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Baicalein induces cervical cancer apoptosis via PTEN/PI3K/AKT pathway modulation

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Abstract: **Background**: Cervical cancer continues to be a major cause of female cancer deaths globally, with dysregulation of the PTEN/PI3K/AKT pathway contributing to disease progression and treatment resistance. Baicalein, a bioactive flavonoid, exhibits anti-cancer properties through incompletely understood mechanisms. **Objectives**: To investigated whether baicalein promotes cervical cancer cell apoptosis by modulating PTEN expression and PI3K/AKT. **Methods**: In this study, we investigated the role of baicalein in promoting apoptosis in SiHa cervical cancer cells, with a focus on its potential modulation of PTEN expression and the PI3K/AKT pathway. Cell viability and apoptosis were assessed following baicalein treatment at various doses and time points. The expression levels of PTEN, PI3K, AKT, and Bcl-2 family proteins were analyzed to elucidate the molecular mechanisms. Additionally, the functional impact of PTEN overexpression, alone or in combination with baicalein, was evaluated. **Results**: We demonstrated that baicalein treatment (IC50 = 53.3 μmol/L) induced dose- and time-dependent cytotoxicity and increased apoptosis through modulation of Bcl-2 family proteins. Mechanistically, baicalein upregulated PTEN expression while suppressing PI3K/AKT pathway components including AKT1 and PDK1. PTEN overexpression alone inhibited PI3K/AKT signaling and induced apoptosis (31.53%). Remarkably, combining baicalein with PTEN overexpression produced synergistic effects, achieving 46.83% apoptosis and maximally suppressing pro-survival signals while activating pro-apoptotic mechanisms. The combination treatment increased the Bax/Bcl-2 ratio 40-fold and reduced AKT1 and PDK1 expression by >80%. **Conclusion**: Our findings reveal that baicalein enhances PTEN tumor suppressor function to inhibit PI3K/AKT signaling, and suggest that combining natural compounds with tumor suppressor restoration represents a promising therapeutic strategy for cervical cancer.

Keywords: Apoptosis; Baicalein; Combination therapy; Cervical cancer; PTEN/PI3K/AKT pathway

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INTRODUCTION

Accounting for an estimated 604,000 incident cases and 342,000 mortalities per year, cervical cancer maintains its position as the world's fourth most frequently occurring cancer in females, representing a substantial disease burden (Zhang *et al*., 2022). Despite advances in prevention through HPV vaccination and screening programs, cervical cancer continues to pose substantial challenges, especially severe in low- and middle-income countries, due to systemic barriers limiting access to preventive care. (Bray *et a*l., 2024). Despite advances in oncology, the prognosis for advanced or recurrent cervical cance remains poor, as current treatments offer limited efficacy, reflected in 5-year survival rates below 20% (Vidal *et a*l., 2024). These statistics underscore the urgent need for novel therapeutic strategies that can effectively target cervical cancer cells while minimizing toxicity.

The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway plays a central role in cervical cancer pathogenesis, regulating crucial cellular processes including proliferation, survival and apoptosis (McCann *et al.,* 2020). Aberrant activation of this pathway, observed in over 60% of cervical cancers, contributes to treatment resistance and poor clinical outcomes (Schwarz *et al.,* 2012). The tumor suppressor PTEN (phosphatase and tensin homolog) serves as the primary negative regulator of PI3K/AKT signaling by dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP3), thereby preventing AKT activation (Lee *et al.,*1999). However, PTEN expression is frequently lost or reduced in cervical cancer through various mechanisms including mutations, deletions and epigenetic silencing (Cheung *et al*., 2004). This loss of PTEN function results in constitutive PI3K/AKT activation, promoting cancer cell survival and resistance to apoptosis.

Restoration of PTEN function represents an attractive therapeutic strategy for cancers with dysregulated PI3K/AKT signaling. However, direct pharmacological activation of tumor suppressors remains challenging, necessitating alternative approaches. Recent evidence suggests that certain natural compounds can modulate PTEN expression and enhance its tumor suppressor activity (Kim *et al*., 2017). Among these, Baicalein (5,6,7-trihydroxyflavone), a flavonoid isolated from Scutellaria baicalensis, is known for its anticancer effects. (Kim *et al*., 2013). Baicalein demonstrates anti-inflammatory, antioxidant and anti-proliferative properties across multiple cancer types. (Zhao *et al*., 2022).

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Previous studies have demonstrated that baicalein can induce apoptosis in cancer cells through multiple mechanisms, including cell cycle arrest, reactive oxygen species generation and modulation of apoptotic proteins (Nguyen *et al*., 2022). In cervical cancer specifically, baicalein has shown growth inhibitory effects, though the precise molecular mechanisms remain incompletely understood (McCann *et al*., 2020). Notably, emerging evidence suggests that baicalein may influence PTEN/PI3K/AKT signaling in certain cancer types (Yu *et al*., 2018), raising the possibility that it could restore pathway balance in PTEN-deficient cervical cancer cells.

Despite baicalein's documented anti-cancer effects and emerging evidence of its influence on PI3K/AKT signaling, three critical gaps remain: (1) Whether baicalein can upregulate PTEN expression in cervical cancer cells-where PTEN loss drives pathway dysregulation-has not been demonstrated; (2) The molecular interplay between baicalein-induced PTEN elevation and downstream apoptotic effectors remains uncharacterized; (3) Most importantly, whether pharmacological PTEN enhancement can potentiate baicalein's therapeutic efficacy through synergistic pathway suppression is unknown. Addressing these gaps is essential because monotherapy with either PTEN restoration strategies or natural compounds alone may be insufficient for effective treatment of PTEN-deficient tumors, while rational combinations targeting convergent pathways could enhance therapeutic responses. Furthermore, whether baicalein can enhance the tumor suppressor effects of PTEN restoration remains unknown. Understanding these interactions could reveal novel therapeutic strategies that combine natural compounds with molecular targeting approaches. We hypothesized that baicalein promotes cervical cancer cell apoptosis by upregulating PTEN expression, thereby inhibiting PI3K/AKT survival signaling. Additionally, we postulated that combining baicalein treatment with PTEN overexpression would produce synergistic anti-cancer effects through convergent targeting of the PI3K/AKT pathway.

This study systematically examined the impact of baicalein on cervical cancer cell viability and apoptosis, elucidating its potential therapeutic mechanisms and its impact on PTEN expression and PI3K/AKT signaling and evaluated whether combining baicalein with PTEN restoration could enhance therapeutic efficacy. Our findings provide mechanistic insights into baicalein's anti-cancer activity and suggest a potential combination strategy for targeting PI3K/AKT-driven cervical cancer.

