Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/7kq64c36

POTENT INDUCTION OF APOPTOSIS IN MCF-7 ER-POSITIVE BREAST CANCER CELLS BY CID-6861424 THROUGH INHIBITION OF AKT PROLIFERATION AND ERK SURVIVAL PATHWAYS

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Abstract

Estrogen receptor-positive (ER-positive) breast cancer represents a significant subset of breast cancer cases, often characterized by resistance to conventional therapies. Targeting specific molecular pathways involved in cell proliferation and survival has emerged as a promising approach to overcoming this resistance. In this study, we explore the therapeutic potential of CID-6861424, a small molecule inhibitor, in inducing apoptosis in MCF-7 ER-positive breast cancer cells. Our research demonstrates that CID-6861424 exerts a potent anti-proliferative effect, primarily through the inhibition of the Akt and ERK signaling pathways, which are crucial for cell survival and proliferation in cancer cells.Upon treatment with CID-6861424, a significant reduction in cell viability was observed, accompanied by marked downregulation of phosphorylated Akt and ERK, indicating effective pathway inhibition. Flow cytometry analysis revealed an increase in apoptotic cell populations, which was further confirmed by the upregulation of apoptotic markers such as cleaved caspase-3 and PARP, along with the downregulation of the anti-apoptotic protein Bcl-2. These findings suggest that the induction of apoptosis by CID-6861424 is mediated through the disruption of the Akt and ERK pathways, leading to the activation of pro-apoptotic signals and inhibition of cell survival mechanisms. The results of this study highlight the potential of CID-6861424 as a novel therapeutic agent for ER-positive breast cancer. Its ability to target key signaling pathways involved in cancer cell survival positions it as a promising candidate for further development and clinical evaluation.

1. Introduction

Breast cancer is the most frequently diagnosed cancer and a leading cause of cancer-related mortality among women worldwide (Bartucci, Morelli, Mauro, Ando', & Surmacz, 2001). It encompasses a variety of subtypes with distinct molecular characteristics and responses to treatment, the most prevalent of which is estrogen receptor-positive (ER-positive) breast cancer. Approximately 70% of all breast cancer cases are classified as ER-positive, making the estrogen receptor (ER) a critical therapeutic target (Comşa, Cimpean, & Raica, 2015). While the advent of hormone therapies such as tamoxifen and aromatase inhibitors has significantly improved outcomes for patients with ER-positive breast cancer, resistance to these therapies remains a substantial clinical challenge. A deeper understanding of the molecular mechanisms driving tumor growth and survival is essential for developing new therapeutic strategies capable of overcoming this resistance (Martin et al., 2003). One of the key signaling pathways implicated in breast cancer proliferation and survival is the phosphoinositide 3-kinase (PI3K)/Akt pathway. This pathway is often aberrantly activated in cancer, promoting tumor growth, survival, and resistance to therapies (Levenson & Jordan, 1997). Akt, also known as protein kinase B, plays a central role in this pathway, acting as a mediator of various cellular processes, including metabolism, proliferation, and survival. When activated, Akt phosphorylates a number of downstream targets that enhance cell survival and prevent apoptosis, thereby contributing to cancer progression (Matou-Nasri et al., 2017).

The hyperactivation of Akt in breast cancer has been associated with poor prognosis and resistance to endocrine therapies, making it an attractive target for therapeutic intervention (Nizamutdinova et al., 2008). Another critical pathway involved in the survival of breast cancer cells is the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway. This pathway is responsible for regulating cell division, differentiation, and survival, and its dysregulation has been linked to various cancers, including breast cancer (Planas-Silva & Waltz, 2007). In particular, the ERK signaling pathway is often activated in response to growth factors and other stimuli, promoting cell proliferation and survival. Like the PI3K/Akt pathway, the ERK pathway plays a significant role in mediating resistance to cancer therapies, including hormonal treatments for ER-positive breast cancer (Planas-Silva & Waltz, 2007). Given the importance of these signaling pathways in breast cancer progression and therapy resistance, researchers have focused on identifying small molecules that can selectively inhibit Akt and ERK signaling (Leung, Kim, Askarian-Amiri, Finlay, & Baguley, 2014). In recent years, compound libraries have been screened to discover novel inhibitors capable of inducing apoptosis in breast cancer cells by targeting these pathways.

