Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.298

RESEARCH ARTICLE

Bufalin Promotes Apoptosis and Autophagy Through the JAK-STAT Signaling Pathway in Myeloid Leukemia

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ARTICLE HISTORY (24-755)

Received: November 23, 2024
Revised: December 22, 2024
Accepted: December 24, 2024
Published online: December 27, 2024

Key words:
Apoptosis
Bufalin
Myeloid leukemia
Autophagy
Transport proteins

A B S T R A C T

Acute myeloid leukemia (AML) is a highly heterogeneous malignancy, characterized by the impaired differentiation of myeloid precursors and the rampant clonal proliferation of bone marrow stem cells, which is often accompanied by symptoms such as infection, fever, anemia, and bleeding. AML constitutes approximately 80% of all adult leukemia cases and is distinguished by its high morbidity and mortality rates. Traditional Chinese Medicine (TCM) exhibits distinct characteristics and advantages in the treatment of contemporary hematological diseases, particularly in the management of relapsed and refractory blood diseases, as well as in addressing complications arising from Western medical treatments. This study investigates the therapeutic potential of Bufalin, the active component of Cinobufotalin derived from Bufo toad venom, in the context of AML. Utilizing the K562 cell line, we investigated the mechanism of bufalin through the GSE6347 database and *in vitro* experiments. Our results indicate that bufalin initially stimulates and subsequently inhibits the JAK-STAT signaling pathway, thereby facilitating apoptosis. Molecular docking with AutoDock Vina suggests that bufalin may induce autophagy by targeting transport proteins. By modulating apoptosis and autophagy via the JAK-STAT pathway, bufalin exhibits a distinctive dual effect on leukemia cells. This study underscores bufalin as a potential therapeutic agent for AML, integrating TCM principles with contemporary molecular techniques. These findings lay the groundwork for further exploration of bufalin's clinical potential and its role as a targeted therapy for modulating cell death in AML.

To Cite This Article: Wu Z, Cui Y, Mao W, Li Y, Memon MA and Lan H, 2024. Bufalin promotes apoptosis and autophagy through the JAK-STAT signaling pathway in myeloid leukemia. Pak Vet J, 44(4): 1142-1152. http://dx.doi.org/10.29261/pakvetj/2024.298

INTRODUCTION

Leukemia, a condition that originates from hematopoietic stem cells, is a malignant clonal disorder that is predominantly bound to acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) (Bernard, 2023; Guo *et al.*, 2024). With the advancement in scientific research, especially the dive of science into molecular studies and immunological dynamics regarding hematopoietic system, the treatment strategies for leukemia have had a great shift. The treatment protocols for leukemia no longer revolve around the administration of cytotoxic agent, now the treatment has been shifted towards protocols that are designed to promote cellular differentiation and apoptosis i.e. programmed cell death of the differentiated cells (Joron *et al.*, 2023; Zhu *et al.*, 2023; Zhu *et al.*, 2024). Leukemia has been studied and documented in both canine and feline populations all over the world, indicating that it's not just a matter of medical significance but it also has substantial veterinary importance (Davis *et al.*, 2018; Skayneh *et al.*, 2019; Tagawa *et al.*, 2020; Marrinhas *et al.*, 2022; Jegatheeson *et al.*, 2023; Lyseight *et al.*, 2024; Stokol *et al.*, 2024). Various types of cancer including leukemia across multiple species (veterinary scope) have been reported in numerous clinical studies and reports by veterinarians (Skayneh *et al.*, 2019; Tagawa *et al.*, 2020; Tong *et al.*, 2022; Jegatheeson *et al.*, 2023; Lyseight *et al.*, 2024;

Stokol *et al.*, 2024; Lyu *et al.*, 2024). Presently, the main focus of research regarding leukemia is to find a therapeutic intervention that works to achieve the target but with minimal or no adverse effects (Wang *et al.*, 2023a; Guo *et al.*, 2024; He *et al.*, 2024). According to the principles of the Traditional Chinese Medicine (TCM), the etiology of myeloid leukemia is directly linked with the presence of certain pathological factors in the body including blood stasis, phlegm-dampness and stagnation. Furthermore, the condition is also associated with the compromised blood flow and low qi (deficient vital energy) flow in the body (Wang *et al.*, 2023b). According to the TCM, the application of some toxic medicinal substances in controlled dosage can target and neutralize these pathogenic factors within an organism (Xu *et al.*, 2020; Yu *et al.*, 2024). By targeting these pathological conditions, unblocking of the meridians and dissipate nodules can be achieved, therefore getting the main objective of treating myeloid leukemia (Fu *et al.*, 2023).

"Bufalin" is a monomeric compound that is derived from the Chinese medicinal substance known as Chan Su (*Venenum bufonis*). Bufalin is characterized as polyhydroxy-steroid, having the molecular formula $C_{24}H_{34}O_4$ (Su *et al.*, 2023). Use of Bufalin aligns with the term "poison" used in the Traditional Chinese Medicine theory, as it stands as the quintessential remedy for the treatment of myeloid leukemia (Li *et al.*, 2023a). Research studies have indicated that bufalin possesses potent antineoplastic effects against various types of tumors (Xu *et al.*, 2020; Soumoy *et al.*, 2022). The antineoplastic effect of bufalin depends upon certain factors that are put in action once used against a tumor; these factors include inhibition of the proliferation of tumor cells, suppression of tumor invasion in surrounding cells and tissues, suppression of metastasis, induction of tumor cell apoptosis and autophagy, reversal of tumor drug resistance and modulation of tumor microenvironment (Pan *et al.*, 2020; Zhang *et al.*, 2024). Its effects encompass the inhibition of tumor cell proliferation, suppression of invasion and metastasis, induction of tumor cell autophagy, reversal of tumor drug resistance, and modulation of the tumor microenvironment. Numerous studies have indicated that Bufalin may potentiate its anti-cancer efficacy by triggering autophagy (Farooqi *et al.*, 2023; Lan *et al.*, 2024) More specifically, the inhibitory effect of bufalin induces a depletion of cellular energy reserves and metabolic disruptions within myeloid leukemia cells, thereby eliciting a cellular autophagic response as a coping mechanism to these stressors (Fu *et al.*, 2023). The autophagy process facilitates the intracellular degradation of organelles and proteins, thereby triggering apoptosis (Li *et al.*, 2019; Mutvei *et al.*, 2023).