While direct plasmid-mediated PTEN overexpression is not currently a clinical reality, this experimental approach serves as a valuable proof-of-concept model for several reasons: (1) It allows mechanistic dissection of whether enhancing PTEN function-through any future therapeutic modality (epigenetic reactivation, gene therapy, protein stabilizers, or small molecule PTEN activators currently in development)-could synergize with baicalein; (2) It tests the hypothesis that dual targeting of PI3K/AKT pathway (PTEN elevation + baicalein's direct effects) produces superior outcomes compared to single interventions; (3) Emerging therapeutic strategies including PTEN gene therapy vectors and demethylating agents (e.g., decitabine) that restore PTEN expression are under active investigation, making our combination rationale increasingly clinically relevant. Our approach thus establishes the mechanistic foundation for future studies combining baicalein with clinically feasible PTEN restoration methods.

MATERIALS AND METHODS

Cell culture and reagents

SiHa cells (obtained fromATCC) were grown in DMEM + 10% FBS, 100 U/mL penicillin and 100μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO2. Cells were routinely tested for mycoplasma contamination and used within 20 passages of thawing. Baicalein (purity >98%) was purchased from Sigma-Aldrich and DMSO to create a 100 mM stock solution, which was stored at -20°C and diluted in culture medium immediately before use. The final DMSO concentration in all experiments was maintained below 0.1%. SiHa cells were selected as they represent an HPV16-positive cervical cancer model with documented PI3K/AKT pathway dysregulation and reduced PTEN expression, making them appropriate for investigating PTEN-mediated pathway modulation. While validation in additional cell lines with diverse HPV backgrounds and PTEN status would strengthen conclusions (as discussed in limitations), SiHa cells provide a well-characterized model for initial mechanistic investigation.

Cell viability assay

SiHa cells were seeded in 96-well plates at a density of 5×10^3 cells per well and allowed to adhere overnight. Cells were then treated with baicalein at concentrations ranging from 20 to 120 μmol/L or vehicle control for 24, 48, or 72 hours. Following treatment, 10 μL of CCK8 reagent (Dojindo Molecular Technologies) was added to each well and incubated for 2 hours at 37°C. Absorbance was measured at 450 nm using a microplate reader (BioTek Synergy H1). Cell viability was calculated as the percentage of absorbance relative to vehicle-treated controls. IC50 values were determined using GraphPad Prism 9.0 software with nonlinear regression analysis.

Plasmid construction and transfection

The human PTEN cDNA was amplified from a HEK293 cDNA library and cloned into the pcDNA3.1(+) expression vector (Invitrogen) using standard molecular biology techniques. The construct was verified by Sanger sequencing. For transfection experiments, SiHa cells were seeded at 2×10^5 cells per well in 6-well plates and

transfected at 70% confluence using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Briefly, 2.5 μg of plasmid DNA (PTEN expression vector or empty vector control) was combined with 5 μL of Lipofectamine 3000 in Opti-MEM medium. After 48 hours of transfection, cells were treated with baicalein (53.3 μmol/L) or vehicle for an additional 72 hours before analysis.

RNA extraction and quantitative RT-PCR

RNA was extracted with TRIzol (Invitrogen), quantified via NanoDrop 2000 (Thermo Fisher) and reverse-transcribed into cDNA using the PrimeScript RT Kit (Takara Bio). qPCR was performed with SYBR Green Master Mix (Applied Biosystems) on a StepOnePlus system. The following gene-specific primers were used: PTEN (forward: 5'-TGGATTCGACTTAGACTTGACCT-3', reverse: 5'-GGTGGGTTATGGTCTTCAAAAGG-3'); AKT1 (forward: 5'-AGCGACGTGGCTATTGTGAAG-3', reverse: 5'-GCCATCATTCTTGAGGAGGAAGT-3'); PDK1 (forward: 5'-CTGTCAACGAGAGCAAGTCC-3', reverse: 5'-AAGCGTGCCATAGTACTCCA-3'); Bax (forward: 5'-CCCGAGAGGTCTTTTTCCGAG-3', reverse: 5'-CCAGCCCATGATGGTTCTGAT-3'); Bcl-2 (forward: 5'-GGTGGGGTCATGTGTGTGG-3', reverse: 5'-CGGTTCAGGTACTCAGTCATCC-3'); β-actin (forward: 5'-CATGTACGTTGCTATCCAGGC-3', reverse: 5'-CTCCTTAATGTCACGCACGAT-3').

PCR conditions consisted of initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Relative gene expression was calculated using the 2^(-ΔΔCt) method with β-actin as the internal reference.

Western blot analysis

Protein concentrations were determined by BCA assay (Pierce) after cell lysis in RIPA buffer (Beyotime) with inhibitors. Equal aliquots (30 μg) underwent SDS-PAGE (10%/12%) and PVDF transfer (Millipore). After 1 h blocking (5% milk/TBST, RT), membranes were probed (4°C, overnight) with: anti-PTEN (CST #9188), anti-AKT1 (#2938), anti-PDK1 (#3062), anti-Bax (#5023), anti-Bcl-2 (#4223) (all 1:1000, CST) and anti-β-actin (1:5000, Sigma #A5441).

Post-TBST washing, membranes were incubated (1 h, RT) with HRP-secondary antibodies (1:5000, Jackson). ECL detection used Bio-Rad reagents, with imaging on ChemiDoc XRS+ and quantification in Image Lab (Bio-Rad), normalized to β-actin.

Flow cytometry analysis of apoptosis

Apoptosis was assessed using the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences). Following treatment, cells were harvested by trypsinization, washed twice with cold PBS and resuspended in binding buffer at a concentration of 1×10^6 cells/mL. Cell suspensions (100 μL) were incubated with 5 μL Annexin V-FITC and 5 μL propidium iodide (PI) for 15 minutes at room temperature in the dark. After adding 400 μL of binding buffer, samples were analyzed within 1 hour using a BD FACSCalibur flow cytometer. Data were acquired from 10,000 events per sample and analyzed using FlowJo software version 10.8.1. Early apoptotic cells were defined as Annexin V-positive/PI-negative populations.

In addition to vehicle-treated negative controls, we included the following control conditions: (1) Unstained cells to set baseline fluorescence; (2) Single-stain controls (Annexin V-only and PI-only) for compensation; (3) Negative control vector-transfected cells to assess transfection-related effects. While a classic apoptosis inducer (e.g., staurosporine) was not included as a separate positive control, our PTEN overexpression group serves as a biological positive control, as PTEN restoration is an established inducer of apoptosis in PI3K/AKT-driven cancers. The dose-dependent apoptotic response to baicalein further validates assay functionality.

Synergy effects analysis

Synergistic effects between baicalein and PTEN overexpression were quantitatively evaluated using the Chou-Talalay method as implemented in CompuSyn software (Version 1.0, ComboSyn, Inc., Paramus, NJ, USA). This method is based on the median-effect principle and provides combination index (CI) values that quantify drug interactions.