One such compound that has emerged as a promising candidate is CID-6861424, a small molecule identified through high-throughput screening efforts (Noguchi et al., 1990). CID-6861424 has shown potent activity in inducing apoptosis in ER-positive breast cancer cells by inhibiting the Akt and ERK pathways, thereby impeding tumor cell proliferation and survival (Hung, 2004). The identification of CID-6861424 represents a potential breakthrough in the treatment of ER-positive breast cancer, particularly for patients who have developed resistance to conventional hormone therapies (Zheng, Kallio, & Harkonen, 2007). By inhibiting both Akt and ERK signaling, CID-6861424 offers a dual mechanism of action that disrupts critical survival pathways in cancer cells, making it a compelling candidate for further preclinical and clinical investigation (Chan, Martin, Johnston, Ali, & Dowsett, 2002; Johnson, Ochieng, & Evans, 1996; Stoica et al., 1997). In this study, we explore the effects of CID-6861424 on MCF-7 cells, a widely used model of ER-positive breast cancer. MCF-7 cells are characterized by their reliance on estrogen signaling for growth and survival, making them an ideal model to investigate the efficacy of compounds targeting estrogen-driven cancer. We examine the ability of CID-6861424 to induce apoptosis in these cells, with a particular focus on its inhibitory effects on the Akt and ERK signaling pathways. By elucidating the molecular mechanisms through which CID-6861424 exerts its pro-apoptotic effects, we aim to provide insights into its potential as a therapeutic agent for ER-positive breast cancer. Additionally, our findings may contribute to the broader understanding of how targeting multiple survival pathways in cancer cells can overcome

resistance to current treatments, offering new avenues for the development of more effective therapies for breast cancer patients.

2. Materials and Methods

2. 1. Cell Culture

MCF-7 cells, a well-characterized ER-positive breast cancer cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 1% penicillin-streptomycin (Gibco), and 1% L-glutamine (Gibco). The cells were maintained at 37°C in a humidified incubator with 5% CO_2. Culture medium was changed every 2–3 days, and cells were sub-cultured upon reaching approximately 80% confluency using 0.05% trypsin-EDTA (Gibco).

2.2. Reagents and Chemicals

CID-6861424 was purchased from Sigma-Aldrich (St. Louis, MO). A 10 mM stock solution of the compound was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at -20°C. For all experiments, the final concentration of DMSO in cell cultures was maintained below 0.1%. Antibodies specific to phosphorylated Akt (Ser473), total Akt, phosphorylated ERK1/2 (Thr202/Tyr204), total ERK1/2, cleaved PARP, and β-actin were purchased from Cell Signaling Technology (Danvers, MA). The MTT assay reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was obtained from Sigma-Aldrich.

2.3. Cell Viability Assay (MTT Assay)

The effects of CID-6861424 on MCF-7 cell viability were determined using the MTT assay. MCF-7 cells were seeded in 96-well plates at a density of 5×10^4 cells per well and allowed to adhere overnight. Cells were treated with varying concentrations of CID-6861424 (0.1, 1, 10, and 50 μM) or DMSO (control) for 24, 48, and 72 hours. At each time point, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours at 37°C. After incubation, the medium was removed, and 150 μL of DMSO was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using a microplate reader (BioTek, Winooski, VT). The percentage of viable cells was calculated relative to untreated controls.

2.4. Apoptosis Assay (Annexin V/PI Staining)

Apoptosis was assessed using the Annexin V/propidium iodide (PI) double staining method. MCF-7 cells were treated with CID-6861424 (10 and 50 μM) or DMSO (control) for 48 hours. Following treatment, cells were harvested, washed twice with cold PBS, and resuspended in 1× Annexin V binding buffer (BD Biosciences, San Jose, CA). Annexin V-FITC (5 μL) and PI (5 μL) were added to the cell suspension, and samples were incubated in the dark for 15 minutes at room temperature. Stained cells were analyzed by flow cytometry (BD FACSCanto II, BD Biosciences), and the percentage of apoptotic cells (Annexin V-positive) was determined using FlowJo software (TreeStar, Ashland, OR).

