RESEARCH ARTICLE

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1'- Acetoxychavicol acetate Suppresses Osteosarcoma Cell Proliferation through the PI3K Pathway: A Molecular Docking and Cytotoxicity Study

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Background: This study aims to investigate anticancer properties of *1'-Acetoxychavicol acetate* (ACA), a phenylpropanoid substance obtained from the rhizomes of the *Alpinia* genus, which has been extensively studied. However research on its cytotoxic effects, particularly against osteosarcoma cells, has never been donenot been conducted. The purpose of the research is to investigate the anticancer potential of ACA to support the its development as a novel therapeutic candidate.
Materials and methods: This study assessed ACA's initial anticancer potential through *in vitro* cytotoxic tests on normal human osteoblast cells (hFOB) and osteosarcoma cells (MG-63) using the MTT assay. Additionally, bioinformatics analyses, including target prediction, gene ontology, hub gene identification, protein-protein interactions (PPI) network construction, Kyoto encyclopedia of genes and genomes (KEGG) pathways analysis, disease association analysis, and molecular docking, were performed.
Results: The cytotoxicity test on normal hFOB showed an IC_{50} of 45.05 µM, while in MG-63 osteosarcoma cells, the IC_{50} was 20.41 µM. In the bioinformatics test, top five target genes identified were SRC, GNAI1, PIK3CD, PIK3CB, PIK3R3. Molecular docking analysis showed that, the native PI3KD ligand showed a strong binding affinity of -10.99 kcal/mol and interacted with more amino acid residues.
Conclusion: Overall, ACA exhibits promise as a treatment option to inhibit osteosarcoma cell proliferation by targeting the PI3K pathway. To develop ACA as a potential osteosarcoma therapeutic candidate, extensive *in vitro* research is needed.

Keywords: *1-Acetoxychavicol acetate, bioinformatics, cytotoxicity, osteosarcoma, PI3K pathway*

Introduction

Osteosarcoma, also knows as osteogenic sarcoma, is a malignant tumor that originates from mesenchymal tissue and is usually detected the metaphyseal region of children's long bones.[1] It is characterized by abnormal bone growth with high malignancy, invasiveness, and rapid progression.[1,2] After lymphoma and brain tumors, osteosarcoma is the most prevalent cancer in teenagers. Its annual incidence in children under age of 15 is roughly 5.6 cases per million and its five-year fatality rate is 80%.[1] While various therapies, including chemotherapy, have been developed to

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treat osteosarcoma, these treatments often lack selectivity, targeting both tumor cells and normal tissues. This leads to acute and chronic damage.[3] Chemotherapy resistance can also reduce the effectiveness of cancer treatment.[4]

The development of new, safe, and selective drugs remains crucial. One promising candidate is *1'-Acetoxychavicol acetate* (ACA), a phenylpropanoid substance obtained from the rhizomes of the *Alpinia* genus, commonly found in plants like ginger, galangal, and kencur.[5] ACA has been shown to have anticancer effects in several kinds of cancer cell line, including breast cancer, myeloma, Ehrlich's ascites tumor, prostate cancer, cervical cancer, and colorectal adenocarcinoma.[6] ACA is known to have strong cytotoxic activity against breast cancer cells (MCF-7) with an IC50 value 23,9 µM.[7] The ACA chemical reduces the growth of breast cancer cells by inducing apoptosis[8], inhibiting cell proliferation, and suppressing cell migration[9]. A previous study has shown that IV administration of the ACA compound greatly lowers tumor volume while maintaining an excellent safety profile and causing no side effects.[9] However, no research has explicitly analyzed ACA's cytotoxic potential against osteosarcoma cells.

The purpose of this research was to investigate the anticancer potential of ACA using *in vitro* tests on normal hFOB and MG-63 osteosarcoma cells, using the MTT assay. Complementary bioinformatics analyses were conducted to identify target genes and pathways associated with ACA's effects on bone cancer. These findings are intended to support the development of ACA as a novel therapeutic candidate for osteosarcoma.

