Evaluation of Benzenesulfonamide Derivatives; Quinazoline and Sulfonamide Selectively Targeting Carbonic Anhydrase XII for Tumor Microenvironment Modulation in Human Breast Cancer Cells

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Abstract:

Tumor cells evolve adaptive mechanisms to function optimally within an acidic microenvironment. This adaptation is driven by the enhanced metabolic activity of tumors, which frequently results in acidosis and hypoxia. Carbonic anhydrase enzymes orchestrate cell homeostasis and affect cancer cell fate. Advances in structure-based design have enabled the identification of selective carbonic anhydrase XII inhibitors. The study aimed to evaluate the anti-proliferative activity of sulfonamide derivatives and elucidate the plausible mode of action. MTT assay was conducted to evaluate cytotoxicity of acetazolamide, parent C3 and its derivatives C4 and C6 against breast cancer cell lines MCF-7, MDA-MB-231 and prostate cancer cells PC3. Further studies for C3, C4 and C6 compounds were conducted against MCF-7. Enzyme-linked immunosorbent assay of carbonic anhydrases IX and XII and fluorescent intracellular pH assay were conducted. Investigated compounds and acetazolamide reduced proliferation of all tested cells after 72 h. C3, C4, and C6 compounds reduced carbonic anhydrase XII concentrations. C3 and C6 did not appear significant reduction in CAIX in MCF-7. Conversely, C4 displayed statistically significant increase. Cells exposed to C3, C4, and C6 revealed a decrease in intracellular pH levels. In conclusion, all three compounds exhibited antiproliferative activities characterized by modulation of tumor pHi/pHe.

Keywords: Benzenesulfonamide derivatives, Carbonic anhydrase, pH, Sulfonamide, Quinazoline

تقييم مشتقات بنزين سلفوناميد؛ الكينازولين والسلفوناميد يستهدفان بشكل انتقائي أنزيم الكربونيك أنهيدراز XII لتعديل بيئة الورم في خلايا سرطان الثدي لدى البشر

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الخلاصة

تطوّر الخلايا السرطانية آليات تكيفية تمكّنها من البقاء والعمل بكفاءة ضمن بيئة حمضية، وهي حالة ناجمة عادةً عن النشاط الأيضي المعزز للأورام، وما يترتب عليه من نقص في الأكسجة (Hypoxia) وتراكم الحموضة. وتُعد إنزيمات الكاربونيك أنهيدريز من العناصر المحورية في الحفاظ على توازن الخلية، كما تسهم في تحديد مصير الخلايا السرطانية. وقد أسهم التقدم في التصميم الدوائي المعتمد على البنية ثلاثية الأبعاد في التوصل إلى مثبطات انتقائية لإنزيم الكاربونيك أنهيدريز XII. هدفت هذه الدراسة إلى تقييم الفعالية المضادة للتكاثر لمشتقات السلفوناميد، واستقصاء آلية عملها المحتملة. تم إجراء اختبار MTT لتحديد السمية الخلوية لكل من الأسيتازولاميد، والمركب الأم C3، ومشتقاته C4 وC6، ضد خطوط خلايا سرطان الثدي MCF-7وMDA-MB-231وسرطان البروستاتPC3 . وركزت الدراسات التكميلية على المركبات C3 و C4وC6باستخدام خلايا MCF-7. تم استخدام اختبار الامتصاص المناعي المرتبط بالإنزيم (ELISA) لتقدير تركيز إنزيمي الكاربونيك أنهيدريز IX وXII، إضافةً إلى اختبار التألق لقياس درجة الحموضة داخل الخلية (pHi). أظهرت النتائج أن جميع المركبات المختبرة، بما في ذلك الأسيتازولاميد، قد أسهمت في تثبيط تكاثر الخلايا بشكل ملحوظ بعد 72 ساعة. كما لوحظ انخفاض في تركيز إنزيم CA XII بعد المعالجة بالمركبات الثلاثة، في حين لم يظهر المركبان C3 و C6تأثيرًا معنويًا على CA IX في خلاياMCF-7 ، بينما أظهر المركب C4 زيادة معنوية في تركيزه. من جهة أخرى، تسببت جميع المركبات في انخفاض ملحوظ في درجة الحموضة داخل الخلية .ختامًا، تشير النتائج إلى أن المركبات C3 و C4وC6تمتلك خصائص مضادة للتكاثر في الخلايا السرطانية، ويرجّح أن هذا التأثير يرتبط بتنظيم توازن الحموضة داخل وخارج الخلية الورمية.