Autophagy represents a pivotal lysosomal degradation and recycling mechanism within the eukaryotic cell, playing a critical role in sustaining cellular functionality and the homeostasis associated with cell survival and cell death (Koller *et al.*, 2023; Pan *et al.*, 2023). Concurrently, autophagy has emerged as a burgeoning focus of interest, representing a novel modality of cellular demise. The immune response (Pinho *et al.*, 2023), hematopoietic stem cell differentiation (Fan *et al.*, 2023), and the drug resistance of tumor cells (Zhang *et al.*, 2023) are all potentially influenced by the process of autophagy. Regulating autophagy could emerge as one of the most promising avenues in the realm of targeted therapeutic strategies. Bufalin exhibits crosstalk with various autophagy-related signaling pathways. For instance, treatment with bufalin may suppress the cellular autophagy process by modulating the mTOR signaling pathway (Su *et al.*, 2021; Mandrioli *et al.*, 2023). Furthermore, bufalin has the potential to modulate autophagy not only by influencing the activity of the NF-κB signaling pathway (Zhan *et al.*, 2020; Wang and Zhou, 2023), but also by governing the expression of various autophagy-regulatory molecules (e.g., Beclin-1 and LC3) (Miao *et al.*, 2013; Zhao *et al.*, 2017).

It is imperative to acknowledge that the interplay between bufalin and autophagy, as well as the precise mechanisms underlying their interaction in the context of myeloid leukemia, remains a subject of considerable debate and ambiguity. Consequently, additional research is essential to achieve a comprehensive understanding of bufalin's role in modulating autophagy and to ascertain how this intricate mechanism can be harnessed to refine and enhance therapeutic strategies for leukemia.

MATERIALS AND METHODS

Cells and Culture: The K562 cell line, a human myelogenous leukemia cell line, was procured from the American Type Culture Collection (ATCC, located in Manassas, Virginia, USA). The cells were subsequently cultured in RPMI 1640 medium, enriched with 10% heat-inactivated fetal bovine serum (FBS) from Gibco, USA, and supplemented with 100 units/mL of penicillin and 100 μg/mL of streptomycin from Invitrogen, California, USA. The incubation was conducted at a temperature of 37°C within a humidified incubator maintaining a 5% CO2 atmosphere.

Western blotting: The total protein was meticulously extracted from the treated K562 cells using the lysis buffer associated with the RNA immunoprecipitation assay. Subsequently, the protein underwent separation via 10% SDS polyacrylamide gel electrophoresis, followed by transfer onto polyvinylidene difluoride membranes. The membranes were then subjected to a blocking procedure using 5% bovine serum albumin. Following this, primary antibodies specific for BCL2 (ab59348, Abcam, Shanghai, China), P62 (ab56416, Abcam), LC3B (ab51520, Abcam), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab9485, Abcam) were incubated with the membranes at 4°C overnight. Subsequent to the primary incubation, secondary antibodies were applied and allowed to react at room temperature for a duration of 1 hour. Ultimately, protein analysis was conducted using enhanced chemiluminescence (GE Healthcare, USA) in conjunction with ImageJ software (National Institute of Health, USA). Additionally, all chemicals, reagents, and equipment used in this study, not specifically mentioned above, were obtained from Beyotime Biotechnology Co., Ltd., under standard conditions recommended by the manufacturer.

GSEA analysis: The log2 (fold change) data retrieved from the GSE6347 dataset was utilized to conduct a pre-

ranked Gene Set Enrichment Analysis (GSEA) employing the JAVA GSEA 3.0 software, by cross-referencing with the gene sets from the Molecular Signatures Database (MSigDB) version 5. To dissect the dataset, Gene Set Variation Analysis (GSVA) was executed using the 'gsva' package in R, with default parameters (method='gsva' and kcdf='Gaussian'), targeting the 50 cancer hallmark gene sets within MSigDB.

Vina autodocking: The protein targets and their corresponding ligand libraries were sourced from Alphafold2 and Pubchem databases. The protein structures underwent meticulous processing using PyMOL software, which involved the elimination of solvent molecules and the identification of the center of mass coordinates for the bound ligand within each structure. Subsequently, the bound ligand was extracted from the protein complex, and concurrently, all duplicate chains within the structure were removed. Furthermore, to ensure a precise and undistracted examination of the active site, all cofactors positioned at a significant distance from the binding pocket were meticulously excised. The ligand search grid was delineated as a cubic volume of 20Å3 dimensions, centered around the mass centroid of the original native ligand. Subsequently, the residual structural components were fine-tuned to a physiological pH level of 7.4 and subsequently transformed into the PDBQT format via the OpenBabel toolkit. Utilizing a scoring function, Vina employs a dual approach of gradient-based optimization and Monte Carlo simulations to identify and rank potential docking poses.

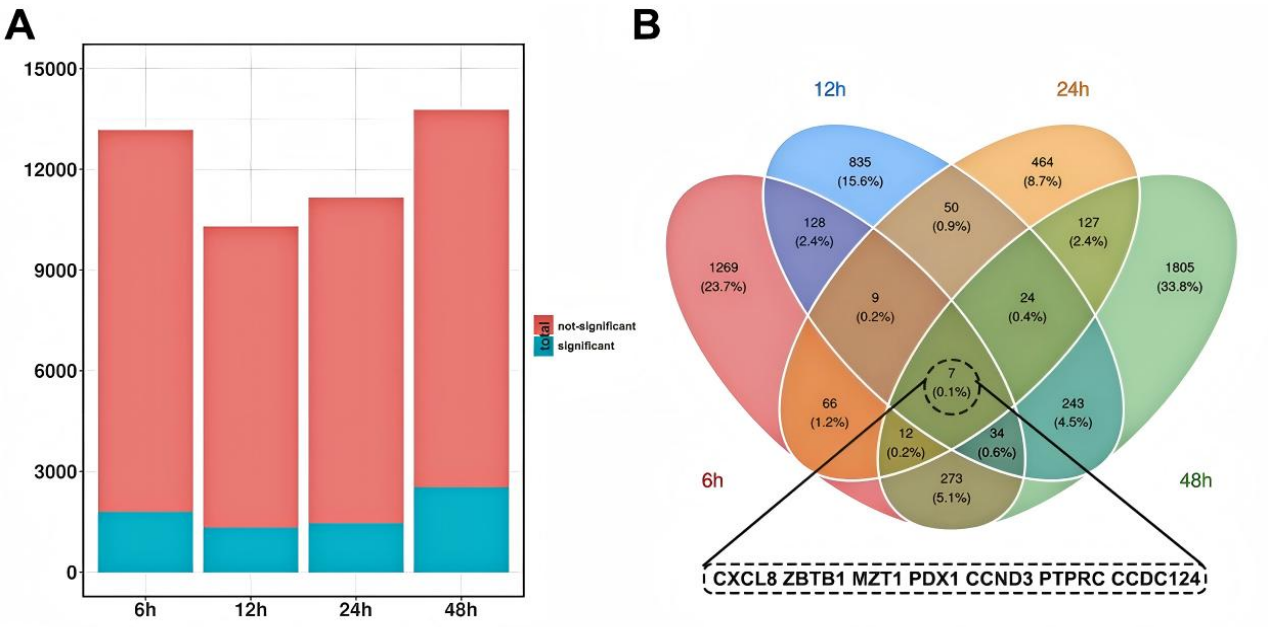
Enrichment Analysis of Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Signaling Pathways: The aforementioned differentially expressed genes (DEGs) were meticulously extracted, and subsequently subjected to a comprehensive Gene Ontology (GO) functional enrichment analysis leveraging the DAVID online database, with Homo sapiens as the biological context. This process yielded the requisite GO function enrichment data. The GO functional enrichment data were meticulously annotated and categorized in accordance with the genes' functions, encompassing biological processes (BP), cellular components (CC), and molecular functions (MF). Concurrently, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was employed to conduct a function enrichment analysis of signaling pathways, with the aim of uncovering potential biological pathways implicated in the study. Parameters were set to a minimum of 10 genes and a maximum of 500 genes, with statistical significance determined by a P-value less than 0.05 and a false discovery rate (FDR) below 0.2. This stringent criterion was used to identify the major enrichment functions and pathways associated with the differential genes. The clusterProfiler package [version 3.14.3] was instrumental in conducting the enrichment analysis. Z-score values were computed using the GOplot package [version 1.0.2], and the org.Hs.eg.db package [version 3.10.0] was utilized for ID conversion. Ultimately, the data were visualized through the creation of bubble and circle plots.