Materials and methods

Cytotoxic Test

hFOB and MG-63 cells were 80% confluence used trypsin-ethylenediaminetetraacetic acid (EDTA) solution 0.25% (Sigma-Aldrich, St. Louis, MO, US). The cells were then grown in 96-well plates until 80% confluence was attained. Various concentrations of ACA were added to the wells, then the cells were incubated overnight at 37°C with 5% CO_2. MTT reagent (Sigma-Aldrich, St. Louis, MO, US) and sodium dodecyl sulfate (SDS) solution (Sigma-Aldrich) were added to measure absorbance using an ELISA reader (Elx 800 BioTek, Winooski, VT, US). The IC_{50} value indicates the concentration needed to stop 50% of cell proliferation, was calculated through linear regression of concentration versus cell viability.[10]

Data Collection and Processing

Predictive target genes of ACA were identified using 7 online databases: Swiss Target Prediction, SEA search, Moltar PRed, Target Net, Binding DB, DINIES, and Hit Pick.[11] The result were analyzed to obtain target genes. Mutated genes associated with bone cancer were retrieved from cBioPortal database (https://www.cbioportal.org/) and analyzed using Microsoft Excel. Venny 2.1.0 was used to determine intersection of predictive targets and bone cancer-associated mutated genes, identifying 69 overlapping genes.[12]

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

DAVID v6.8 was used to analyzed GO for biological processes, molecular functions, and cellular components, with a p-value threshold of <0.05. Kyoto pathway enrichment analysis was performed using WebGestalt (http://www.webgestalt.org/), and bar charts were generated for visualization. An FDR-adjusted p-value of <0.05 was considered statistically significant.[13]

Interactions Between Proteins of Hub Gene Contructs and Predictive Biomarkers

Protein-protein interaction (PPI) networks were constructed using search tool for the retrieval of interacting genes/proteins database (STRING-DB) (https://string-db.org/) with a confidence score of >0.4. The parameters used included a maximum depth of 100, degree cutoff of 2, node score cutoff of 0.2, and k-score of 2.[12]

Molecular Docking

Protein structures for SRC (PDB ID: 3D7T), PIK3CD (PDB ID: 5T8F), and PI3K (PDB ID: 2RED) were obtained from the protein data bank (PDB: https://www.rscb.org/). The ACA ligand structure was retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/). Molecular docking simulation were performed using AutoDock v4.2. AutoDock was used for molecular docking, PyMOL and Discovery Studio Visualizer were used for molecular visualizations. The default parameters used in molecular docking included setting the X, Y, Z positions and grid box volume, with a number of points set to 20 and set to spacing 0.375 Å. The volume of the grid box used is 40×40×40 Å, with coordinate points at SRC (x: -64.635; y: 38.965; z: -41.545), PIK3CD (x: 37.873; y: 14.374; z: 33.957), and PI3K (x: -2.087; y: 40.70, z: 2.592). An anaysis was conducted on binding

affinity and hydrogen bonds formed.[14-16]

Statistical Analysis

Result were presented as mean±standard deviation (SD) Data were performed in triplicates and analyzed using Mann-Whitney's post hoc test, with a *p*-value of ≤ 0.05 indicating a statistically significant difference.

Results

ACA Induced Concentration-Dependent Reduction in Viable Cell Count of Normal hFOB and Osteosarcoma MG-63 Cells

The cytotoxicity of ACA was evaluated against normal hFOB cells and osteosarcoma MG-63 cells. The absorbance values used to calculate the IC_{50} value, which represents the concentration needed to inhibit 50% of cell proliferation. The IC_{50} value for normal hFOB cells was 45.05 µM, while for MG-63 cells it was 20.41 µM (Figure 1). The cytotoxicity test revealed that as the concentration of ACA increased, the percentage of viable cells in normal HFOB cells and MG-63 cells decreased. This demonstrates a dose-dependent cytotoxic effect.[17]

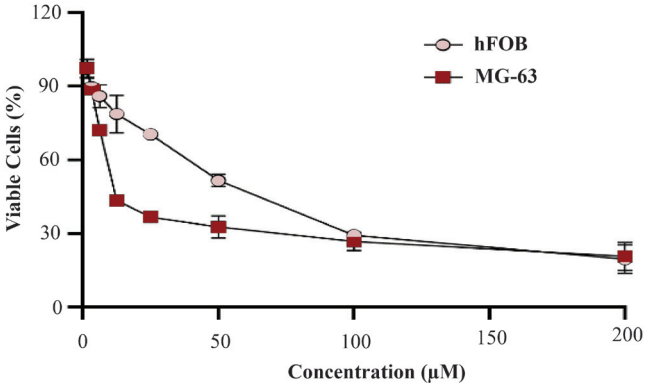
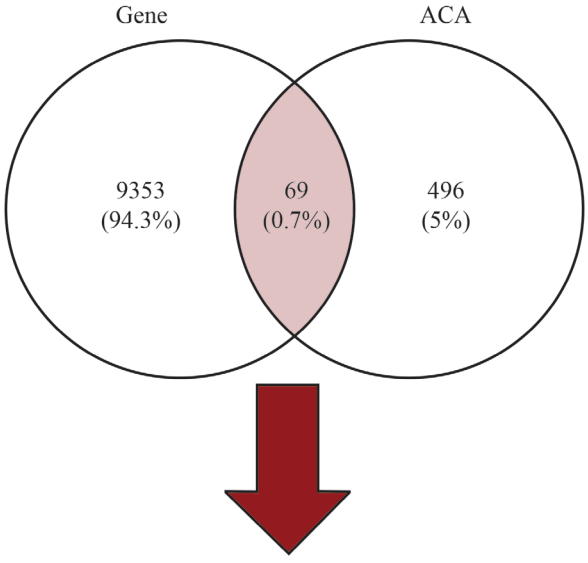