For synergy analysis, the following experimental data were used:

- Baicalein alone: IC_{50} concentration (53.3 μmol/L) for 72 hours
- PTEN overexpression alone: standard transfection protocol
- Combination: baicalein (53.3 μmol/L) + PTEN overexpression

Apoptotic rates (determined by Annexin V-FITC/PI flow cytometry) from control, single-agent and combination treatments were entered into CompuSyn software. The fraction affected (Fa) was calculated as: Fa = (apoptotic rate in treatment group - basal apoptotic rate in control) / (100% - basal apoptotic rate in control).

CI values were calculated according to the equation: $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$

where $(D)_1$ and $(D)_2$ are the doses of baicalein and PTEN (represented by transfection effect) in combination that produce a given effect and $(Dx)_1$ and $(Dx)_2$ are the doses of each agent alone that produce the same effect.

CI values were interpreted as follows:

- CI < 0.9: synergism

- $0.9 \leq CI \leq 1.1$: additive effect
- $CI > 1.1$: antagonism

Isobologram analysis was performed to visualize synergistic interactions, where the combination data point falling below the line of additivity indicates synergism. Dose-reduction index (DRI) values were calculated to determine the fold-reduction in dose that could be achieved while maintaining the same effect level when used in combination compared to each agent alone.
Additionally, we calculated the predicted additive effect using the Bliss independence model: $E(A+B) = EA + EB - (EA \times EB)$, where EA and EB are the fractional effects of agents A and B individually. The observed combination effect was compared to this predicted additive effect using Student's t-test.

Statistical analysis
Experiments were repeated at least three times (mean ± SD). Statistical analyses were performed in GraphPad Prism 9.0: two-group comparisons used unpaired t-tests, multi-group comparisons used ANOVA with Tukey's post-hoc test ($P < 0.05$). Synergy was evaluated via CompuSyn (Chou-Talalay method).

Sample sizes (n=3 biological replicates per experiment) were determined based on preliminary data and established practices in cell-based apoptosis studies, where biological triplicates with technical replicates typically provide adequate statistical power to detect large effect sizes (Cohen's $d \geq 0.8$). Formal a priori power analysis was not conducted, which represents a limitation. Post-hoc power analysis using observed effect sizes and variance from our apoptosis data indicates that our sample size provided >90% power to detect the observed differences between combination treatment and single treatments ($\alpha=0.05$, two-tailed), suggesting adequate statistical power for our primary conclusions. However, future studies should incorporate prospective power analysis to optimize sample sizes.

RESULTS

Baicalein exhibits dose- and time-dependent cytotoxicity in cervical cancer cells
To evaluate the anti-cancer potential of baicalein in cervical cancer, we first examined its effects on SiHa cell viability using CCK8 assays. Baicalein treatment resulted in a concentration- and time-dependent reduction in cell viability (Fig. 1). After 24 hours of treatment, cell viability decreased modestly at concentrations up to 60 μmol/L, with more pronounced effects observed at higher doses. This cytotoxic effect was amplified with longer exposure times. At 48 hours, substantial reductions in viability were evident at concentrations ≥40 μmol/L, while 72-hour treatment showed marked cytotoxicity across all tested concentrations. The calculated IC50 value at 72 hours was 53.3 μmol/L, which was selected as the working concentration for subsequent mechanistic studies. These findings establish baicalein as a potent inhibitor of cervical cancer cell growth.

Baicalein induces apoptosis through modulation of mitochondrial pathway proteins
Having established baicalein's cytotoxic effects, we investigated whether apoptosis contributed to the observed cell death. Western blot analysis revealed that treatment with baicalein at the IC50 concentration (53.3 μmol/L) for 72 hours significantly altered the expression of key apoptotic regulators (Fig. 2). Specifically, baicalein treatment induced a 2.1-fold increase in the pro-apoptotic protein Bax ($P < 0.05$) while simultaneously reducing the anti-apoptotic protein Bcl-2 by approximately 50% ($P < 0.05$). This shift in the Bax/Bcl-2 ratio, a critical determinant of mitochondrial membrane permeabilization and apoptosis initiation, suggests that baicalein promotes cervical cancer cell death through activation of the intrinsic apoptotic pathway.

PTEN overexpression suppresses PI3K/AKT signaling in cervical cancer cells
Given the known dysregulation of PTEN/PI3K/AKT signaling in cervical cancer, we examined whether restoring PTEN expression could modulate this pathway. Transfection of SiHa cells with a PTEN overexpression vector resulted in a 3.2-fold increase in PTEN mRNA and a corresponding 2.5-fold elevation in protein levels compared to controls (Figs. 3a-c). This PTEN restoration led to significant downstream effects: AKT1 expression decreased by 45% at both mRNA and protein levels, while PDK1, a key activator of AKT, was reduced by 48% (all $P < 0.001$ versus control). The negative control vector had no significant effect on any measured parameters, confirming the specificity of PTEN-mediated pathway suppression. These results demonstrate that PTEN overexpression effectively inhibits PI3K/AKT signaling in cervical cancer cells.

Baicalein enhances PTEN expression and synergizes with PTEN overexpression to suppress PI3K/AKT pathway
To elucidate the molecular mechanisms responsible for baicalein's anti-tumor activity, with particular emphasis on its regulatory effects on the PTEN/PI3K/AKT signaling pathway., we performed comprehensive gene expression analysis across multiple treatment conditions (Fig. 3). First, we confirmed that transfection with PTEN overexpression vector successfully increased PTEN mRNA by 3.2-fold compared to controls, while the negative control vector showed no effect. This PTEN restoration alone significantly suppressed downstream targets, reducing AKT1 and PDK1 expression by approximately 45% and 48%, respectively ($P < 0.001$).

Interestingly, baicalein treatment alone increased endogenous PTEN mRNA by 1.5-fold while reducing AKT1 and PDK1 expression by approximately 30% each. The combination of PTEN overexpression and baicalein produced remarkable synergistic effects: PTEN mRNA increased 4.8-fold (versus 3.2-fold with PTEN alone), while AKT1 and PDK1 were suppressed by 75% and 80%, respectively ($P < 0.001$ for all comparisons).

Parallel changes occurred in apoptotic gene expression. Baicalein alone increased pro-apoptotic Bax mRNA 2.3-fold and decreased anti-apoptotic Bcl-2 by 45%. The combination treatment amplified these effects, with Bax increasing 4.5-fold and Bcl-2 decreasing by 85% compared to controls. These transcriptional changes demonstrate that baicalein not only modulates apoptotic genes directly but also enhances PTEN's tumor suppressor function, creating a coordinated anti-cancer response.