الكلمات المفتاحية: مشتقات بنزين سلفوناميد، أنهيدراز الكربونيك، الرقم الهيدروجيني، سلفوناميد، كينازولين

1. Introduction

Cancer is a leading cause of death, with cases expected to reach 19.3 million by 2025. Due to resistance to single-agent therapies, research now focuses on combination treatments targeting specific cancer pathways (1)(2)(3). Targeted therapies offer promise in overcoming cancer drug resistance driven by tumor heterogeneity and a hostile microenvironment. Hypoxia and acidosis in tumor microenvironment promote glycolysis-dependent, treatment-resistant cancer cell survival and cancer progression. HIF-1/2 help cancer cells adapt by regulating metabolism, pH, and angiogenesis (4)(5)(6)(7)(5)(8). The bicarbonate buffer system is a fundamental cellular mechanism for maintaining pH homeostasis. Carbonic anhydrases (CAs; EC 4.2.1.1.) serving pivotal enzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and protons (9)(10). In oncology, carbonic anhydrases IX and XII are overexpressed under tumor hypoxia, helping regulate the acidic microenvironment that supports cancer progression. Their distinct expression and localization make them promising targets for therapies aimed at disrupting tumor growth (11)(12). Acetazolamide inhibits carbonic anhydrases but lacks isoform selectivity. Current research aimed to develop selective inhibitors to better target cancer-related CAs and improve treatment outcomes. (13). Carbonic anhydrase XII (CA XII) differs from CA IX in cancer regulation; unlike CA IX, its expression in breast tumors is less dependent on HIF-1 and shows weaker induction under hypoxia (14). CA XII is notably upregulated in several cancers, especially estrogen receptor-positive breast carcinomas, where its expression is driven by estrogen rather than hypoxia. It is also found in gastric, ovarian, lung, and brain cancers, but remains low in most normal tissues (15)(16). CA XII promotes tumor growth, invasion, and drug resistance by regulating pH and P-glycoprotein. Its silencing enhances chemosensitivity in resistant cells (17)(18). Elevated CA XII levels in breast cancer cells, including paclitaxel-resistant variants, suggest its

involvement in cancer development and therapy resistance (19). CA XII is also linked to epithelial-mesenchymal transition (EMT) and interacts with chloride/bicarbonate exchangers like AE2, further promoting cancer progression and drug resistance (20). Quinazolines and quinazolinones are recognized for their diverse pharmacological activities, including anti-inflammatory and anticancer effects, with some derivatives approved as drugs for conditions such as cancer and benign prostatic hyperplasia (21)(22). Lapatinib has been shown to be efficacious in combination therapy for breast cancer (23). Sulfonamides also hold promise due to their wide-ranging biological properties, notably in cancer treatment, where they inhibit carbonic anhydrase and disrupt the cell cycle (24)(25). Recent studies have synthesized hybrid sulfonamido-quinazoline compounds, with certain analogs showing cytotoxicity against cancer cell lines like MDA-MB-231 (26). Researchers at the College of Pharmacy, Mustansiriyah University, synthesized a series of compounds derived from 4-(2-mercapto-4-oxoquinazolin-3(4H)-yl) benzene sulfonamide 3 and investigated their toxic effects on the AMJ-13 Iraqi breast cancer cell line (27). Our study focused on investigating the molecular pharmacological pathways in MCF-7 human breast cancer of three of these chemical compounds illustrated in figure (1); the parent C3 and two derivatives C4 and C6 chemical compounds.