Figure 1. ACA induced concentration-dependent reduction in viable cell count of normal hFOB and Osteosarcoma MG-63 Cells. Viable cell count was measured after treatment with ACA at different concentrations for 24 hours. The results are presented as mean±SD (n=3). *Statistical significance ($p<0.05$) was determined using Mann-Whitney's post hoc test when compared to the Sham group.

ACA Enhanced Bone Cancer Treatment by Targeting 69 Overlapping Genes

RSV Predictive target genes of ACA were identified using 7 online databases, resulting in 9,353 potential target genes. Each target gene was represented by a mutations list. Venny 2.1.0 was used to determine the potential target genes from ACA compound against osteosarcoma. These were cross-



IGF1R, GABRB3, GABRA2, PIM3, HRH2, CA5B, CHRM2, F7, ECE1, CYP1A2, CHRM3, PLAU, HMGCR, CETP, S3, ADK, CA2, HTR4, REN, PLAT, PTPN7, BCL2, JAK3, P2RY1, CA6, PLG, MET, MMP13, HCK, MC3R, VCAM1, GPR35, ROCK1, SSTR4, LCK, CA1, FYN, CHRM4, PDE10A, CCNB2, PDE2A, PTGDR, AKT2, STAT1, NFKB1, NOS3, SRD5A1, GABRA1, HDAC2, SSTR1, PLD2, CA12, SCN5A, CYP2C19, HTR1E, MDM2, SYK, DHFR

Figure 2. The prediction of potential ACA target genes. The diagram illustrates the overlap between the predicted target genes of ACA and mutated genes associated with osteosarcoma, yielding 69 overlapping genes as potential targets.

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Table 1. Top five potential target genes of ACA compounds and their associated pathways.

Term	*p*-value	Gene
Biological Process		
GO:0007187~G protein-coupled receptor signaling pathway, coupled to cyclical nucleotide second messenger	1.19E+04	CHRM2, CHRM3, MC3R, HTR1E, HRH2, CHRM4, SSTR1, HTR4, SSTR4
GO:0006730~one-carbon metabolism process	1.25E+08	CA12, DHFR, CA1, CA5B, CA2, CA6
GO:0098664~G protein-coupled serotonin receptor signaling pathway	2.27E+09	CHRM2, CHRM3, HRH2, CHRM4, HTR4
GO:0007186~G protein-coupled receptor signaling pathway	1.70E+10	CHRM2, CHRM3, HTR1E, GPR35, PTGER2, CXCR4, ECE1, HTR4, SSTR4, PIK3CG, BRS3, P2RY1, PTGDR, TGM2
GO:0016310~phosphorylation	9.87E+09	HCK, SYK, ROCK1, PLAU, LCK, AKT2, ADK, FYN, JAK3, MET, PIK3CG, IGF1R
Molecular Function		
GO:0016907~G protein-coupled acetylcholine receptor activity	2.54E+11	CHRM2, CHRM3, CHRM4
GO:0004089~carbonate dehydratase activity	3.24E+08	CA12, CA1, CA5B, CA2, CA6
GO:0005102~signaling receptor binding	3.14E+11	F7, HCK, SYK, LCK, REN, FYN, PLG, PLAT
GO:0019899~enzyme binding	3.29E+12	HDAC2, STAT1, CYP1A2, MDM2, FYN, PLG, SCN5A, CYP2C19
GO:0001784~phosphotyrosine residue binding	9.12E+11	HCK, SYK, LCK, FYN
Cellular Component		
GO:0098685~Schaffer collateral - CA1 synapse	2.95E+12	CDC42, ROCK1, FYN, PLG, PLAT
GO:0009986~cell surface	2.68E+12	VCAM1, ITGB5, PLAU, P2RY1, CD38, CXCR4, PLG, PLAT, SCN5A, MET
GO:0045202~synapse	2.29E+12	GABRB3, CHRM2, GABRA2, GABRA1, CHRM3, HTR1E, HRH2, CHRM4, HTR4
GO:0045211~postsynaptic membrane	4.91E+10	GABRB3, CHRM2, GABRA2, GABRA1, CHRM3, CHRM4, P2RY1
GO:0031234~extrinsic components of cytoplasmic side of plasma membrane	2.00E+10	HCK, LCK, FYN, JAK3