Statistical analysis: All data are presented as mean values accompanied by their respective standard deviations, as computed through a meticulous analysis utilizing the GraphPad Prism 6.0 software suite (GraphPad Prism, USA). This analytical process involved the application of the Student's t-test for the comparison of two distinct groups or the one-way Analysis of Variance (ANOVA) for the comparison of more than two groups, followed by the implementation of Tukey's post hoc test to ascertain the statistical significance among individual pairs of groups. Within this framework, a P-value less than 0.05 was deemed to signify a statistically significant difference.

RESULTS

To explore the complex mechanisms through which bufalin influences myeloid leukemia, our research team embarked on the task of accessing the publicly available gene expression dataset known as GSE6347. Upon acquiring this dataset, we conducted a thorough analysis to examine the molecular pathways and genetic alterations that may be modulated by bufalin within the framework of this specific form of leukemia. The dataset elucidates the gene expression profiles of HL60 cells at 6, 12, 24, and 48 hours post-treatment with bufalin at a concentration of 12.5nM, identifying DEGs at various time points through comparative analysis. The total number of genes detected at these intervals were 13,166, 10,291, 11,152, and 13,762, respectively. A noteworthy observation was that the expression levels of 1,798, 1,330, 1,454, and 2,525 genes, respectively, were altered by a factor of≥2-fold (|log2foldchange|≥1) at 6, 12, 24, and 48 hours post-treatment (Fig. 1A). Intriguingly, the number of DEGs did not exhibit a consistent increase or decrease with time but instead peaked immediately post-treatment, reached a base at 12 hours, and subsequently rose over time. This phenomenon may suggest that bufalin induces a robust stress response in cells, which subsequently self-regulates to maintain homeostasis within the cellular milieu. Ultimately, as treatment duration accumulates, bufalin induces alterations in gene transcription levels, upon which subsequent cellular responses are predicated. Additionally, we observed intersections of DEGs at different time points post-bufalin treatment (Fig. 1B), with seven genes common to all four time points. Further scrutiny revealed that these genes are pivotal hub genes within the Cell adhesion molecules (CAMs), Cytokine-cytokine receptor interaction, JAK-STAT signaling pathway, and Wnt signaling pathway, focusing on the primary category of Environmental information processing (EIP). This indicates that bufalin modulates cellular mechanisms through these five pathways (Fig. 1C).

In order to probe deeper into the underlying mechanisms of bufalin in the context of myeloid leukemia, we undertook a GSEA on the DEGs identified in our previous screening. Upon selecting the signaling pathways within the EIP classification, we observed that the activity levels of these pathways varied at distinct time points (Fig. 2A, 2B). It is quite evident that both the JAK-STAT signaling pathway and the Wnt signaling pathway are pivotal in the cellular signaling cascades influenced by bufalin, correlating with the outcomes of the common genes pathway previously discussed. Furthermore, we detected a robust negative correlation between the activity of the JAK-



C

gs_exact_source	gene_symbol	gs_description	main_class
hsa04514	PTPRC	Cell adhesion molecules (CAMs)	Environmental Information Processing
hsa04514	PTPRC	Cell adhesion molecules (CAMs)	Environmental Information Processing
hsa04060	CXCL8	Cytokine-cytokine receptor interaction	Environmental Information Processing
hsa04630	CCND3	Jak-STAT signaling pathway	Environmental Information Processing
hsa04310	CCND3	Wnt signaling pathway	Environmental Information Processing

Fig. 1: (A) Mathematical analysis of DEGs. (B) The Venn diagram illustrates the overlap of DEGs across various time points; common genes present in all samples are depicted below the diagram. (C) Pathway analysis of the primary class of EPI, within which the common genes are located.

STAT signaling pathway and the differential expression of genes at various time intervals post-bufalin treatment, suggesting that the JAK-STAT signaling pathway is the principal signaling cascade activated by bufalin in AML, in contrast to the Wnt signaling pathway, which exhibited a lesser degree of correlation (Fig. 2C, 2D, 2E, 2F).

Consistent with expectations, the expression levels of BCL2 exhibited discernible variations in response to varying concentrations of bufalin, with a progressive decline observed as the concentration of bufalin increased. However, upon reaching a concentration of 25nM, cell proliferation appeared to be maximally suppressed, and any further escalation in bufalin concentration did not result in a concomitant reduction in BCL2 protein expression (Fig. 3A). Subsequently, we assessed the expression levels of JAK2 and STAT3, pivotal proteins situated within the JAK-STAT signaling pathway, in K562 cells subjected to diverse concentrations of bufalin to ascertain the alterations within the cellular JAK-STAT pathway. Our findings indicated that the JAK-STAT pathway was indeed activated subsequent to bufalin treatment. Nevertheless, the expression of JAK2 protein peaked at a concentration of 12.5nM and subsequently diminished with increasing bufalin concentrations. In contrast, STAT3, a downstream effector within the pathway, displayed a straightforward linear correlation with concentration, exhibiting a gradual decline as bufalin concentration rose. It is postulated that the non-linear alteration in JAK2 expression may be attributed to the cell stress response elicited by bufalin, which stimulates JAK2 transcriptional expression. This, in turn, modulates the establishment of a comprehensive JAK-STAT signaling cascade by activating STAT3, ultimately culminating in cellular apoptosis. However, when the treatment concentration or duration exceeds a certain threshold, the cellular emergency response induced by bufalin is either modulated by the cell's intrinsic negative feedback mechanisms to revert to a homeostatic state or bufalin surpasses the cellular tolerance threshold, resulting in a downregulation of overall cellular activity and a subsequent reduction in JAK2 expression levels.