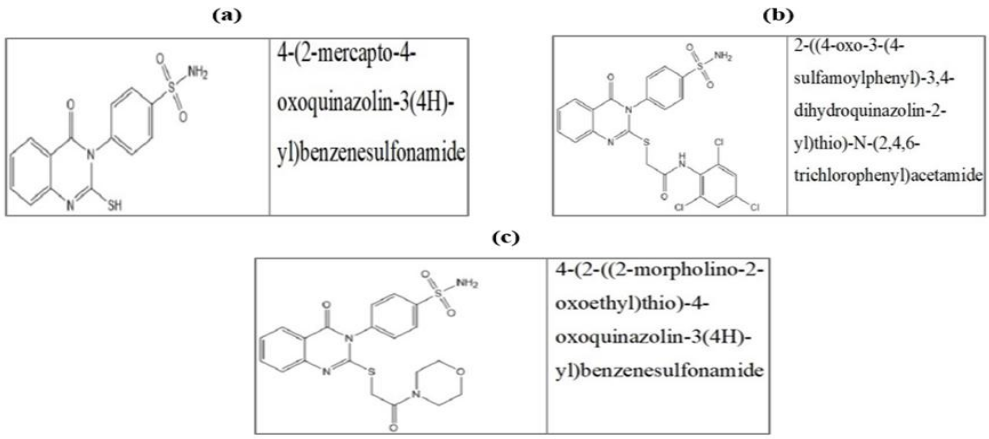

Figure (1) Illustrates (a) parent compound C3 (b) derivative compound C4 and (c) derivative compound C6 (27)

2. Materials and Methods

2.1. Cell Culture Preparation

The cell lines MCF-7, MDA-MB-231 and PC3 were purchased from (ATCC, USA) and cultured in MEM (Minimum Essential Medium) (US Biological, USA), DMEM (Dulbecco's Modified Eagle Medium) (Capricorn), Roswell Park Memorial Institute 1640 (RPMI 1640) medium enriched with Fetal bovine serum (FBS) at 10% (v/v) and supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin, both sourced from Capricorn Scientific, Germany. The cultures were maintained at 37 °C in a 5% CO_2, 95% humidified atmosphere. Cells in the exponential growth phase were selected for subsequent experiments (28).

2.2. Chemical Compounds

Sulfonamide derivatives C4, and C6 were synthesized from the parent C3 and previously detailed in the literature (27), with

their structures displayed in Figure (1-1). Dimethyl sulfoxide (DMSO) was used to prepare 50 mM stock solutions of the compounds C3, C4, C6, and the reference acetazolamide to conduct in vitro assays. All solutions were stored at −20 °C. To achieve the target concentrations, the media used for dilution included DMEM (Gibco) or RPMI 1640. The preparations, retained at −20 °C.

2.3. Cell Proliferation and Viability Assay

Cytotoxicity of C3, C4, C6, and acetazolamide was assessed on MCF-7, MDA-MB-231, and PC3 cancer cell lines using the MTT assay. Cells (5×10^4 cells/mL) were seeded in 96-well plates and treated with varying concentrations (12.5–400 µM) for 72 h. Post-treatment, MTT reagent (10 µL of 5 mg/mL) was added and incubated for 4 h. Formazan crystals were dissolved in 100 µL DMSO, and absorbance was measured at 560 nm. IC_{50} values were calculated using GraphPad Prism 10.01. All tests were done in triplicate (29)(30).

2.4. Bradford Protein Colorimetric Assay

Total protein content was measured using the Bradford assay for normalization. The chromogenic reagent was prepared by mixing 50 µL of stock with 200 µL of distilled water per well. A 1 mg/mL standard solution was diluted with saline to prepare concentrations ranging from 0 to 0.6 mg/mL. Fresh standard dilutions were prepared for each run. MCF-7 cells (1×10^6) were harvested, washed, homogenized, and centrifuged at 10,000×g for 10 minutes at 4 °C. The supernatant was collected, and absorbance was measured at 595 nm using a microplate reader. Figure (2) illustrates the standard curve.