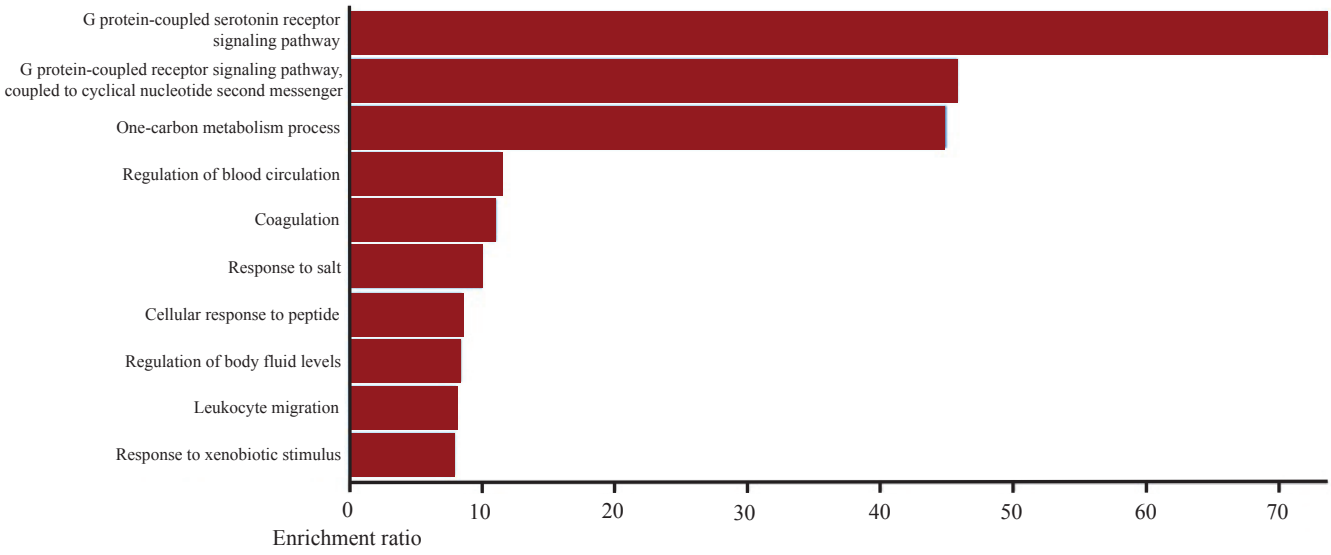

Figure 3. KEGG pathway analysis of ACA target genes.

referenced with mutated genes in bone cancer, yielding 69 overlapping genes as potential targets (Figure 2).

ACA Target Genes Enhanced Osteosarcoma Treatment by Regulating Critical Pathways

GO categorized 69 potential target genes into 3 categories: biological processes, cellular component, and molecular functions. The top pathways included G protein-coupled receptor signaling, one-carbon metabolic processes, and phosphorylation (Table 1). KEGG pathways analysis indicated these genes are involved in multiple cancer-related pathways (Figure 3). Specifically, the GO analysis revealed that ACA target genes regulate biological processes through mechanisms such as one-carbon metabolic processes and G protein-coupled receptor signaling pathways. These genes are also located in cellular components like phosphotyrosine residue binding sites and are involved in molecular functions related to the cell surface and synapses. These finding suggest that ACA may exert its anticancer effects by modulating multiple key pathways and cellular processes.

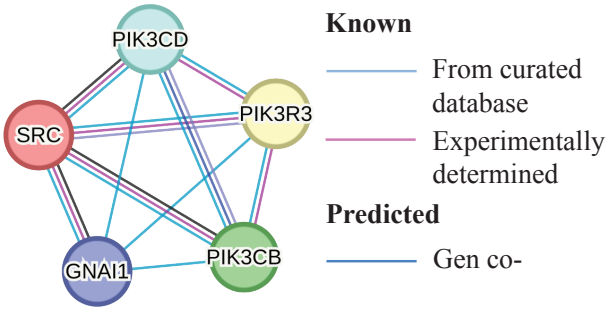

Figure 4. The top five hub gen based on degree score were SRC, PIK3R3, PIK3CD, PIK3CB, and GNAI1. Edges in the figure represent protein-protein.

