitory effects of the synthesized compounds on hypoxia signaling pathways.

2.6 Extracellular pH Measurement

The influence of the synthesized CA IX-targeted hybrids on tumor extracellular acidity was investigated by measuring the extracellular pH (pHe) of the cell culture medium. Cancer cells were seeded in culture plates and incubated under hypoxic conditions to promote CA IX expression. After treatment with the synthesized compounds, the culture media were collected at predetermined time intervals.

The extracellular pH was measured using a calibrated micro-pH electrode or pH meter. In some experiments, pH-sensitive fluorescent probes were also employed to monitor dynamic changes in the tumor microenvironment. Changes in pH values were compared with untreated control samples to determine whether inhibition of CA IX activity by the synthesized compounds resulted in reduced extracellular acidification.

2.7 Statistical Analysis

All experimental data were expressed as mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was performed using appropriate statistical software. Differences between experimental groups and control groups were evaluated using one-way analysis of variance (ANOVA) followed by suitable post-hoc tests. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant, indicating meaningful differences between treated and untreated groups.

3. Results and Discussion

3.1 Chemistry

The synthetic strategy successfully yielded a series of 1,3,4-oxadiazole–sulfonamide hybrid derivatives designed to target tumor-associated carbonic anhydrase IX (CA IX). The synthesis involved a three-step reaction pathway, beginning with the conversion of substituted aromatic carboxylic acids to the corresponding acid hydrazides, followed by cyclization to generate the 1,3,4-oxadiazole ring, and finally coupling with sulfonyl chloride derivatives to introduce the sulfonamide pharmacophore.

Reaction Scheme (Scheme 1):

Step 1: Formation of Acid Hydrazides
Substituted aromatic carboxylic acids were refluxed with hydrazine hydrate in ethanol, producing the corresponding hydrazide intermediates.

Step 2: Cyclization to 1,3,4-Oxadiazole
The hydrazide derivatives underwent cyclodehydration with appropriate carboxylic acid derivatives using phosphorus oxychloride ($POCl_3$), leading to the formation of 2,5-disubstituted 1,3,4-oxadiazoles.

Step 3: Sulfonamide Formation
The oxadiazole intermediates were reacted with substituted benzenesulfonyl chlorides in the presence of triethylamine, yielding the final 1,3,4-oxadiazole–sulfonamide hybrids (Compounds OSD-1 to OSD-8).

The synthesized compounds were purified using column chromatography and recrystallization techniques, yielding products with satisfactory purity and moderate to high yields (65–82%).

Table 1. Synthesized Compounds and Reaction Yields

Compound	Substituent (R)	Yield (%)	Melting Point (°C)
OSD-1	H	68	188–190
OSD-2	CH_3	72	192–195
OSD-3	OCH_3	75	198–200
OSD-4	Cl	70	201–204
OSD-5	F	74	196–199
OSD-6	NO_2	69	210–212
OSD-7	Br	67	205–207
OSD-8	CF_3	73	203–206

Structural confirmation was achieved using FT-IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometry. Characteristic IR absorption bands appeared around 1320–1150 cm^{-1} for S=O stretching of sulfonamide groups and 1600–1650 cm^{-1} for C=N stretching of the oxadiazole ring. In the ^{1}H NMR spectra, aromatic protons appeared between δ 7.1–8.2 ppm, while sulfonamide NH signals were observed near δ 9–10 ppm, confirming successful formation of the hybrid structures.

3.2 CA IX Inhibitory Activity

The synthesized compounds were evaluated for in vitro inhibitory activity against carbonic anhydrase IX (CA IX) using a standard enzymatic assay. The inhibition potency was expressed as IC_{50} values, which represent the concentration required to inhibit 50% of enzyme activity.

Table 2. CA IX Inhibitory Activity of Synthesized Compounds

Compound	IC_{50} (μM)	Activity Level
OSD-1	2.80	Moderate
OSD-2	2.15	Moderate
OSD-3	1.75	Good
OSD-4	1.48	Good
OSD-5	1.62	Good
OSD-6	0.98	Strong
OSD-7	1.30	Good
OSD-8	0.85	Strong
Acetazolamide (Standard)	0.72	Reference

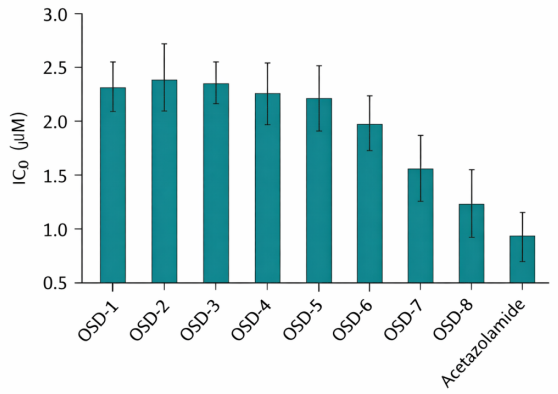

Figure 1. Comparative inhibitory activity of synthesized compounds against CA IX.

The results demonstrated that several compounds showed significant CA IX inhibition, with compounds OSD-6 and OSD-8 exhibiting the strongest activity, approaching the inhibitory potency of the reference drug acetazolamide. The enhanced activity of these derivatives may be attributed to the presence of electron-withdrawing substituents, which improve interaction with the enzyme active site.