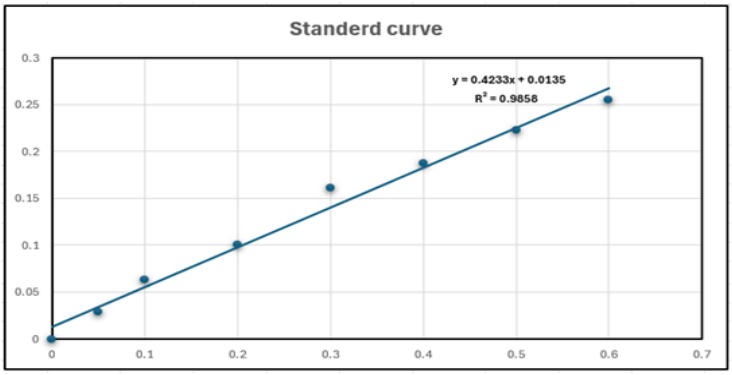

Figure (2) Bradford standard curve

2.5. Enzyme-Linked Immunosorbent Assay for Carbonic Anhydrase XII and IX Detection

The human CA12 (RayBio®.US) and human CA9 (Elabscience®. US) ELISA assay was performed as follow; Cells were washed with cold PBS, trypsinized, and centrifuged at 1000×g for 5 min. After three PBS washes, 1×10^6 cells were suspended in 200 µL cold PBS and lysed by repeated freeze-thaw cycles. Lysates were centrifuged at 1500×g for 10 min at 2–8 °C, and supernatants were collected. For ELISA, 100 µL of standards and samples were added to wells and incubated at 37 °C for 90 min. After adding biotinylated antibody and HRP conjugate with washing steps, substrate was added, and absorbance was measured at 450 nm (31)(32). The standard curve of CAIX is illustrated in figure (3)

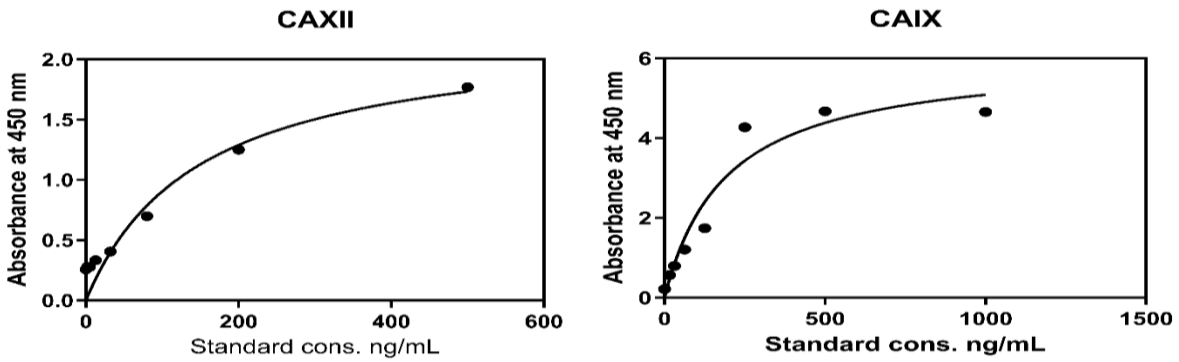

Figure (3) ELISA standard curve of CAXII and CAIX for MCF-7 cell lines

2.6. Fluorometric Assay for Measuring Intracellular pH

Intracellular pH was measured using the fluorometric Intracellular pH Assay Kit (MAK-150, Sigma Aldrich) per the manufacturer's instructions. MCF-7 cells (4×10^4/well) were seeded in black 96-well plates and incubated for 24 h. Cells were then loaded with BCFL-AM dye in HBS buffer and incubated at 37 °C for 30 min in the dark. Test compounds (C3, C4, C6 at IC_{50}) were added, and fluorescence was measured within 5 minutes (excitation: 490 nm, emission: 535 nm) to evaluate pH changes (33).

2.7. Data Analysis and Statistics

Data from the in vitro experiments were reported as the mean with standard deviation (mean ± SD). For statistical analysis, one way and two-way ANOVA to examine variations among groups test. All analyses were executed using GraphPad Prism software, version 10, on Windows. Values of $p < 0.05$ were considered statistically significant (* $p < 0.05$; ** $p < 0.001$; *** $p = 0.0001$; **** $p < 0.0001$).