ACA Target Genes Enhance Protein-Protein Interactions and Play Critical Roles in Human Biology

Sixty-nine target genes were subjected to protein-protein interaction analysis using STRING-DB. The highest five hub genes based on degree score, were SRC, GNAI1, PIK3CD, PIK3CB, and PIK3R3. The edges represent protein interactions derived from curated databases, most of which are from experimentally determinated and gene eco-occurence (Figure 4). This associations suggest that the proteins work together to contribute to a common function, but do not imply that they are physically binding another. Based on their known functions, these five hub genes play critical roles in human biology (Table 2).

Gene Expression of PIK3CD and PIK3R3 Enhanced Prognostic Outcomes in Osteosarcoma

Gene expression analysis using GEPIA indicated that PIK3CD and PIK3R3 were highly expressed in osteosarcoma tissues compared to normal tissues (Figure 5). Prognostic analysis further demonstrated a strong correlation between elevated PIK3R3 expression and improved survival rates in osteosarcoma patients (Figure 6).

Molecular Docking

Molecular docking simulations showed that ACA interacted effectively with the target proteins SRC, PIK3CD, and PI3K, with binding affinity approaching that of the native ligand. This indicates strong and stable ligand-receptor interactions.

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Table 2. Role and functions of ACA- correlated genes.

Protein	Role	Function	Ref.
SRC	Non-receptor tyrosine kinase	Migration, transformation, apoptosis, cell adhesion, and cell cycle progression	18,19
GNAI1	G-protein subunit	Transducer in several signaling cascades downstream of G protein-coupled receptors (GPCRs)	20–22
PIK3CD	PI3K regulatory subunit	Development and immune cells activity, cell survival and proliferation	20,23,24
PIK3CB	PI3K regulatory subunit	Act downstream receptor tyrosine kinases	25–27
PIK3R3	PI3K regulatory subunit	Stimulated protein tyrosine kinases, controls kinase activity via SH2 domain	28

Ref: References.

Native The native ligand of PIK3CD has a higher binding affinity than the ACA molecule, which is consistent withthis accordance with of the amino acid residues in the native ligand of PIK3CD, which containing 6 hidrogen bonds and five non-hydrogen bonds, further confirms this (Table 3).

Based on connections between amino acid residues, the native ligand of PIK3CD has more interactions than ACA's compounds. Non-hydrogen residues (Tyr 813, Ile 825, Met 900) and hydrogen residues (Asp 911; Val 828) bind with ACA compound. According to the reference, key amino acids in PIK3CD receptor (PDB ID: 5T8F) include Tyr813, Glu 472, dan Leu 474.[29] This indicates that ACA compounds and the native ligands of PIK3CD bind at the same site and are expected to exhbit similar affinity in inhibiting PIK3CD activity.

In drug discovery, good permeability and solubility are crucial. Lipinski's Rule of Five is a widely used guidline to assess drug-likeness based on these properties. ACA satisfies all five of Lipinski's Rule criteria, suggesting that it has favorable pharmacokinetic properties for oral bioavailability (Table 4).

Discussion

The cytotoxic test was performed using the MTT assay, which evaluates cell viability by measuring the reduction of tetrazolium salt by the mitochondrial enzyme succinate reductase. Increasing ACA concentration led to a decrease in the percentage of viable cells in both hFOB and MG-63 cells, demonstrating a dose-dependent effect. The American National Cancer Institute (NCI) categorizes compounds with an IC_{50} value < 30 µg/mL as active. Therefore, ACA is classified as a strongly cytotoxic agent against MG-63 cells (IC_{50}: 20.41 µM) and moderately cytotoxic against hFOB