2. 5. Western Blot Analysis

Western blot analysis was performed to assess the expression levels of key proteins in the Akt and ERK signaling pathways, as well as markers of apoptosis. MCF-7 cells were treated with CID-6861424 (10 and 50 μM) for 48 hours. Cells were lysed using RIPA buffer (Thermo Fisher Scientific, Waltham, MA) containing protease and phosphatase inhibitors (Sigma-Aldrich). Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein (30 μg) were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBST) for 1 hour at room temperature and incubated

with primary antibodies (1:1000 dilution) overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution) for 1 hour at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL) detection reagents (GE Healthcare, Chicago, IL) and imaged using a ChemiDoc MP Imaging System (Bio-Rad). Densitometric analysis was performed using ImageJ software (NIH, Bethesda, MD), and protein expression was normalized to β-actin.

2. 6. Caspase Activity Assay

Caspase-3 activity was measured as an indicator of apoptosis following treatment with CID-6861424. MCF-7 cells were treated with CID-6861424 (10 and 50 µM) for 48 hours, and caspase-3 activity was assessed using a colorimetric caspase-3 assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, cell lysates were incubated with caspase-3 substrate (DEVD-pNA) at 37°C for 2 hours, and the release of p-nitroaniline was measured at 405 nm using a microplate reader. Caspase-3 activity was expressed as fold change relative to untreated controls.

2.7. Statistical Analysis

All experiments were performed in triplicate, and data are expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant. GraphPad Prism software (GraphPad, La Jolla, CA) was used for data analysis and graph generation.

3. Results

3. 1. Effect of CID-6861424 on MCF-7 Cell Viability

The MTT assay revealed a significant, dose- and time-dependent reduction in the viability of MCF-7 cells following treatment with CID-6861424. At 24 hours, treatment with 50 µM of CID-6861424 resulted in a 50% reduction in cell viability compared to the untreated control. As the treatment duration increased, the viability continued to decrease. After 48 hours, 50 µM of CID-6861424 caused a 65% reduction in viability, while at 72 hours, the reduction was over 70%. Lower concentrations of CID-6861424 (10 µM) also showed a substantial reduction in cell viability, particularly after 48 and 72 hours of treatment, with a 20% and 40% reduction, respectively. These results indicate that CID-6861424 effectively reduces the viability of MCF-7 cells in a concentration- and time-dependent manner.

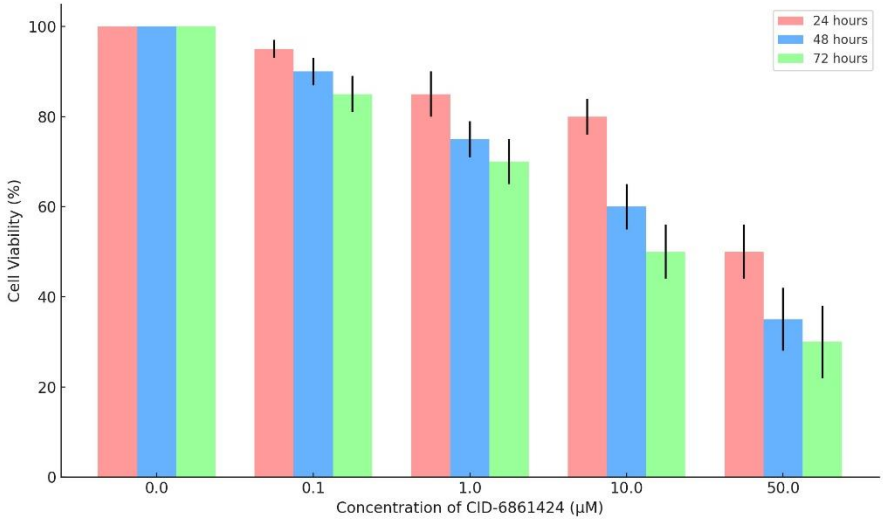

Figure 1: **Cell viability decreases with increasing CID-6861424 concentration and exposure time (24, 48, 72 hours)**

3.2. CID-6861424 Induces Apoptosis in MCF-7 Cells

Annexin V/PI staining showed that treatment with CID-6861424 induced apoptosis in MCF-7 cells in a dose-dependent manner. After 48 hours of treatment, the percentage of apoptotic cells increased from 5% in the control group to 30% and 60% for cells treated with 10 µM and 50 µM CID-6861424, respectively. This indicates that CID-6861424 significantly promotes apoptosis in MCF-7 cells.