Protein-level validation confirms synergistic effects of combination treatment

To confirm that transcriptional changes translated to altered protein expression, we performed comprehensive Western blot analysis across all treatment conditions (Fig. 4). The protein expression patterns closely mirrored the mRNA findings. Combination treatment produced supra-additive PTEN elevation (5.2-fold vs. 3.9-fold predicted from individual effects), suggesting that baicalein not only independently upregulates PTEN but may also enhance stability or reduce degradation of ectopically expressed PTEN. This enhanced PTEN activity translated to near-complete suppression of survival signaling nodes (AKT1, PDK1 reduced >80%), effectively disabling the PI3K/AKT pro-survival machinery and shifting the cellular balance decisively toward apoptosis.

The apoptotic protein profile showed similar synergistic modulation. While PTEN overexpression or baicalein alone moderately affected Bax and Bcl-2 levels, their combination resulted in a 4.8-fold increase in Bax and an 88% reduction in Bcl-2 protein expression. The Bax/Bcl-2 ratio, a key indicator of apoptotic potential, increased 40-fold in the combination treatment group compared to controls. These protein-level changes confirm that baicalein and PTEN overexpression synergistically reprogram cervical cancer cells toward an apoptotic phenotype.

Combined treatment maximizes apoptotic cell death through pathway convergence

To determine the functional consequences of the observed molecular changes, we quantified apoptosis using flow cytometry (Figs. 5A and B). Consistent with the molecular data, PTEN overexpression alone increased early apoptotic cells from 8.27% to 31.53% ($P < 0.001$), while baicalein treatment alone induced 24.57% apoptosis. Remarkably, the combination of PTEN overexpression and baicalein treatment resulted in 46.83% early apoptotic cells, significantly exceeding either individual treatment ($P < 0.001$ versus PTEN alone or baicalein alone). The negative control groups showed apoptotic rates similar to untreated controls, confirming the specificity of the observed effects. Fig. 5c summarizes the molecular mechanism by which baicalein promotes apoptosis in SiHa cells through PTEN elevation and subsequent PI3K/AKT pathway inhibition.

Quantitative synergy analysis confirms supra-additive effects of baicalein and PTEN overexpression

To rigorously evaluate whether the observed enhancement represents true synergy versus simple additive effects, we performed Chou-Talalay combination index (CI) analysis using CompuSyn software. The combination of baicalein (53.3 μmol/L) with PTEN overexpression yielded a CI value of 0.73, indicating synergism ($CI < 0.9$) (Fig. 6A).

Isobologram analysis provided geometric visualization of this synergistic interaction (Fig. 6B). The experimental combination data point (red dot) fell substantially below the line of additivity (diagonal line connecting the two single-agent effective doses), confirming synergistic rather than additive interaction. The distance of the data point from the additivity line represents the degree of synergy, with our combination showing strong departure from additivity.

Dose-reduction index (DRI) analysis demonstrated that the combination strategy allows for significant dose-sparing effects (Fig. 6C). The DRI value of 2.1 for baicalein indicates that a 2.1-fold lower concentration of baicalein would be required in combination with PTEN overexpression to achieve the same apoptotic effect as baicalein monotherapy, suggesting potential for reduced toxicity in therapeutic applications.

We further validated synergy by comparing observed versus predicted additive effects using the Bliss independence model (Fig. 6D). The observed apoptotic rate in the combination treatment (46.83 ± 1.14%) significantly exceeded the predicted additive effect (42.15%, calculated as: 31.53% + 24.57% - [31.53% × 24.57%/100%]) ($P < 0.01$), providing additional evidence of supra-additive interaction. When normalized to control baseline (8.27% apoptosis), the fraction affected (Fa) values were: baicalein alone Fa = 0.178, PTEN alone Fa = 0.254, combination Fa = 0.420, confirming that the combination effect (Fa = 0.420) exceeds the sum of individual effects.

Collectively, these quantitative analyses-including CI value, isobologram positioning, DRI calculation and Bliss model comparison-provide robust evidence that baicalein and PTEN overexpression interact synergistically to promote cervical cancer cell apoptosis through convergent targeting of PI3K/AKT survival signaling.

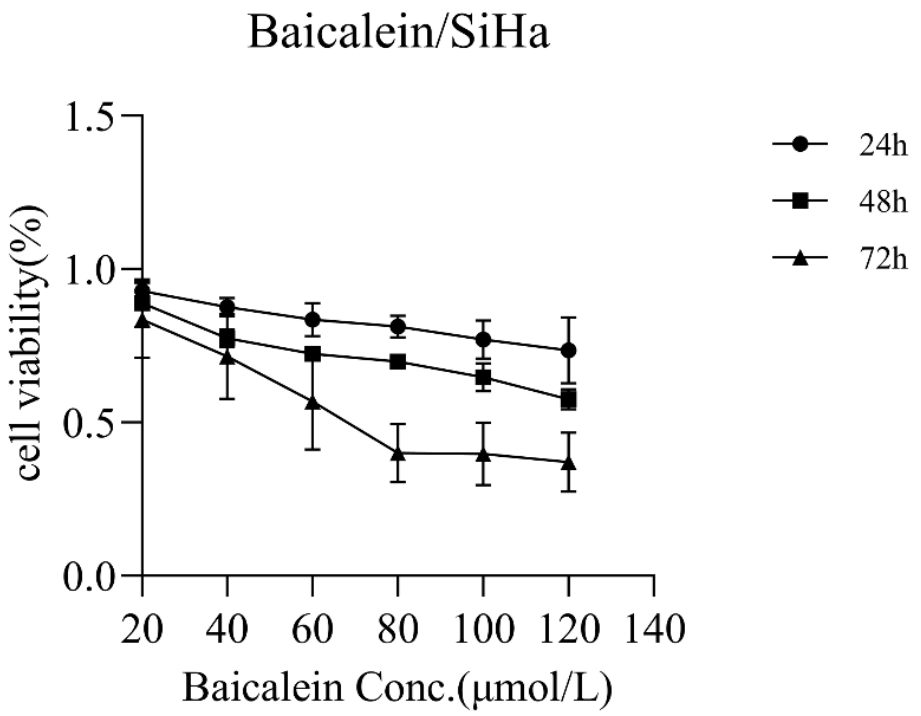

Fig. 1: Baicalein reduces SiHa cervical cancer cell viability in a dose- and time-dependent manner.
Cell viability was assessed using CCK8 assay after treatment with baicalein at concentrations ranging from 20 to 120 μmol/L for 24, 48, and 72 hours. The IC50 value of baicalein in SiHa cells at 72 hours was determined to be 53.3 μmol/L. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group at corresponding time points.