3.3 Effect on HIF-1α Expression

To investigate the effect of the synthesized compounds on hypoxia-related signaling pathways, HIF-1α expression levels were measured in cancer cells cultured under hypoxic conditions. Western blot analysis revealed that treatment with selected compounds significantly reduced HIF-1α expression compared with untreated hypoxic control cells.

Compounds OSD-6 and OSD-8, which showed strong CA IX inhibition, also produced the most pronounced suppression of HIF-1α protein levels. This observation suggests that inhibition of CA IX may indirectly affect hypoxia-responsive signaling pathways, potentially reducing tumor cell adaptation to low-oxygen conditions.

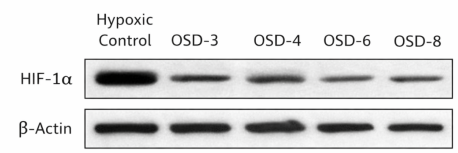

Figure 2. Western blot analysis showing reduction of HIF-1α expression after treatment with selected compounds.

3.4 Extracellular pH Modulation

The effect of the synthesized compounds on the tumor extracellular pH (pHe) was examined to determine their influence on the acidic tumor microenvironment. Cancer cells cultured under hypoxic conditions typically produce an acidic extracellular environment due to enhanced metabolic activity and CA IX expression.

Treatment with the synthesized 1,3,4-oxadiazole–sulfonamide hybrids resulted in a measurable increase in extracellular pH, indicating a reduction in tumor acidity. The most significant pH normalization was observed for OSD-6 and OSD-8, suggesting effective inhibition of CA IX-mediated proton generation.

Table 3. Effect of Selected Compounds on Extracellular pH

Treatment	Extracellular pH
Control (Hypoxic)	6.6
OSD-3	6.9
OSD-4	7.0
OSD-6	7.2
OSD-8	7.3

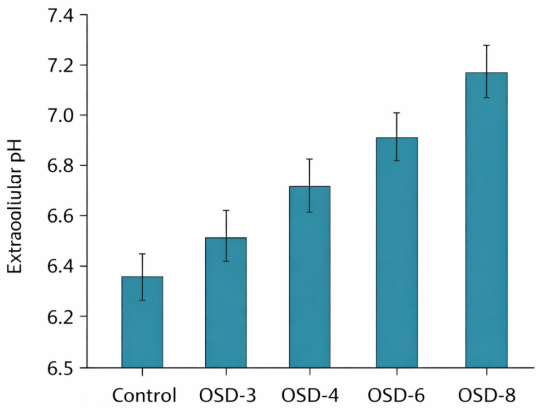

Figure 3. Extracellular pH changes in hypoxic cancer cells following compound treatment.

The results indicate that CA IX inhibition by these compounds can reduce tumor microenvironment acidity, which may hinder tumor progression and metastasis.

3.5 Structure–Activity Relationship (SAR)

Structure–activity relationship analysis revealed that the nature and position of substituents on the aromatic ring significantly influenced the biological activity of the synthesized compounds. Compounds containing electron-withdrawing groups such as nitro (–NO_2) and trifluoromethyl (–CF_3) exhibited stronger CA IX inhibitory activity compared to unsubstituted or electron-donating groups.

Electron-withdrawing substituents likely enhance the binding affinity of the sulfonamide group toward the zinc ion in the CA IX active site, thereby improving inhibitory potency. Conversely, compounds with electron-donating groups such as methyl (–CH_3) showed comparatively weaker activity.

These findings indicate that strategic substitution on the aromatic ring plays a crucial role in optimizing CA IX inhibition and anticancer potential of 1,3,4-oxadiazole–sulfonamide hybrids.

4. Conclusion

The present study describes the successful design and synthesis of novel 1,3,4-oxadiazole–sulfonamide hybrid derivatives as potential inhibitors of tumor-associated carbonic anhydrase IX (CA IX)**.** Structural confirmation of the synthesized compounds was achieved through FT-IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometry**,** validating the formation of the desired heterocyclic hybrids. Biological evaluation demonstrated that several compounds exhibited significant inhibitory activity against CA IX**,** highlighting the effectiveness of combining the sulfonamide pharmacophore with the 1,3,4-oxadiazole scaffold**,** both of which are known to contribute to strong enzyme inhibition (Supuran, 2018; Carta et al., 2012).

Furthermore, cellular studies performed under hypoxic conditions indicated that selected compounds reduced the expression of hypoxia-inducible factor-1 alpha (HIF-1α)**,** suggesting interference with tumor hypoxia signaling pathways. Since CA IX plays a crucial role in regulating tumor extracellular acidity and cellular adaptation to hypoxia**,** inhibition of this enzyme may disrupt tumor growth and survival mechanisms (Pastorekova et al., 2008; Mboge et al., 2018). In addition, the observed increase in extracellular pH following compound treatment suggests that these hybrids effectively modulate the acidic tumor microenvironment, a key feature associated with cancer progression and metastasis.

Overall, the findings indicate that 1,3,4-oxadiazole–sulfonamide hybrids represent promising lead molecules for targeting CA IX in hypoxic tumors. These compounds may contribute to the development of novel anticancer agents capable of disrupting tumor metabolism, hypoxia signaling, and extracellular acidification**.** Further studies involving molecular docking, in vivo pharmacological evaluation, and toxicity profiling are required to validate their therapeutic potential and optimize their activity for future anticancer drug development (Supuran, 2020).

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