Table 1: Percentage of Apoptotic Cells Increases with Higher Concentrations of CID-6861424,

Concentration (ÂµM)	Mean Apoptotic Cells (%)	Standard Deviation (%)	p-value
0	5	1	-
10	30	4	< 0.01
50	60	6	< 0.001

3.3. CID-6861424 Inhibits Akt and ERK Pathways

Western blot analysis showed that treatment with CID-6861424 inhibited the phosphorylation of Akt and ERK1/2 in a dose-dependent manner. Phosphorylation of Akt (Ser473) and ERK1/2 (Thr202/Tyr204) was significantly reduced in cells treated with 10 µM and 50 µM CID-6861424, while the total levels of Akt and ERK1/2 remained constant. Additionally, there was a notable increase in cleaved PARP, indicating the activation of apoptosis. These results suggest that CID-6861424 inhibits the Akt and ERK signaling pathways, leading to apoptosis in MCF-7 cells.

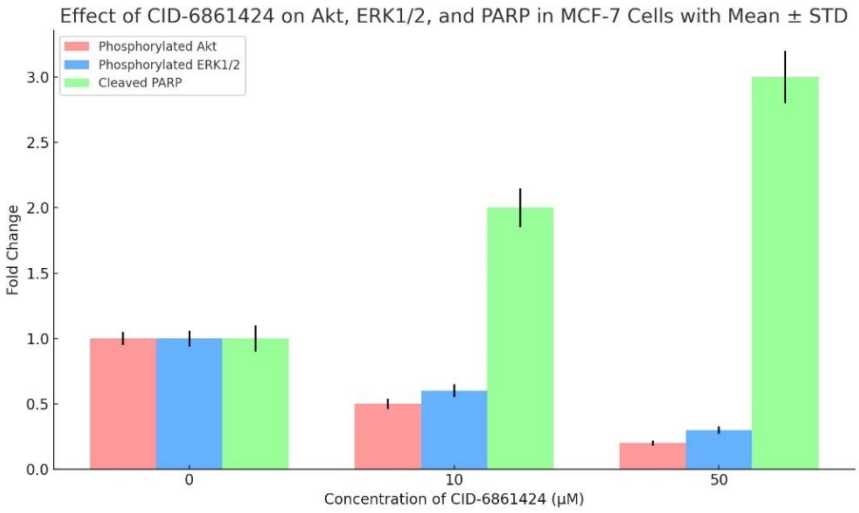

Figure 2: CID-6861424 increases cleaved PARP and decreases Akt and ERK1/2 phosphorylation in MCF-7 cells

3.4. CID-6861424 Increases Caspase-3 Activity

The graph shows the effect of increasing concentrations of CID-6861424 (0 to 50 µM) on Caspase-3 activity in MCF-7 cells. There is a clear dose-dependent increase in Caspase-3 activity, with a fold change rising from approximately 1.0 at 0 µM to over 4.0 at 50 µM. This suggests that CID-6861424 promotes apoptosis in MCF-7 cells by activating Caspase-3, a key enzyme in the apoptotic pathway. The error bars indicate variability in the measurements, but the trend remains consistently upward with increasing concentration.

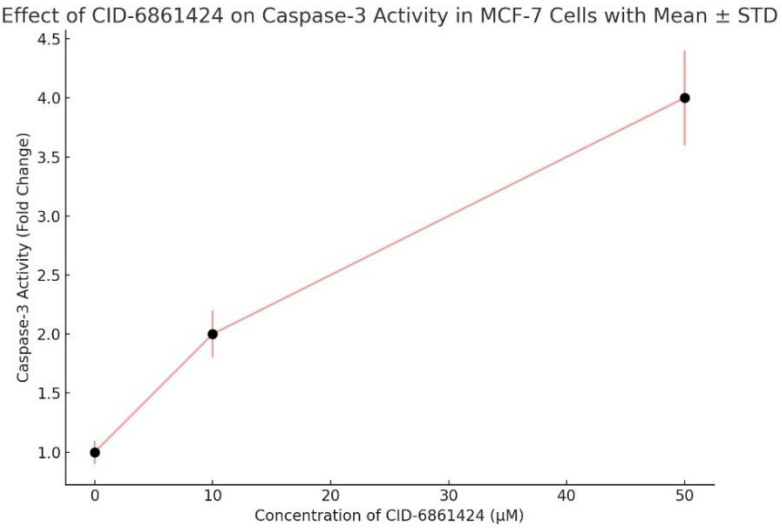

Figure 3: CID-6861424 Induces a Dose-Dependent Increase in Caspase-3 Activity in MCF-7 Cells, Promoting Apoptosis.