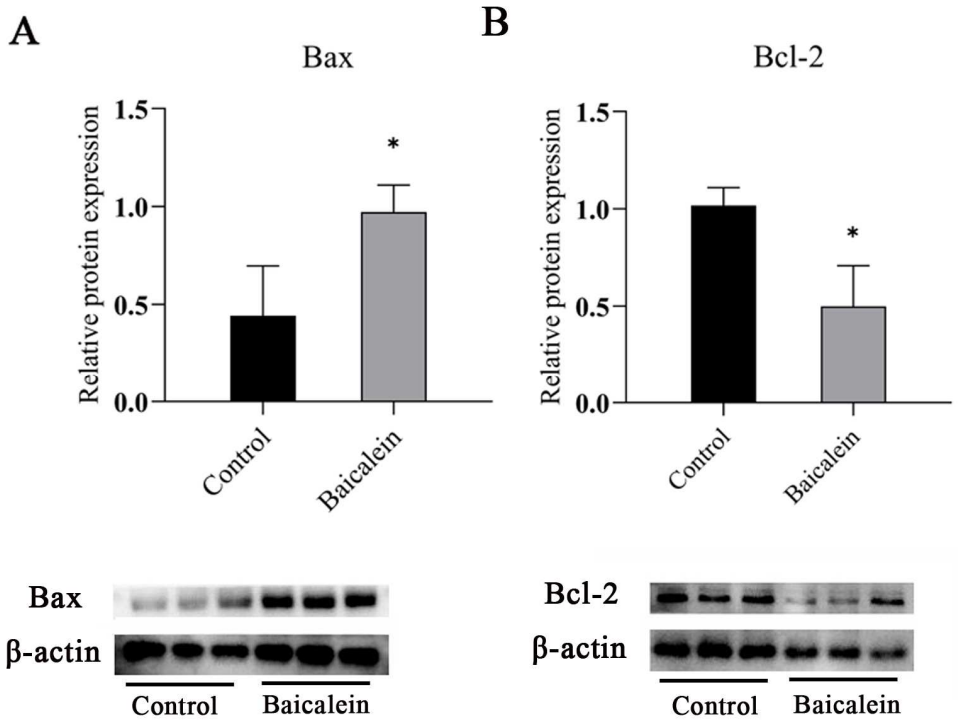

Fig. 2: Baicalein promotes apoptosis through modulation of Bcl-2 family proteins in SiHa cells.
Western blot analysis of Bax (A) and Bcl-2 protein expression (B) in SiHa cells treated with 53.3 μmol/L baicalein or vehicle control for 72 hours. β-actin served as loading control. Densitometry analysis shows relative protein expression normalized to β-actin. *P < 0.05 versus control group.

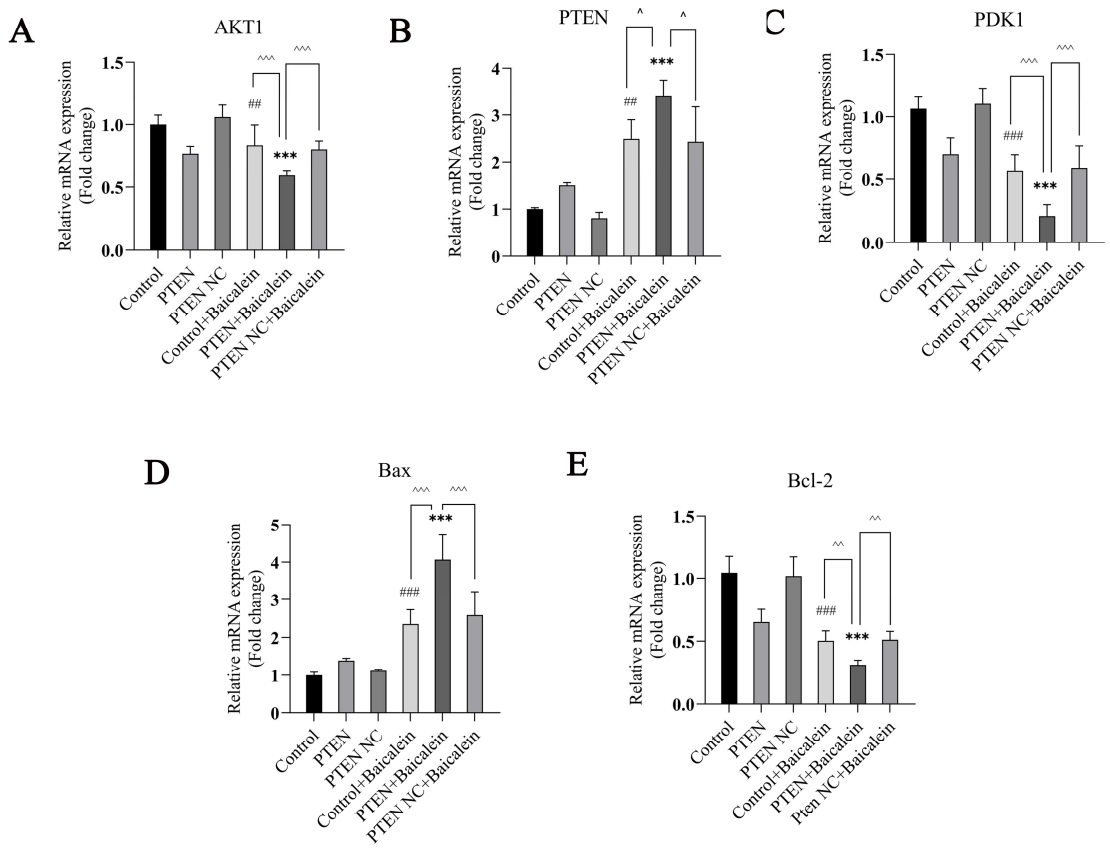

Fig. 3: Baicalein enhances PTEN-mediated suppression of PI3K/AKT pathway at the transcriptional level.
RT-qPCR analysis of (A) AKT1, (B) PTEN, (C) PDK1, (D) Baxs and (E) Bcl-2 mRNA expression in SiHa cells across six experimental groups: Control, PTEN overexpression, PTEN NC, Control+Baicalein (53.3 μmol/L), PTEN+Baicalein, and PTEN NC+Baicalein. Expression levels were normalized to β-actin. *P < 0.05, **P < 0.01, ***P < 0.001; ##P < 0.01, ###P < 0.001; ^^P < 0.01, ^^^P < 0.001 (specific comparisons detailed in figure).

The synergistic induction of apoptosis by combination treatment (combination index < 1.0) demonstrates that baicalein and PTEN target complementary nodes in the survival signaling network. While PTEN directly antagonizes PI3K/AKT signaling, baicalein appears to both enhance PTEN expression and independently modulate apoptotic proteins, creating a multi-pronged assault on cancer cell survival mechanisms.