It is evident that bufalin elicits cellular apoptosis via the JAK-STAT signaling pathway; however, the precise influence of bufalin on the pathway's activity remains enigmatic. To elucidate the mechanism of action with greater specificity, we conducted simulations and analyses to assess the congruence between the molecular architecture of bufalin and various protein structures. By comparing binding energies, we evaluated bufalin's direct target protein and postulated its potential targets and mechanisms of action (Fig. 4A, 4B). Utilizing the median binding energy as the criterion for selection, proteins with binding energies below the median were filtered out and subsequently employed as a dataset for comprehensive KEGG and GO analyses (Fig. 4C, 4D). The outcomes depicted in Fig. 4C reveal that ABC transporters exhibit the highest level of confidence. Moreover, several transport signaling pathways are implicated in the GO results. All these results suggest that bufalin may directly influence cell transporters and mediate the regulation of downstream pathways.

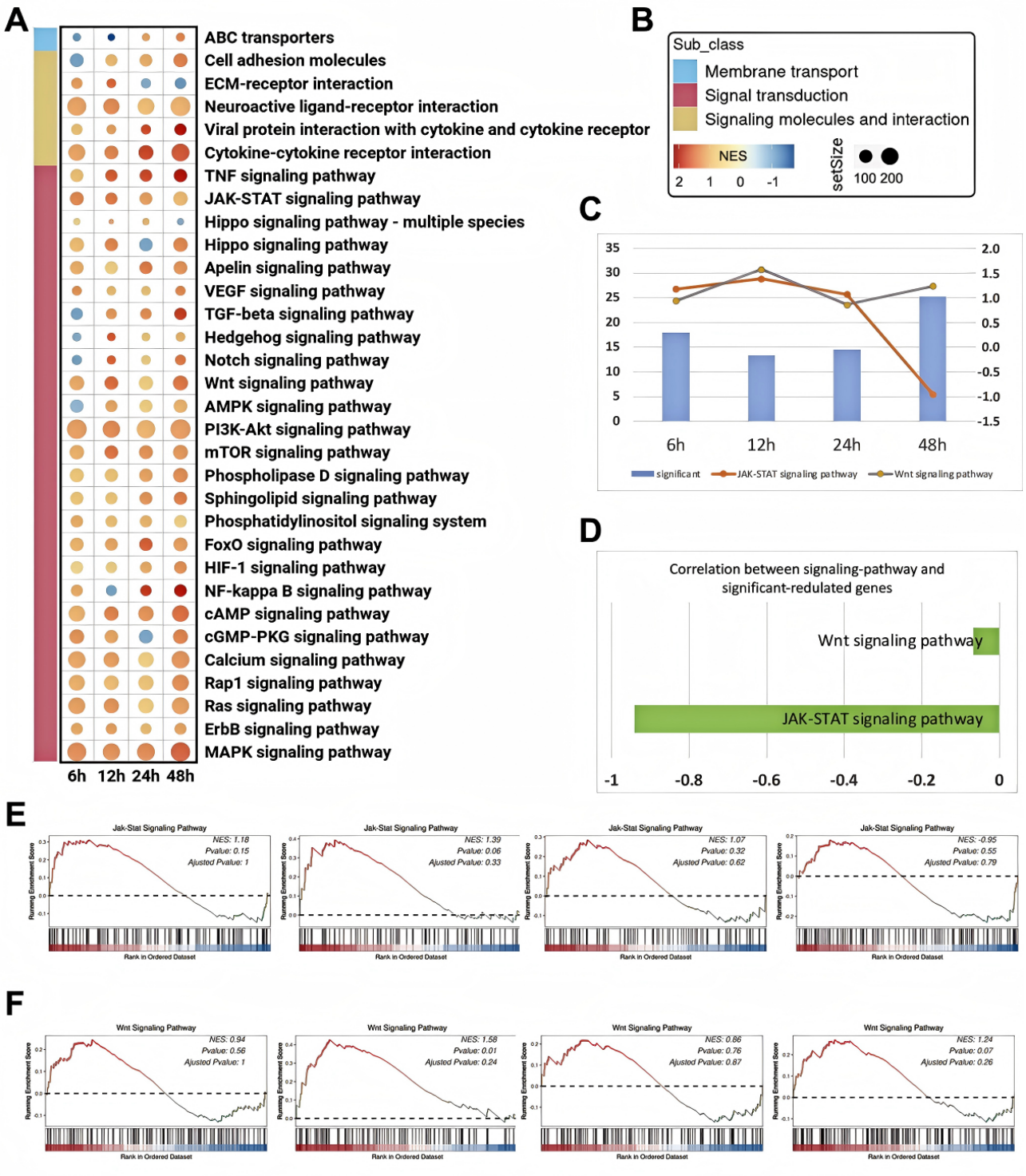


Fig. 2; (A) Hierarchical clustering tree heatmap of genes in samples from GSE6347, illustrating up-regulated (red dots) and down-regulated (blue dots) differentially expressed genes. (B) Legend for (A). (C) Lines represent the NES) for the Jak-STAT signaling pathway and Wnt signaling pathway; bar plots indicate significant genes at various time points for the samples. (D) Correlation between signaling pathways and significantly regulated genes. (E) GSEA reveals the significantly enriched JAK-STAT pathway between control and bufalin-treated samples at 6h, 12h, 24h, and 48h, respectively. The p-value was determined using a one-sided permutation test, comparing observed NES. The FDR was controlled using the Benjamini-Hochberg method. (F) The same as (E), but for the Wnt-signaling pathway.