3. Results

3.1. Evaluation of Antiproliferative Activity of Acetazolamide and Chemicals (C3, C4, and C6)

The in vitro cytotoxicity of C3, C4, C6, and the reference inhibitor acetazolamide (ACZ) was assessed using the MTT assay in MCF-7, MDA-MB-231, and PC3 cancer cell lines. All compounds showed dose-dependent cytotoxic effects after 72 hours of treatment. In MCF-7 cells, C4 exhibited the highest potency (IC_{50} = 104.7 µg/mL), followed by C3 (190.7 µg/mL), C6 (193.8 µg/mL), and ACZ (203.9 µg/mL). In MDA-MB-231, C4 showed the strongest effect (60.76 µg/mL), followed by C3 (94.19 µg/mL), C6 (106.7 µg/mL), and ACZ (120.5 µg/mL). In PC3 cells, C4 had the lowest IC_{50} (73.8 µg/mL), indicating the highest potency, while ACZ, C3, and C6 had IC_{50} values of 108.9, 108.1, and 129.8 µg/mL, respectively. Across all cell lines, reduced optical density with increasing compound concentration indicated decreased cell viability. See figures (4)(5)(6)

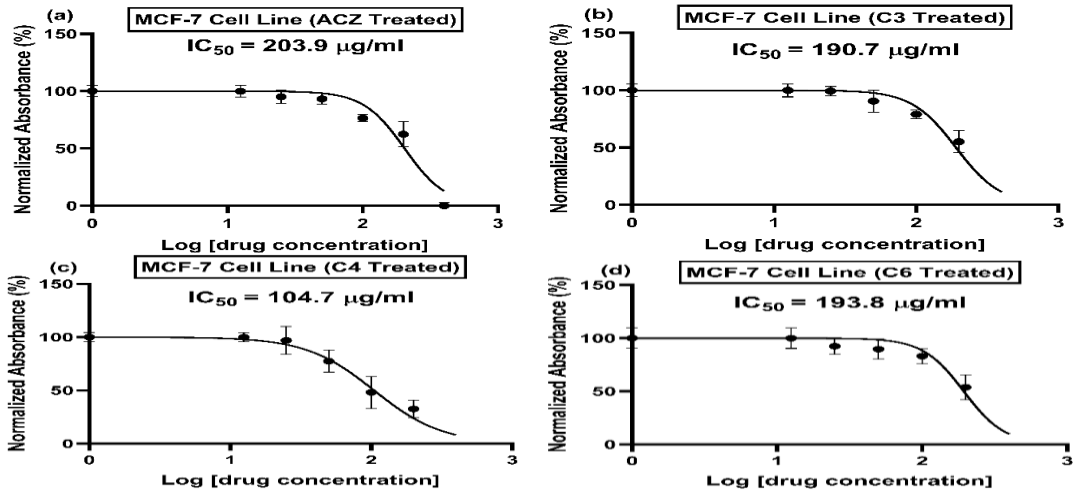

Figure (4) Dose-response curves determined the IC_{50} of (a)ACZ (b)C3 (c)C4, and (d)C6 on MCF-7 cells after 72 h (MTT assay). The x-axis shows log concentration (µg); the y-axis, normalized absorbance (%). IC_{50} represents the log dose reducing cell viability by 50%. Analysis used GraphPad Prism 10.