DISCUSSION

This study provides compelling evidence that baicalein promotes cervical cancer cell apoptosis through PTEN elevation and subsequent PI3K/AKT pathway inhibition. Our findings reveal a novel mechanism whereby baicalein not only directly modulates apoptotic proteins but also enhances PTEN expression, creating a multi-targeted approach to cancer cell elimination. The synergistic effects observed with combined baicalein treatment and PTEN overexpression highlight the potential for developing combination therapies that simultaneously restore tumor suppressor function and inhibit survival signaling.

The cytotoxic effects of baicalein on SiHa cells, with an IC50 of 53.3 μmol/L at 72 hours, are consistent with previous reports in other cancer types (Kim *et al*., 2013). This concentration falls within the pharmacologically achievable range, as plasma concentrations of 20-100 μmol/L have been reported following oral administration in animal models (Tian *et al.,* 2012). However, human pharmacokinetic data remain limited, with conventional oral dosing typically yielding plasma concentrations of 1-10 μM-substantially below our experimental IC50. This represents a translational challenge requiring alternative delivery strategies: local/regional administration (intravaginal, direct tumor injection), nanoformulation for enhanced bioavailability, or combination regimens exploiting synergistic effects at lower doses.

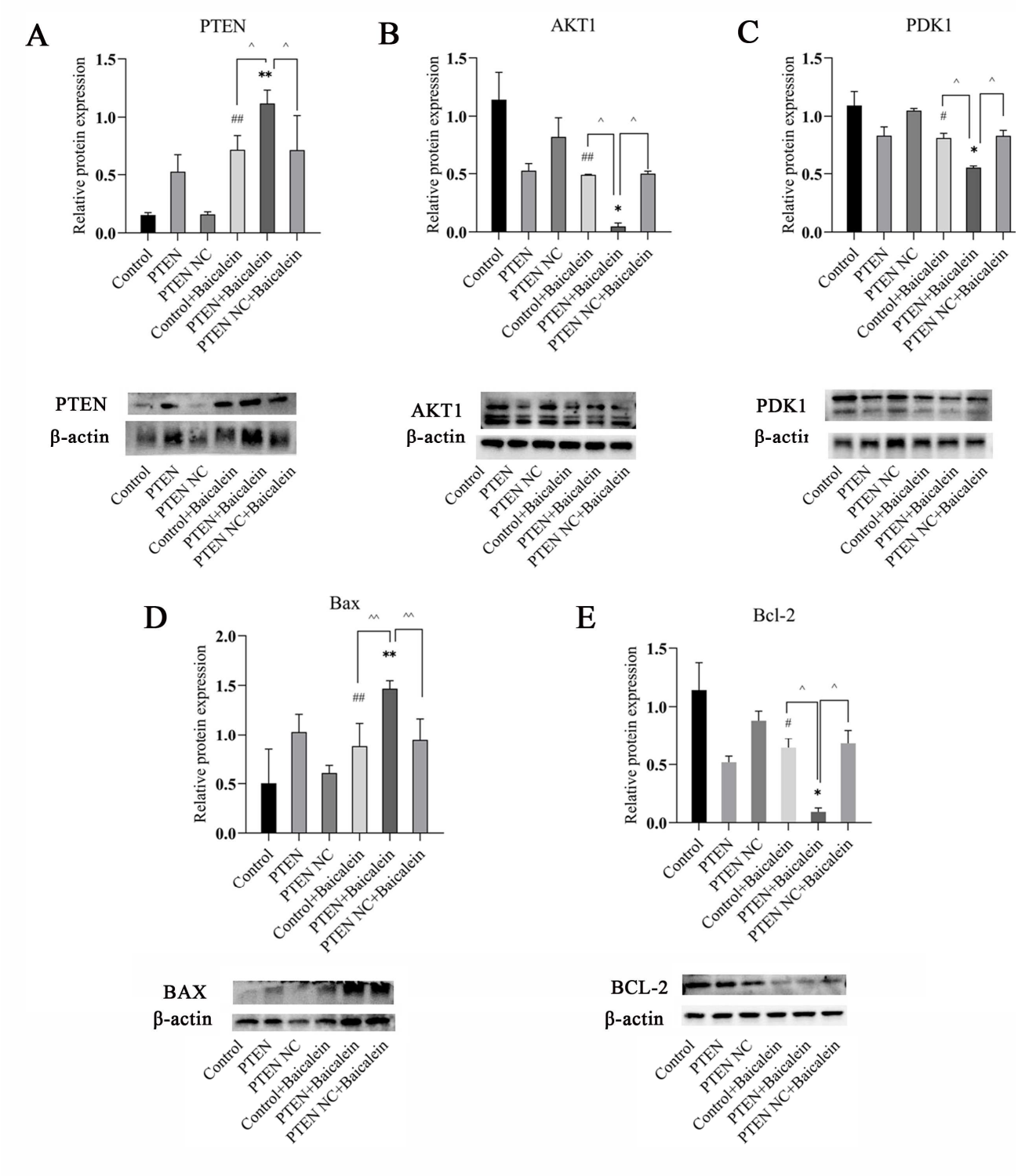

Fig. 4: Baicalein synergizes with PTEN overexpression to modulate apoptotic signaling at the protein level.
Representative Western blot images showing expression of PTEN (A), AKT1 (B), PDK1 (C), Bax (D) and Bcl-2 (E) proteins across all six experimental groups. β-actin served as loading control. (Upper panel) Densitometry quantification of PTEN, AKT1, PDK1, Bax, and Bcl-2 protein levels normalized to β-actin.