In order to elucidate the specific mechanisms by which bufalin modulates transporter signaling, we conducted simulations and analyses to investigate the interaction between bufalin and transporter proteins (PDB:5YW7). The compound's two-dimensional structure was meticulously crafted using ChemDraw, and subsequently, it was imported into ChemDraw 3D for three-dimensional modeling. Energy minimization was executed employing the MM2 module to ascertain the most favorable conformation characterized by minimal energy, which was then saved in mol2 format. The protein structure was retrieved from the Protein Data Bank (PDB) and visualized independently with the aid of PyMOL. Subsequently, the ligand and receptor were saved as separate pdbqt files using Mgtools 1.5.6, after undergoing dehydrogenation, hydrogenation, charge calculation, and

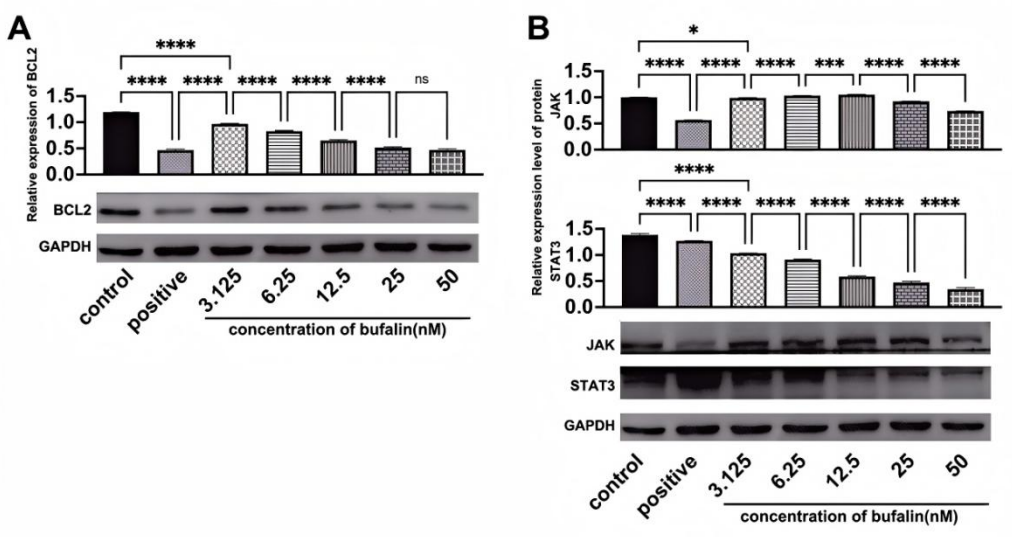


Fig. 3: (A) The levels of BCL2 protein in K562 cells treated with varying concentrations of bufalin were assessed using Western blot analysis, with serial dilutions of whole cell extracts employed for semi-quantitative evaluation. GAPDH served as a loading control. Statistical significance was denoted as follows: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. (B) The procedure was identical to (A), except that the analysis focused on Jak and STAT3 proteins.

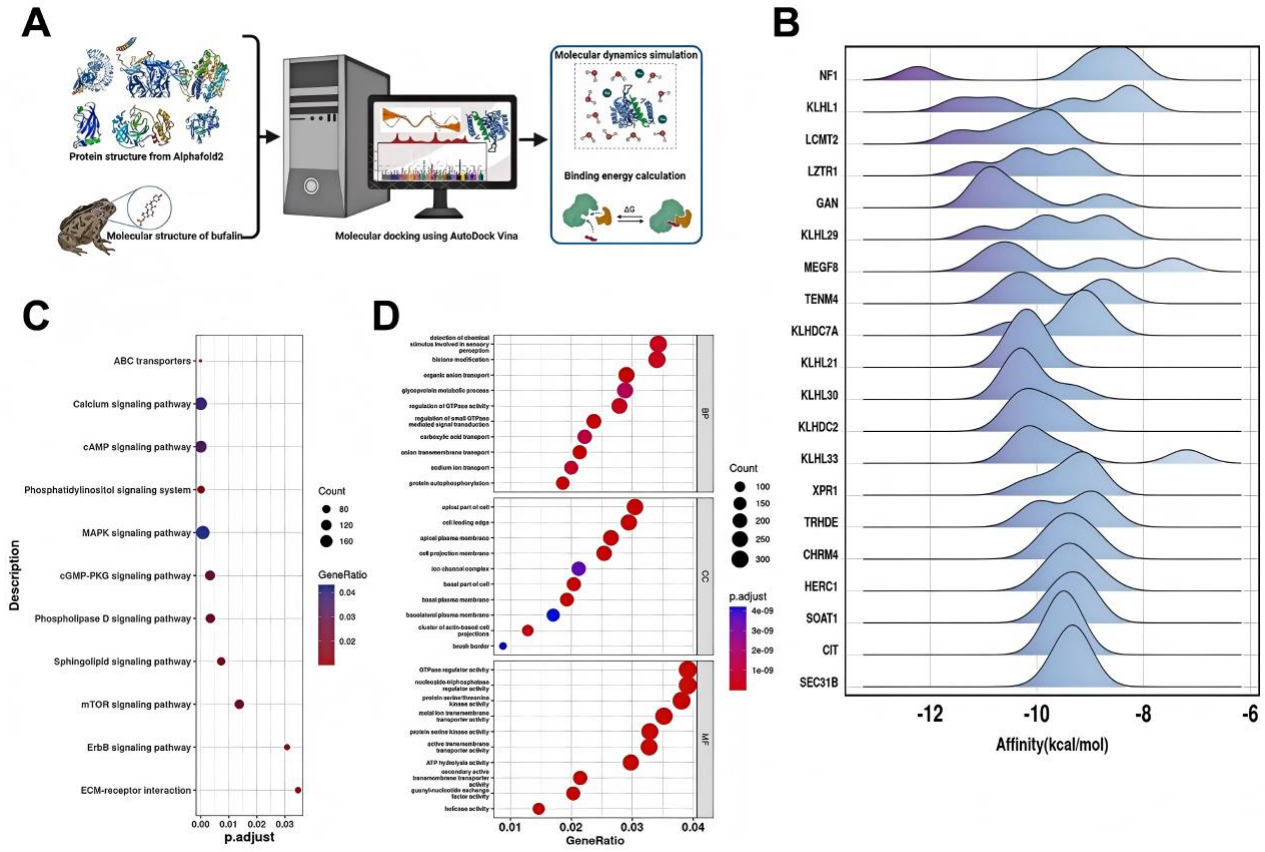


Fig. 4: (A) Analysis diagram of AutoDock Vina. (B) Protein affinity analysis using AutoDock Vina, with the top 20 results displayed. (C) Enrichment analysis of KEGG pathways within the classification of EPI. (D) Enrichment results for the initial 10 GO terms, where the X-axis represents the proportion of DEGs enriched within each GO term. The color intensity corresponds to the magnitude of the corrected p-value, with a redder color indicating a larger value. The size of each dot signifies the count of enriched genes, encompassing biological process (BP), cellular component (CC), and molecular function (MF).