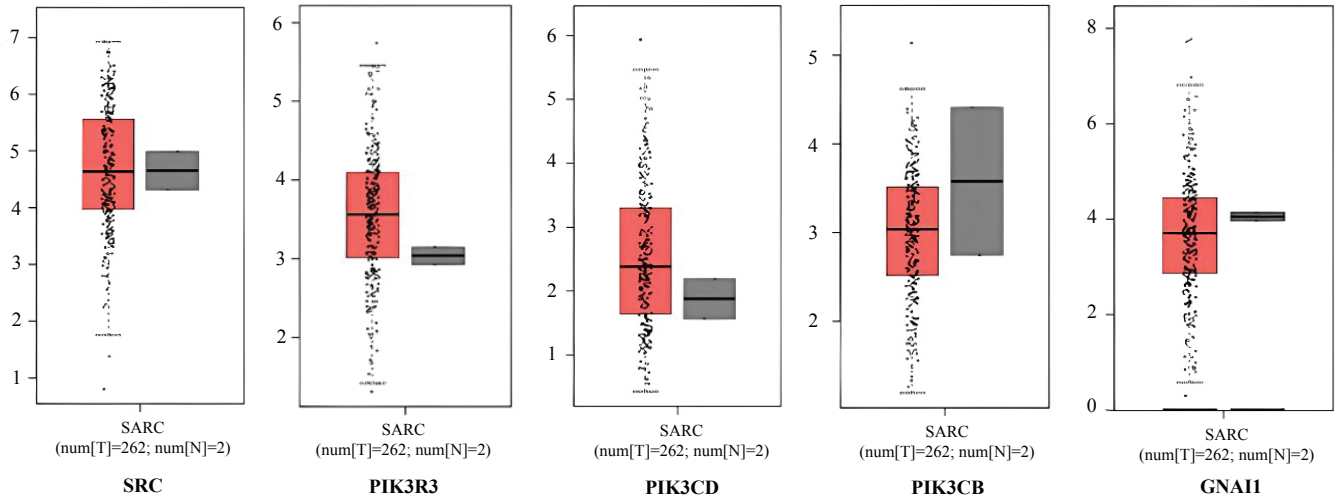

Figure 5. Gene expression analysis of SRC, PIK3R3, PIK3CD, PIK3CB, and GNAI1.