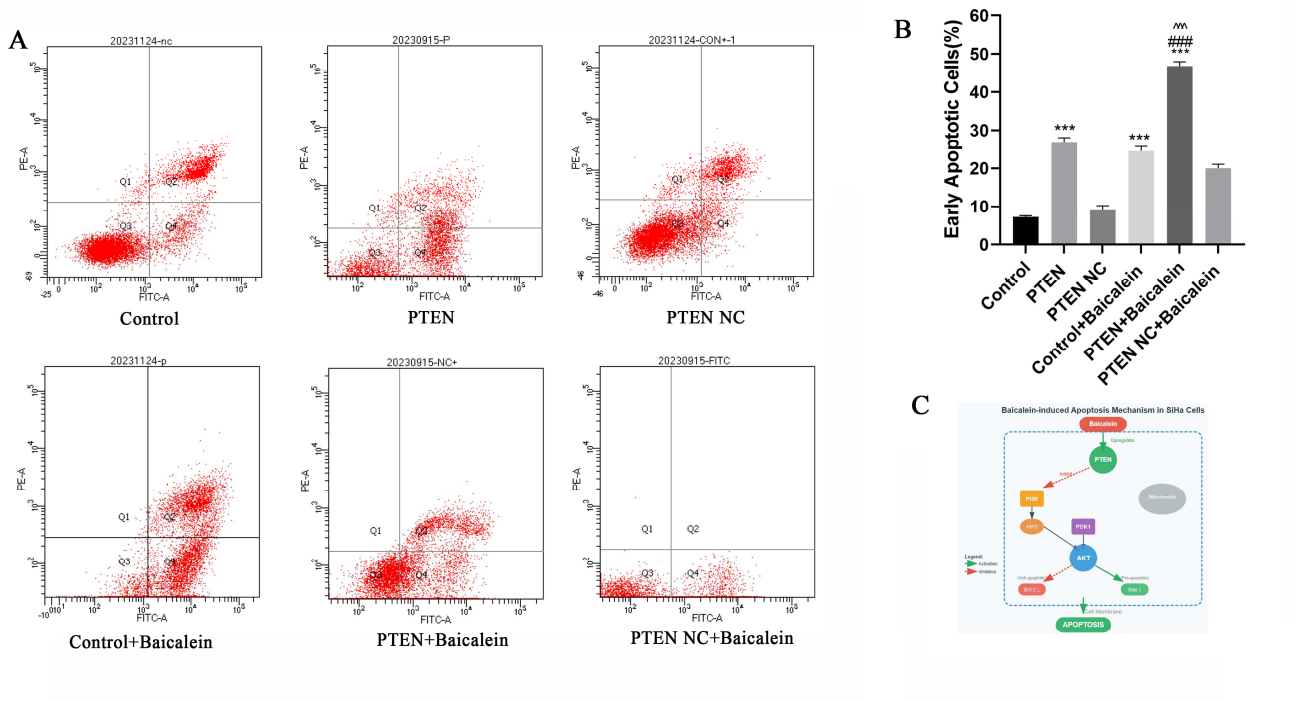

Fig. 5: Combination of baicalein and PTEN overexpression synergistically induces apoptosis in SiHa cells.
(A) Representative flow cytometry plots showing apoptosis analysis using Annexin V-FITC/PI double staining. Early apoptotic cells are shown in the Q3 quadrant (Annexin V+/PI-). (B) Quantification of early apoptotic cell percentages across all treatment groups. Blue bars represent control conditions, green bars represent baicalein-treated groups. ***P < 0.001 versus Control; ###P < 0.001 versus PTEN; ^^^P < 0.001 versus Control+Baicalein. The combination of PTEN overexpression and baicalein treatment showed the highest apoptotic rate (46.83±1.14%), demonstrating significant synergistic effects compared to either treatment alone. (C) Schematic illustration of baicalein-induced apoptosis in cervical cancer SiHa cells via the PTEN/PI3K/AKT signaling pathway. Baicalein upregulates PTEN expression, which inhibits PI3K activity and subsequent PIP3 production. This leads to reduced AKT activation by PDK1, resulting in downregulation of anti-apoptotic Bcl-2 and upregulation of pro-apoptotic Bax. The shift in Bcl-2/Bax ratio triggers mitochondrial-mediated apoptosis. Green solid arrows indicate activation/upregulation; red dashed arrows indicate inhibition/downregulation.

Comprehensive human pharmacokinetic studies coupled with formulation optimization will be essential for clinical translation. The time- and dose-dependent nature of baicalein's effects suggests cumulative cellular damage, possibly involving multiple death pathways. Importantly, our observation that baicalein modulates the Bax/Bcl-2 ratio confirms activation of the intrinsic apoptotic pathway, a mechanism previously reported in hepatocellular carcinoma and breast cancer cells (Nguyen *et al*., 2022).

However, translational challenges must be acknowledged. The micromolar concentrations required for efficacy substantially exceed plasma levels typically achieved with oral baicalein in humans (1-10 μM), limiting immediate clinical applicability. Strategies to bridge this gap include: (1) alternative delivery routes enabling higher local concentrations (intravaginal formulations, intratumoral injection); (2) nanoparticle-based formulations demonstrated to enhance flavonoid bioavailability 5-10 fold; (3) exploitation of synergistic combinations (our DRI analysis suggests 2.1-fold dose reduction is achievable) to lower required concentrations. Our findings should thus be viewed as mechanistic proof-of-concept requiring substantial pharmaceutical development before clinical translation.

The principal discovery is that baicalein enhances PTEN expression at both mRNA and protein levels. While prior studies have shown baicalein's inhibitory effects on PI3K/AKT signaling in cervical cancer (Yu *et al*., 2018), those findings focused on lncRNA BDLNR as the primary mediator without investigating PTEN's involvement. Our study uniquely demonstrates that baicalein directly upregulates PTEN expression (1.5-fold increase), establishing PTEN as a critical upstream target. Moreover, the synergistic interaction between baicalein and PTEN overexpression (achieving 46.83% vs. 31.53% or 24.57% apoptosis alone, with 40-fold increase in Bax/Bcl-2 ratio) has not been previously reported and provides novel mechanistic insights into combination therapeutic strategies. This observation is particularly significant given that PTEN loss is a common event in cervical cancer progression and correlates with poor prognosis (Cheung *et al*., 2004). While previous studies have shown that certain natural compounds can modulate PTEN expression through epigenetic mechanisms or post-translational