merging of nonpolar hydrogens. The docking of the ligand to the receptor was accomplished using Autodock Vina 1.1.2, with the docking parameters specified below. The higher-scoring conformations were visualized using PyMOL and Discovery Studio (Fig. 5A). Bufalin exhibited a robust binding affinity to the protein's active pocket, with a binding energy of -9.2 kcal/mol, which is lower than the -8 kcal/mol threshold, indicating a superior hydrophobic interaction with the protein-binding pocket (Fig. 5B). Further analysis revealed that the hydrophobic moieties of bufalin engaged in hydrophobic interactions with amino acid residues LEU434, TRP430, PHE433, ILE381, and TYR377, culminating in the formation of a complex where the ligand is bound to the protein's active pocket. These findings suggest that bufalin has a strong affinity for the active pocket of the transporter protein. This implies that bufalin may bind with considerable ease to the transporter protein, rendering it challenging for the compound to dissociate once it encounters the active pocket, thereby potentially obstructing the transporter protein's normal functional activity.

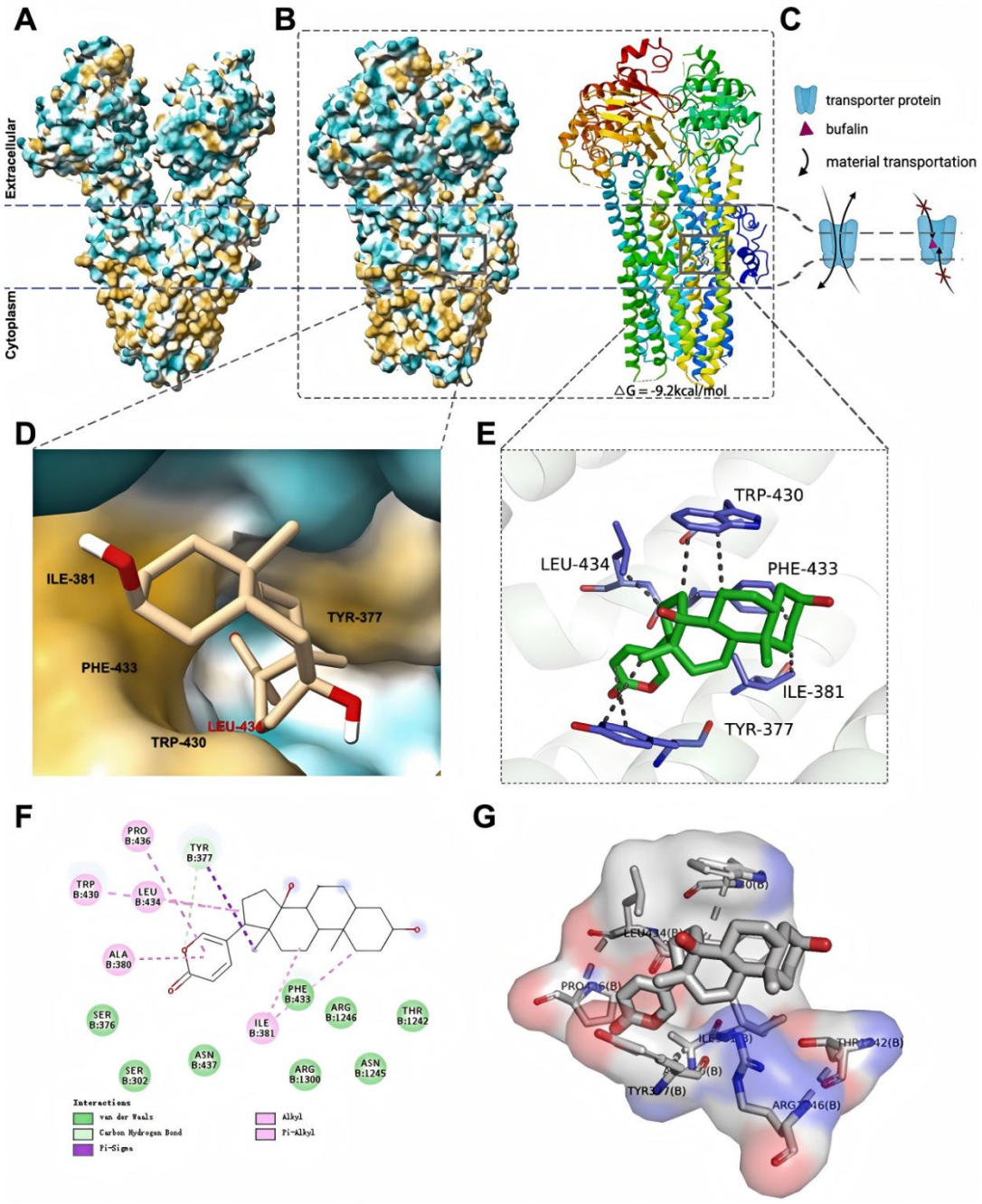


Fig. 5: (A) Three-dimensional structure of ABC3C protein. (B) Lateral view of (A). (C) Interaction bonds between bufalin and proteins. (D) Schematic representation of bufalin target sites and the interaction forces with collar proteins. (E) Schematic representation of bufalin binding to the transporter and its impact on the transporter's normal functional conformation. The left side illustrates the normal driving function of the transporter, while the right side depicts the blockage of the protein transport function following the occupation of the transporter's active pocket by bufalin.

To find out the involvement of autophagy in the mechanism of cell survival, which is influenced by bufalin, we conducted an analysis of autophagic activity using K562 cell line. During the process two renowned indicators of autophagy are used and we observed the following: 1) P62 (Turco *et al.*, 2021) an ubiquitin-binding receptor protein which is targeted by the autophagic process for degradation (Fig. 6A), 2) LC3A/B (Nath *et al.*, 2014), which is a specific form of LC3 which becomes integrated into the membrane of phagophore (precursor to the autophagosomes) after conjugation with the phosphatidylethanolamine followed by the induction of autophagy (Fig. 6B), and 3) ULK1 (Torii *et al.*, 2020), which is a catalytic element of the pre-initiation complex of autophagy that enhances autophagy by phosphorylating ATG14 between other substrates (Fig. 6C). Our results were particularly unexpected, as bufalin was strikingly observed to modulate the induction of autophagy in K562 cells, as verified by the alterations in P62 levels and the ratio of LC3-II/I.