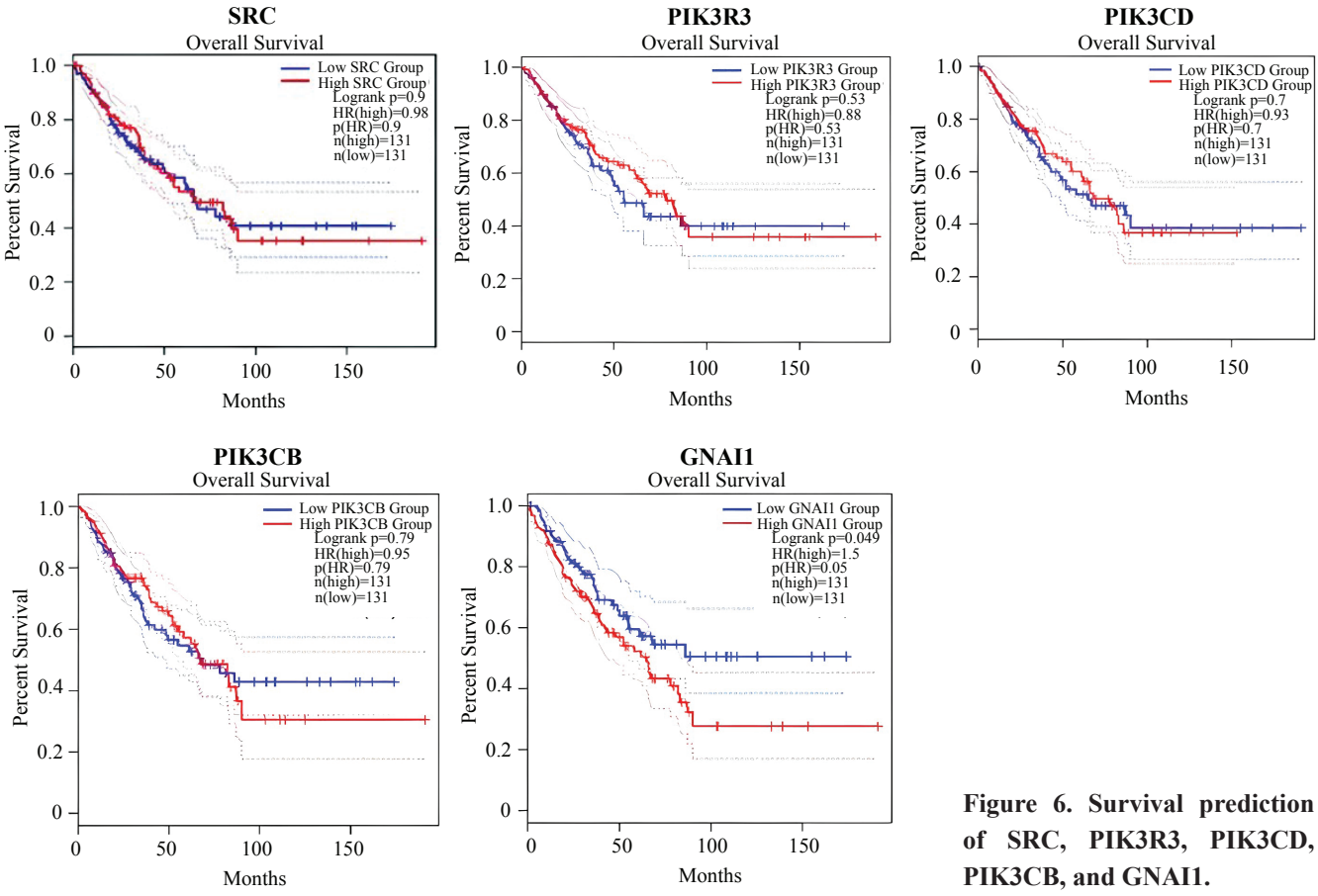

Figure 6. Survival prediction of SRC, PIK3R3, PIK3CD, PIK3CB, and GNAI1.

cells (IC_{50}: 45.05 µM). These results suggest that ACA may selectively inhibit cancer cells while exerting less impact on normal cells. These findings align with previous research demonstrating ACA's anticancer activity in various cancer cell lines, such as breast, prostate, and lung cancer cells.

Previous research indicates that ACA exhibits anticancer activity in several cell types, including breast cancer cells. Specifically, ACA has been reported to have active cytotoxic activity against MCF-7 breast cancer cells, with an IC_{50} value of 23.9 µM.[7] Similarly, a previous study[10] reported that ACA from Pasar Legi Surakarta galangal extract exhibited active cytotoxic activity against T47D, HeLa, and MCF-7 cancer cells, with IC_{50} values of 12.50 µg/ml, 13.20 µg/ml, and 15.80 µg/ml, respectively. Additionally, another study[30] demonstrated that ACA exhibits an active cytotoxic effect on MDA-MB 231 breast cancer cells, with an IC_{50} value of 4.8 µM. In addition to breast cancer cells, ACA compounds have been shown to exhibit cytotoxic effect againts lung and prostate cancer cells. According to a previous study[30], ACA exhibits active cytotoxic activity against PC-3 prostate cancer cells, with an IC_{50} value of 26.7 µM. ACA also exhibits active cytotoxic activity in A549 and SK-LU-1 lung cancer cells, with IC_{50} values of 29.2 µM and 25 µM, respectively. Similar research was also conducted on blood cancer cells (HL-60) which stated that ACA compounds have active cytotoxic activity with a IC_{50} value of 2 µM.[31] Furthermore, a study[32] found the IC_{50} value of various human cancer cells, including oral aquamous carcinoma (HSC-2), hepatocyte carcinoma (HepG2), and epidermoid cervical carcinoma (CaSKi) were 5 µM, 18 µM, and 17 µM. Based on the literature review, ACA exhibits significant anticancer activity. Cytotoxic testing data, supported by bioinformatics and molecular docking assays, suggests the potential development of ACA as a chemotherapeutic treatment for osteosarcoma.

In the bioinformatics analysis, 69 overlapping genes were identified as potential ACA targets in osteosarcoma. These genes are involved in critical biological processes, including the PI3K/AKT, SRC and GNAI1 which control cell viability, differentiation, and cell growth. The PI3K subunits (PIK3CD, PIK3CB, and PIK3R3) demonstrated significant roles in cell proliferation and survival, highlighting their importance as therapeutic targets. Activation of the PI3K/AKT/mTOR pathway involving these subunits enhances cell proliferation and tumor growth and contributing to chemoresistance. Inhibiting

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Table 3. Docking scores of ACA with native ligand.

Protein	ID	ΔG Binding (kCal/mol)		Interaction of Amino Acid Residues			
				Native Ligand		ACA	
		Native Ligand	ACA	Hydrogen	Non Hydrogen	Hydrogen	Non Hydrogen
SRC	3D7T	5.62	123.85	Lys 271, Ser 273, Asp 276, Ala 270	Gly 272, Val 209, Ile 201	Cys 498	Leu 516, Phe 515, Ala 367, Leu 512
PIK3CD	5T8F	-10.99	-6.54	Asp 911, Val 828, Lys 779, Ser 831, phe 908, leu 784	Tyr 813, Ile 825, Met 900, Trp 760, Pro 758,	Asp 911, Val 828, Ser 831,	Tyr 813, Met 900, Ile 825, Ile 910
PI3K	2RED	-4.96	-3.27	Pro1486, Arg 1503, Glu1506, Val 1499	-	Ser 1428, Lys 1504, Val 1429, Arg 1447	-