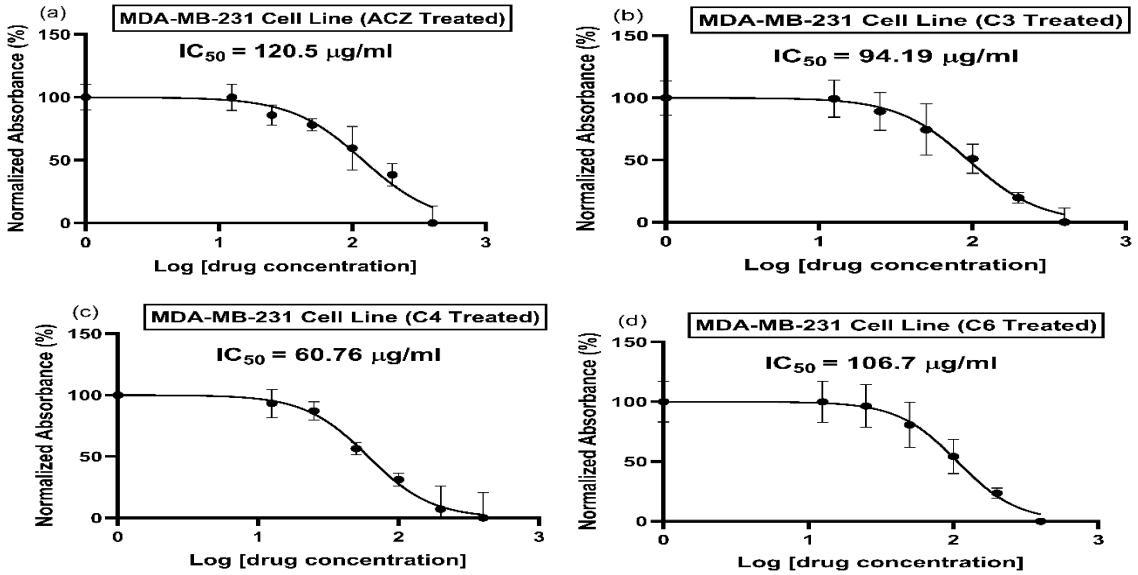

Figure (5) Dose-response curves determined the IC_{50} of (a)ACZ (b)C3 (c)C4, and (d)C6 on MDA-MB-231 cells after 72 h (MTT assay). The x-axis shows log concentration (µg); the y-axis, normalized absorbance (%). IC_{50} represents the log dose reducing cell viability by 50%. Analysis used GraphPad Prism 10.

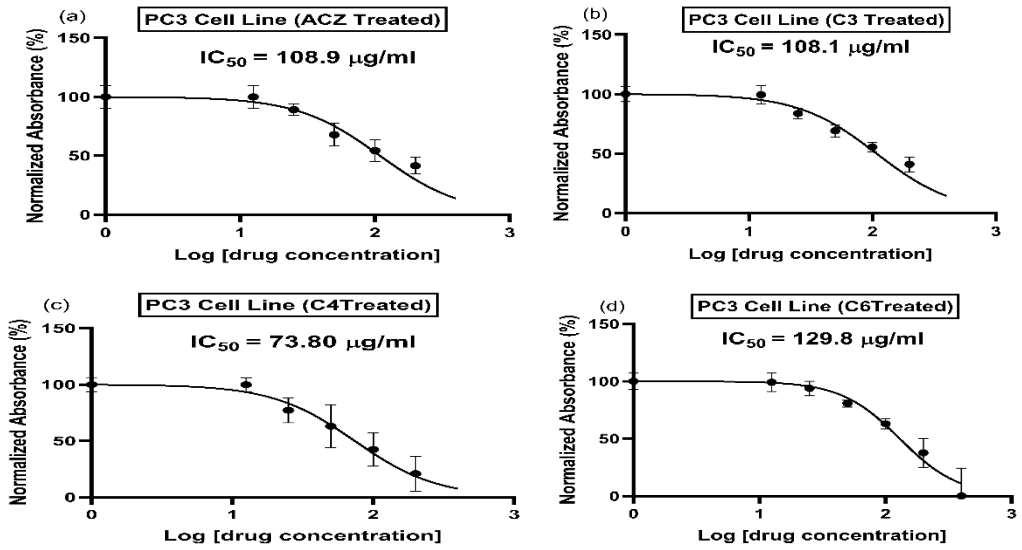

Figure (6) Dose-response curves determined the IC_{50} of (a)ACZ (b)C3 (c)C4, and (d)C6 on PC3 cells after 72 h (MTT assay). The x-axis shows log concentration (µg); the y-axis, normalized absorbance (%). IC_{50} represents the log dose reducing cell viability by 50%. Analysis used GraphPad Prism 10.

3.2. Quantification of Human Carbonic Anhydrase XII and IX Concentration Through Enzyme-Linked Immunosorbent Assay

Figure (7) illustrates the concentrations of carbonic anhydrase XII (CAXII) and IX (CAIX) in MCF-7 cells under different treatments. In (a), CAXII levels were significantly reduced in the C3, C4, and C6 groups compared to the control. The reductions in C3 and C6 were highly significant ($p = 0.0003$ and $p = 0.0009$, respectively), while C4 also showed a significant decrease ($p < 0.001$). In contrast, (b) shows that CAIX levels remained similar in the control, C3, and C6 groups, with a significant increase observed only in C4-treated cells. See tables (1) and (2)