DISCUSSION

Bufalin has revealed remarkable efficacy against myeloid leukemia which is basically derived from the venom of the toad *Bufo bufogargarizans*(Pan *et al.*,

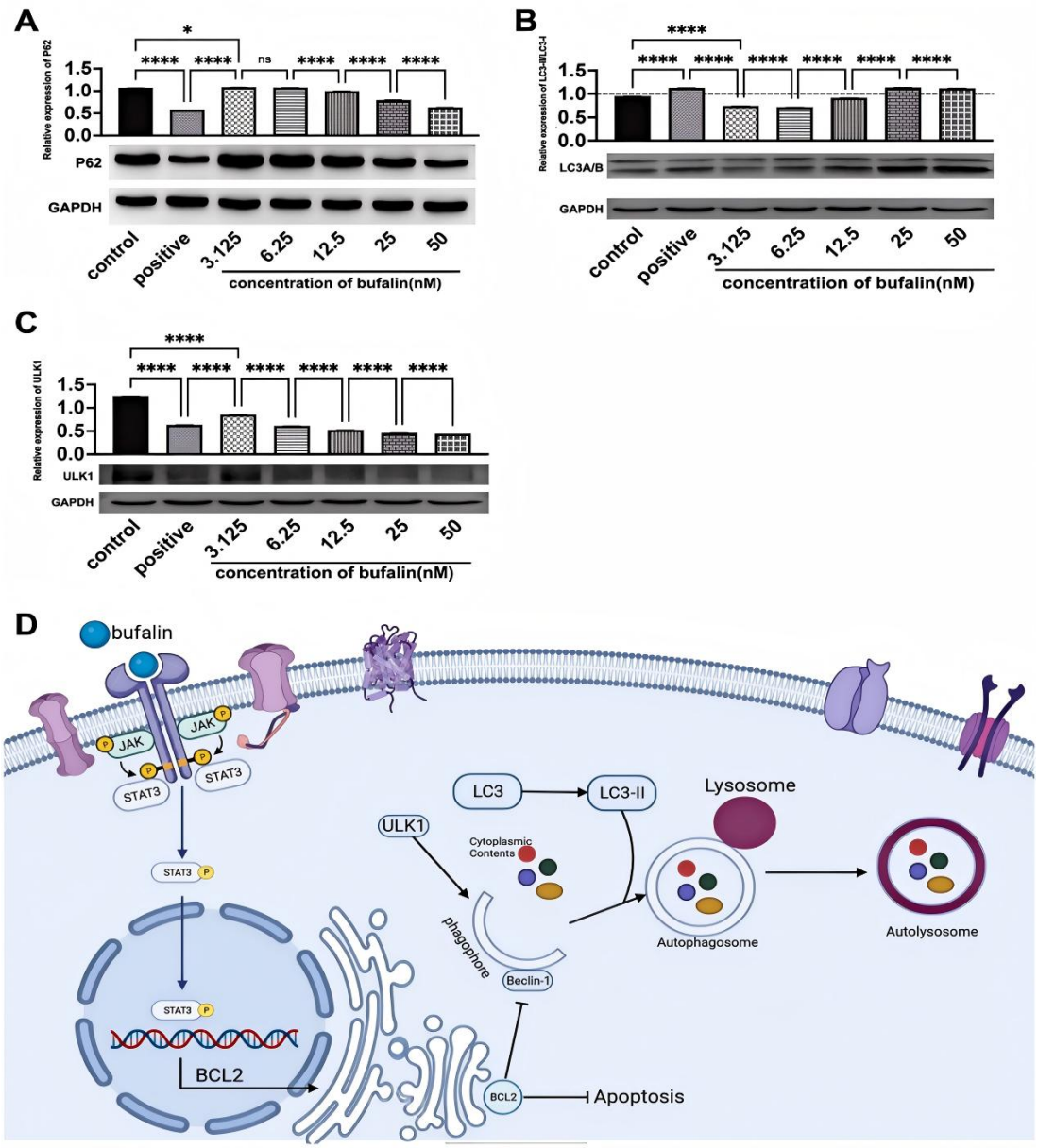


Fig. 6: (A) The protein levels of P62 in K562 cells treated with varying concentrations of bufalin were assessed using Western blot analysis with serial dilutions of whole cell extracts for semi-quantitative analysis. GAPDH served as a loading control. Statistical significance was denoted as follows: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. (B) The procedure was identical to (A), but for the detection of LC3A/B. (C) The procedure was identical to (A), but for the detection of ULK1. (D) Schematic representation of the research methodology.

2020). This type of cancer is generally linked to abnormal regulation of signaling pathways, mainly the JAK-STAT pathway, which helps the expansion of cell mass sustains survival of the cell while avoiding apoptosis (Li *et al.*, 2018; Deng *et al.*, 2024). The JAK-STAT signaling pathway, regularly hyperactive in leukemia because of different mutations or due to the overproduction of cytokines, is important for the expression of genes that enhances cell survival, especially those that regulate the synthesis of anti-apoptotic proteins for example BCL-2 and MCL-1. Consequently, the anti-leukemic effect of the bufalin is basically attributed to its capacity to disrupt the JAK-STAT signaling network, as a result of which is inducing apoptosis and autophagy in leukemic cells (Soumoy *et al.*, 2022).

The reactivation of JAK1 and JAK2 protein phosphorylation, together with their target proteins JAK3 and JAK5, makes bufalin ineffective against cells, consequently allowing for cell survival (Lin *et al.*, 2016; Peng *et al.*, 2023). Bufalin not only induces the apoptotic factors, but it also disrupts the potential of the inner mitochondrial membrane, followed by the release of cytochrome c into the cytoplasm and initiation of the intrinsic apoptotic pathway. After the release of cytochrome c into the cytoplasm, it activates caspase-9 which provokes the activation of effector caspase-3. After activation, caspase-3 deactivates poly (ADP-ribose) polymerase (PARP), followed by irreversible cell death. Based on these events, bufalin profoundly increases STAT3 and STAT5-mediated apoptosis in myeloid leukemia cells, leading to the deprivation of survival signals in these cells.

Besides apoptosis, bufalin also triggers autophagy which is a process of cellular degradation and recycling which also plays a somewhat contraindicated role in cancer. As a result of which, the level of mTOR activity

which is an inhibitor of autophagy is decreased because of the blockade of the JAK-STAT pathway by bufalin. Autophagic flux is also increased which can be calculated by the presence of autophagic markers which includes LC3-II and Beclin-1. The autophagic process induced by bufalin can be coordinated with apoptosis to remove leukemic cells. For instance, the extreme stimulation of autophagy can exceed the invasive stress tolerance threshold of the cells which ends up into cell death due to autophagy. Moreover, the bufalin-induced apoptotic process together with enhanced ROS generation, may represent a notable regulatory molecular event upstream which makes cancer cells to go through both processes, developing a self-destructive loop in leukemic cells (Miao *et al.*, 2013; Zhao *et al.*, 2017).