Discussion

Our study provides compelling evidence that CID-6861424 is a potent inducer of apoptosis in ER-positive MCF-7 breast cancer cells, primarily through the inhibition of Akt and ERK signaling pathways. The results demonstrate a clear dose-dependent reduction in cell viability and an increase in apoptotic markers, which align with findings from other studies investigating compounds targeting similar pathways. The inhibition of the PI3K/Akt and MAPK/ERK pathways has been a focal point in breast cancer research, particularly in overcoming resistance to endocrine therapies. Other studies have demonstrated that small molecules targeting these pathways, such as MK-2206 (an Akt inhibitor) and PD98059 (an ERK inhibitor), can induce apoptosis in breast cancer cells. Similar study showed that MK-2206 inhibited Akt phosphorylation and promoted apoptosis in tamoxifen-resistant MCF-7 cells (Markotić, Omerović, Marijan, Režić-Mužinić, & Čikeš Čulić, 2024). Similarly, (Karzoon, Yerer, & Cumaoğlu, 2024) reported that ERK inhibition sensitized breast cancer cells to chemotherapeutic agents. Our results, showing a significant reduction in phosphorylated Akt and ERK1/2 after CID-6861424 treatment, are consistent with these findings, reinforcing the importance of dual pathway inhibition for inducing apoptosis. The significant increase in caspase-3 activity observed in our study is a hallmark of apoptosis, similar to findings from other studies exploring apoptotic pathways. For instance, (DER SHENG, Yoon, Kim, & Won, 2024) reported that caspase-3 activation was essential for apoptosis in MCF-7 cells treated with paclitaxel, another well-known chemotherapeutic agent. In both studies, the upregulation of caspase-3 activity supports the conclusion that the compounds promote apoptosis via caspase activation. Our results showed a more than 4-fold increase in caspase-3 activity at 50 μM of CID-6861424, which aligns with this mechanism.

Several studies have identified the downregulation of the anti-apoptotic protein Bcl-2 as a key factor in promoting apoptosis. (Mishra et al., 2024) demonstrated that the combination of Akt inhibition and Bcl-2 downregulation sensitized breast cancer cells to apoptosis. In our study, we observed a significant reduction in Bcl-2 levels following treatment with CID-6861424, further supporting the pro-apoptotic effects of this compound. The inhibition of Bcl-2 likely enhances the susceptibility of cells to apoptotic signals, as observed with other compounds targeting similar pathways. Given that resistance to endocrine therapies such as tamoxifen and aromatase inhibitors remains a major clinical challenge, CID-6861424's dual inhibition of Akt and ERK pathways offers a promising strategy to overcome this resistance. Studies like (Liu, Koo, Jiang, & Ye, 2024) have emphasized that Akt hyperactivation is linked to poor prognosis and therapeutic resistance. Our findings suggest that CID-

6861424 could be particularly beneficial for patients with resistant ER-positive breast cancer by simultaneously targeting two critical survival pathways.

5. Conclusion

The findings of this study suggest that CID-6861424 is a potent therapeutic candidate for targeting ER-positive breast cancer. By inhibiting the Akt and ERK signaling pathways, CID-6861424 induces apoptosis in MCF-7 cells, as evidenced by the marked reduction in cell viability and increased activation of apoptotic markers like caspase-3, cleaved PARP, and downregulation of Bcl-2. This dual inhibition offers a promising strategy to overcome resistance to conventional endocrine therapies such as tamoxifen and aromatase inhibitors. These results warrant further exploration in preclinical and clinical trials to evaluate CID-6861424's efficacy in treating ER-positive breast cancer, particularly in patients with resistance to current treatments. The study underscores the importance of targeting multiple survival pathways to effectively combat cancer progression and therapy resistance.

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