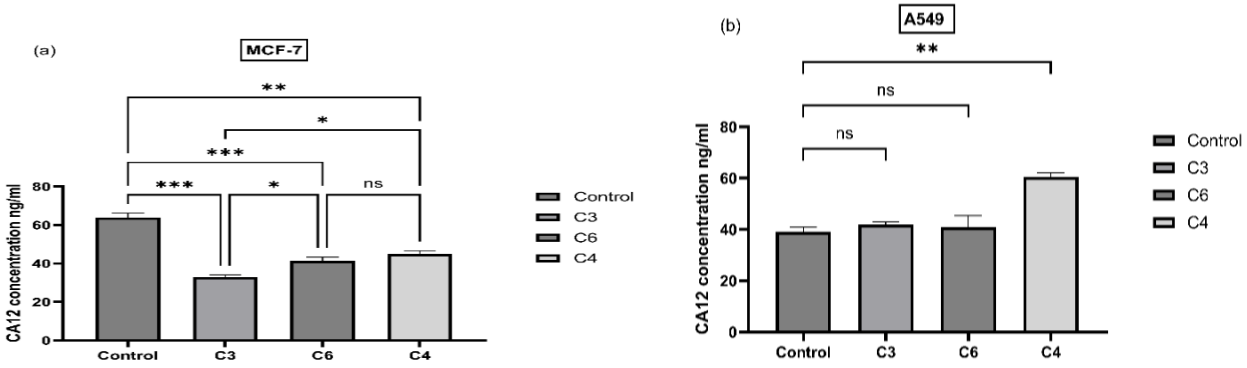

Figure (7) depicts CAs level in MCF-7 cancer cells determined by ELISA assay (a) CAXII (b) CAIX. Results were shown as mean ± SD. ANOVA with post-hoc tests (GraphPad Prism 10) was used, with significance at *p < 0.05; **p < 0.001; ***p = 0.0001; ****p < 0.0001.

Table (1) Tukey's multiple comparisons test of CAXII in MCF-7.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
Control vs. C3	30.91	23.31 to 38.51	Yes	***	0.0003
Control vs. C6	22.54	14.94 to 30.14	Yes	***	0.0009
Control vs. C4	18.91	11.31 to 26.51	Yes	**	0.0019
C3 vs. C6	-8.37	-15.97 to -0.7690	Yes	*	0.0365
C3 vs. C4	-12	-19.60 to -4.399	Yes	*	0.0103
C6 vs. C4	-3.63	-11.23 to 3.971	No	ns	0.3397

Data were expressed as mean ± SD. Statistical analysis used one-way ANOVA with post-hoc tests via GraphPad Prism 10 (Windows). Significance was set at *p < 0.05; **p < 0.001; ***p = 0.0001; ****p < 0.0001.

Table (2) Tukey's multiple comparisons test of CAIX in MCF-7.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
Control vs. 3	-0.9015	-4.031 to 2.228	No	ns	0.6718
Control vs. 6	-1.694	-4.823 to 1.435	No	ns	0.2645
Control vs. 4	-5.936	-9.065 to -2.806	Yes	**	0.0052
3 vs. 6	-0.7924	-3.922 to 2.337	No	ns	0.7433
3 vs. 4	-5.034	-8.163 to -1.905	Yes	**	0.0097
6 vs. 4	-4.242	-7.371 to -1.113	Yes	*	0.0179

Data were expressed as mean ± SD. Statistical analysis used one-way ANOVA with post-hoc tests via GraphPad Prism 10 (Windows). Significance was set at *p < 0.05; **p < 0.001; ***p = 0.0001; ****p < 0.0001.

3.3. The Impacts of Compounds C3, C4, And C6 on Intracellular pH Balance

Figure 8 shows the effects of C3, C4, and C6 on intracellular pH in MCF-7 cells, measured by fluorescence intensity. All three compounds significantly reduced intracellular pH compared to the control. C3 induced the greatest reduction ($p < 0.0001$), followed by C4 ($p < 0.006$) and C6 ($p < 0.03$). The difference between C4 and C6 was not statistically significant, indicating similar effects on intracellular pH. See table (3)