The above mentioned mechanisms have been authenticated by experimental studies. *In vitro* targeting of different myeloid leukemia models which were treated with bufalin showed remarkable reduction in cell viability and also the induction of apoptotic features which includes chromatic condensation and initiation of caspases within the treated cells. This represents the therapeutic capability of bufalin against myeloid leukemia because it significantly induces programmed cell death in these cancer models. Also the increased level of LC3-II (an autophagy marker) shows that bufalin seems to have worked on both pathways (Lan *et al.*, 2024). *In vivo* experiments also represented that the model with animals having leukemia was able to survive longer and also had decreased tumor burden after treatment with bufalin along with the lower-level activity of the JAK-STAT signaling pathways and enhanced rates of apoptosis together with autophagy.

The JAK-STAT signaling pathways highlights the potential therapeutic efficacy of bufalin for myeloid leukemia, as it has been revealed to evoke apoptosis and autophagy. To decrease the limitations of conventional treatments of targeting leukemic cells and their resistance profiles, bufalin is considered as a captivating option. Its potential to modulate both ways of programmed cell deaths shows its potential use in synergistic treatments, supplementing the efficacy of already present chemotherapy regimes or JAK pathway inhibitors. These results emphasize the necessity for further research into bufalin's application for myeloid leukemia therapy (Bernard, 2023).

Organisms usually activate autophagy-related pathways after they come into contact with toxic medicinal herbs, to overcome the toxicity of the herbs and maintain the homeostasis of striatal cells (Yang *et al.*, 2023). LC3-I and LC3-II show two isoforms of LC3, and LC3-II/LC3-I ratio is represented as a biomarker for autophagy (Mu *et al.*, 2023). P62 works as a bridge which connects ubiquitinated protein aggregates to LC3, helping in degradation of autophagic proteins, along with its collection which is inversely proportional to autophagic activity (Ray *et al.*, 2023). Our study shows a significant upregulation of p62, along with a downregulation of LC3-II/I in K562 cells exposed to bufalin. This indicates that bufalin induces autophagy in the early stages such as during the phagophore and autophagosome formation and disrupts P62-mediated degradation in the later stages of autophagy for example autolysosome.

Autophagy demonstrates a crucial mechanism of elimination of misfolded or dysfunctional proteins and organelles inside cells, and prevention of this mechanism leads to cell death. To understand the pathogenesis and progression of Leukemia, it is important to find out the mechanism of blood cell death. BAX which is distributed in the cell cytosol, relocate to mitochondria when receiving signals of apoptosis followed by release of cytochrome c from mitochondria into the cytosol and inducing cell death (Li *et al.*, 2023b). BCL2 maintains the stability of membrane permeability and stops the release of cytochrome c from mitochondria (Gourisankar *et al.*, 2023). The proapoptotic BAX and the antiapoptotic Bcl-2 work together to maintain homeostasis of mitochondrial permeability. The present study represented that bufalin triggers apoptosis in K562 cells by decreasing BCL2 expression (Yin *et al.*, 2012; Dai *et al.*, 2018; Ding *et al.*, 2018; Huang and Zhang *et al.*, 2018; Xie *et al.*, 2019; LingHu *et al.*, 2020).

AML and CML are two different forms of myeloid leukemia, distinguished by differences in pathological characteristics, clinical manifestations, and therapeutic approaches (Sanz *et al.*, 2023). An AML-specific experimental model which gives that GSE6437 dataset utilized HL60 cells, we try to explore more that whether the JAK-STAT signaling pathway is also modulated by bufalin to show stress responses and apoptosis in CML. To this end, we used K562 cells which are hematopoietic cell line obtained from the bone marrow of a 53-year-old female with CML in blast crisis in 1999 (Bressin *et al.*, 2023), to assess alterations in the expression levels of key proteins within the JAK signaling pathway, our main experimental subject. This makes us to discover similarities and differences between CML and AML with the reference of bufalin-induced mechanistic responses. Furthermore, we compared cellular changes between different concentrations of bufalin treatment to get a more detailed understanding of mode of action on bufalin. At first, we select 48-hour time point to assess the expression levels of the BCL2, an apoptosis-related protein, while using different concentrations of bufalin treatment.

ABC transporters are classified into seven subfamilies, named ABCA through to ABCG, where transporters aiding the exchange between intracellular and extracellular spaces establish the main mechanism for regulating cell metabolism and signal transduction (Flatt *et al.*, 2023). This process is adapted by transporters which are located on the plasma membrane or intracellular membranes enveloping different organelles, in reaction to environmental signals. Autophagy demonstrates as a highly protected process in which surplus or dysfunctional macromolecules are transported to the lysosomes for degradation to produce basic components, which are finally recycled to assist survival among different stressors for example nutrient shortage (Bernard *et al.*, 2023).

Additionally, our experimental findings show that bufalin significantly stimulates the JAK-STAT signaling pathway, indicating its importance in the induction of apoptosis by bufalin. Molecular docking analyses show that bufalin can act directly to modulate protein activity on transporters, deriving in cellular stress responses. Various studies have shown a strong relationship between

the inhibition of transport pathways and cellular autophagy, as evidenced by Miao *et al.* (2013). It is likely to consider whether bufalin effect cellular transport, consequently inducing autophagy. Afterwards, another question arises: does bufalin activate the JAK-STAT signaling pathway to activate autophagy before its occurrence, as a result of that assisting cell apoptosis, or it is triggered after autophagy to sustain cellular homeostasis? Additional investigation is needed to confirm whether there is a direct correlation between JAK-STAT signaling pathway and autophagy (Zhao *et al.*, 2017).

Author contribution: Zelei Wu and Muhammad Azhar Memon contributed equally to the conceptualization, experimental design, and data analysis of the study. Zelei Wu and Yunqing Cui conducted the primary experiments and drafted the manuscript. Weixin Mao performed data analysis, including GSEA and KEGG pathway enrichment studies. Yingjun Li carried out molecular docking and protein-ligand interaction studies. Muhammad Azhar Memon provided critical technical insights and reviewed the manuscript for intellectual content. Lan Hai supervised the research, provided guidance throughout the study, and was responsible for correspondence and revisions to the manuscript. All authors read and approved the final version of the manuscript.

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