Some amino acid residues colored red are similar to key amino acid residues.

these targets could therefore be a promising strategy for osteosarcoma treatment. SRC is a proto-oncogene encoding a non-receptor tyrosine kinase that promotes migration, growth cell, and survival by activating the PI3K/Akt and MAPK/ERK pathways. This highlights ACA's potential in modulating multiple oncogenic pathways. GNAI1 is also known to play important roles in several human biological functions. GNAI1 (guanine nucleotide-binding protein G) is one of the genes that codes for α-type heterotrimeric G proteins that may impact the growth and spread of cancer cells, and GNAI1 also regulates the activity of several cell surface receptors, which contributes to cellular signaling pathways.[33]

A lower binding affinity value indicates that the native ligand of PIK3CD has a stronger docking outcome than ACA molecule, according to molecular docking data. Binding affinity indicates a ligand's ability to attach to a receptor.[34] Moreover, based on interactions between amino acid residues, the native ligan of PIK3CD has more interactions than ACA's compounds. The receptor tyrosine kinase pathway plays a very important role in osteosarcoma.[35] This pathway modulates many pathways such as MAPK, PI3K/Akt, and JAK/STAT which contribute to cancer malignancy, metastasis, and angiogenesis.[9] In osteosarcoma, PI3K/Akt pathway regulates cell growth, differentiation, and survival.[36] Based on the analysis, there are non-hydrogen (Tyr 813, Ile 825, Met 900) and hydrogen (Asp 911; Val 828) residues that bind with ACA compound. According to reference, key amino acids in PIK3CD receptor (PDB ID: 5T8F) include Tyr813, Glu 472, dan Leu 474.[29] This indicates that ACA compounds and native ligands PIK3CD bind to the same place and are expected to provide the same affinity in inhibiting the work of PIK3CD.

In drug discovery, good permeability and solubility are crucial. Based on solubility and permeability characteristics, Lipinski created a rule known as the Lipinski rule or rule of five (RO5) to expedite the drug discovery and development process.[37] Lipinski's rule predicts a compound's bioavailability, or ability to be absorbed and circulated in the body if administered orally. A compound is considered potential if it can be absorbed well and has high permeability (ability to penetrate cell membranes). Lipinski's rule

Table 4. Lipinski's Rule of Five data for ACA.

Lipinski's Rule of Five	Native Ligand			ACA
	SRC	PIK3CD	PI3K	
Molecular weight	312	312	312	234
Hydrogen donor	5	5	5	0
Hydrogen acceptor	6	6	6	4
LogP	-0.05	-0.05	-0.05	2.40
Topological polar surface area (TPSA)	77.14	77.14	77.14	62.45
Druglikeness	Yes	Yes	Yes	Yes

indicates that compounds with a molecular weight of more than 500 Da can cause inhibition of permeability in the digestive system and central nervous system. In addition, the lipophilicity of a compound is measured by LogP. LogP is the solubility value of a compound in octanol and water solvents, if a compound's logP less than 5 it can pass through lipid bilayer-based cell membranes. The difficulty of breaking through the lipid bilayer barrier is indicated by the quantity of hydrogen donors and acceptors, this is because it has the potential to be partitioned in solvents with strong hydrogen bonds such as water.[38,39] Drugs that are given orally (through the mouth) must be removed from the intestine through the intestinal wall to reach the bloodstream.[40] Considering table 4, the ACA compound's Lipinski test outcomes satisfy the requirements of Lipinski Rule, so it can be stated as a potential drug candidate for the development of oral drugs.

Futher *in vitro* studies, such as proliferation, cell cycle, apoptosis, and western blot assay, are needed to support the development of ACA as a novel treatment candidate for osteosarcoma, as this research was limited to cytotoxicity testing, bioinformatics, and molecular docking.

Conclusion

1'Acetoxychavicol acetate (ACA) significantly supressed osteosarcoma cells. Through molecular docking and cytotoxic activity, potential target genes of ACA in osteosarcoma mostly involve the PI3K pathway.

Acknowledgments

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Authors' Contributions

PG performed data collection, data analysis, and wrote the manuscript. MD contributed to the main idea of the work, validated the data, participated in the final version of the manuscript, and secured funding. FW conducted data analysis, supervised the work, participated in the final version of the manuscript, and provided comments and additional scientific information.

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