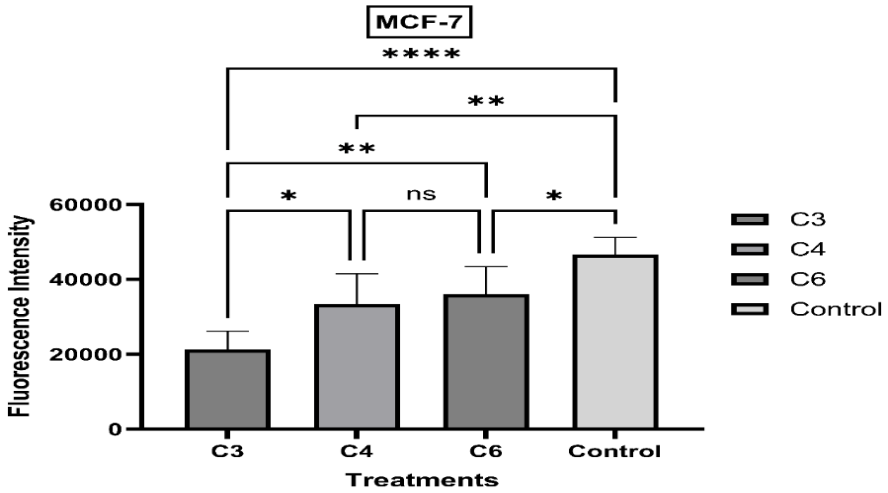

Figure (8) Intracellular pH assay results in MCF-7 cells were shown as mean ± SD. ANOVA with post-hoc tests was used (GraphPad Prism 10), with significance at *p < 0.05; **p < 0.001; ***p = 0.0001; ****p < 0.0001.

Table (3): Tukey's multiple comparisons test of pH assay for MCF-7.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
C3 vs. C4	-12134	-23103 to -1164	Yes	*	0.027
C3 vs. C6	-14713	-25682 to -3743	Yes	**	0.0065
C3 vs. Control	-25385	-35273 to -15497	Yes	****	<0.0001
C4 vs. C6	-2579	-13548 to 8391	No	ns	0.9103
C4 vs. Control	-13251	-23139 to -3364	Yes	**	0.0065
C6 vs. Control	-10672	-20560 to -784.8	Yes	*	0.0316

Data were expressed as mean ± SD. Statistical analysis used one-way ANOVA with post-hoc tests via GraphPad Prism 10 (Windows). Significance was set at *p < 0.05; **p < 0.001; ***p = 0.0001; ****p < 0.0001.

4. Discussion

The differential effects of compounds C3, C4, and C6 on CAXII and CAIX across the breast cancer cell suggest that these inhibitors may exert their effects through varied mechanisms depending on the cellular context. This selective inhibition of CAXII, particularly in the estrogen receptor-positive MCF-7 cell line, points to a potential therapeutic advantage in targeting carbonic anhydrase pathways in specific cancer types. The study underscores the complexity of carbonic anhydrase regulation in cancer cells, reflecting both cell-specific factors and the interplay between different carbonic anhydrase isoforms (15)(16). The observed upregulation of CAIX levels in C4-treated cells could be explained by the potential cross-talk between CAXII and CAIX, where inhibition of one carbonic anhydrase isoform leads to the compensatory upregulation of another (34)(35)(36). The findings from the intracellular pH assay showed that compounds C3, C4, and C6 were effective in reducing intracellular pH in MCF-7 cancer cells, suggests a direct link to the inhibition of carbonic anhydrase XII (CAXII). C3, which showed the strongest inhibitory effect on CAXII, was also the most effective in

reducing intracellular pH, aligning with its potential for significant impact on cancer cell metabolism. C4 and C6 also inhibited CAXII and correspondingly reduced intracellular pH, supporting the idea that targeting CAXII can alter intracellular environments critical for cancer cell survival (37). By inhibiting CAXII, the intracellular pH may become more acidic, which can potentially enhance the efficiency of oxidative phosphorylation and improve mitochondrial function. Moreover, in MCF-7 cells, estrogen signaling can be influenced by changes in cellular pH. CAXII inhibition could enhance estrogen receptor signaling, leading to a more robust different response (38).

5. Conclusions

The investigated compounds manifested noteworthy antiproliferative activities concomitant with restoration of extracellular and intracellular pH (pHe/pHi) to baseline values. All three compounds effectively reduced CAXII. The lack of impact on CAIX levels indicates specificity for CAXII inhibition in MCF-7 cells. The acidification, could be leveraged in combination with other therapies for more effective cancer treatment.

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Conflict of Interests

Conflicts of interest do not exist.

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