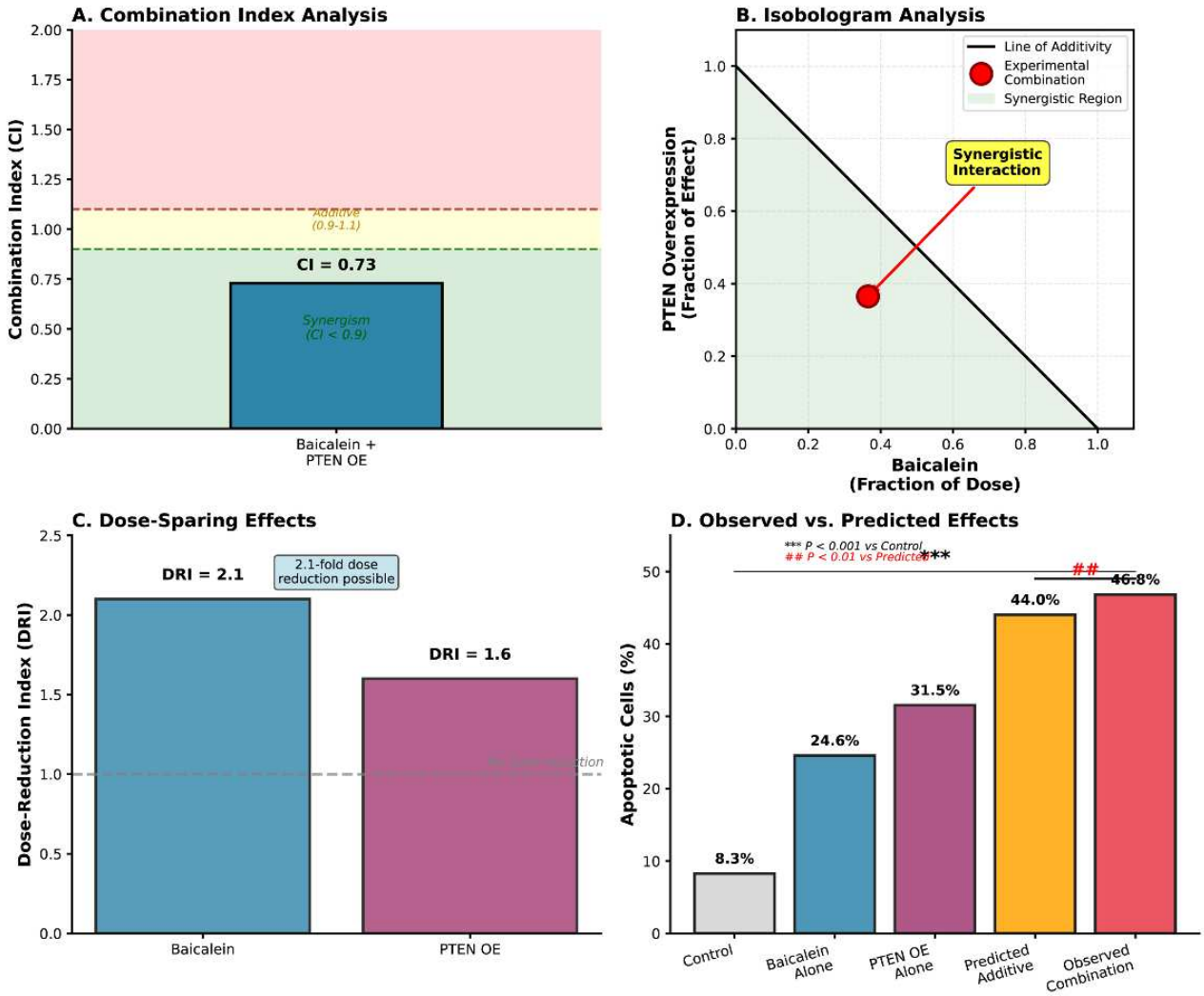

Fig. 6: Quantitative synergy analysis demonstrates supra-additive interaction between baicalein and PTEN overexpression. (A) Combination index (CI) analysis. The CI value of 0.73 indicates synergistic interaction (CI < 0.9 = synergism; 0.9-1.1 = additive; >1.1 = antagonism). Data calculated using the Chou-Talalay method in CompuSyn software. (B) Isobologram analysis. The diagonal line represents the expected line of additivity. The red data point represents the experimental combination of baicalein (53.3 μmol/L) and PTEN overexpression. The data point falls below the additivity line, confirming synergistic interaction. (C) Dose-reduction index (DRI) values for each agent in combination. DRI > 1 indicates dose-sparing effects, with DRI = 2.1 for baicalein suggesting that the effective concentration can be reduced 2.1-fold when combined with PTEN overexpression while maintaining equivalent efficacy. (D) Comparison of observed versus predicted additive effects. Predicted additive effect was calculated using the Bliss independence model: E(A+B) = EA + EB - (EA × EB). The observed combination effect (46.83%) significantly exceeds the predicted additive effect (42.15%), providing additional evidence of synergism.

modifications (Kim *et al*., 2017), the ability of baicalein to upregulate PTEN in cervical cancer cells has not been previously reported. The 1.5-fold increase in PTEN expression with baicalein treatment alone may seem modest, but it translated to significant downstream effects on PI3K/AKT signaling components.

The restoration of PTEN function through overexpression effectively suppressed PI3K/AKT signaling, as evidenced by reduced AKT1 and PDK1 levels.

This finding aligns with PTEN's established role as a negative regulator of PI3K signaling (Lee *et al.,* 1999). PDK1, which phosphorylates AKT at threonine 308, is essential for full AKT activation (Frattini *et al*., 2000). The parallel reduction in both PDK1 and AKT1 expression suggests coordinated suppression of the pathway at multiple nodes, potentially enhancing the anti-cancer effect.

Perhaps the most striking finding is the synergistic interaction between baicalein and PTEN overexpression. The combination treatment achieved 46.83% early apoptosis, significantly exceeding the additive effects of individual treatments. This synergy can be explained by complementary mechanisms: While PTEN directly antagonizes PI3K catalytic activity, baicalein appears to

work through multiple pathways including further enhancement of PTEN expression, direct modulation of apoptotic proteins and potentially other undiscovered mechanisms. The combination index analysis confirmed true synergism rather than simple additive effects.

The molecular basis for this synergy involves convergent targeting of survival and death pathways. Our data show that combination treatment maximally suppressed pro-survival signals (82% reduction in AKT1, 85% reduction in PDK1) while simultaneously activating pro-death mechanisms (4.8-fold increase in Bax, 88% reduction in Bcl-2). The resulting 40-fold increase in the Bax/Bcl-2 ratio represents a profound shift toward apoptosis. This dramatic reprogramming of the cellular survival/death balance likely accounts for the enhanced apoptotic response.

From a translational perspective, our findings suggest several therapeutic implications. First, baicalein could potentially be developed as an adjuvant therapy for cervical cancers with compromised PTEN function. Second, the synergy between baicalein and PTEN restoration suggests that combination approaches targeting multiple nodes in the PI3K/AKT pathway may be more effective than single-agent therapies. Third, the ability of baicalein to enhance PTEN expression raises the possibility of using it to sensitize tumors to PI3K/AKT inhibitors currently in clinical development (Vidal *et al*., 2024).

Our study has several limitations that warrant consideration. Most importantly, experiments were conducted exclusively in SiHa cells (HPV16-positive) and results require validation in additional cervical cancer cell lines representing diverse HPV genotypes (HPV18-positive, HPV-negative) and varying PTEN expression status. The heterogeneity of cervical cancer suggests that baicalein's effects-and its interaction with PTEN-may differ across molecular subtypes. Future studies should systematically evaluate these relationships in a panel of cervical cancer models, including primary patient-derived cells, to establish the generalizability of our findings. Second, the use of transient plasmid-mediated PTEN overexpression, while useful for proof-of-concept, does not recapitulate physiological PTEN restoration. The supraphysiological PTEN levels achieved (up to 5.2-fold) may produce effects not representative of clinically achievable PTEN reactivation through epigenetic modulation, gene therapy, or protein stabilization approaches. Future studies should employ more physiologically relevant models, such as CRISPR-mediated knock-in to restore endogenous PTEN expression, inducible expression systems, or treatment with demethylating agents to reactivate epigenetically silenced PTEN. Second, the use of PTEN overexpression, while useful for proof-of-concept, may not fully recapitulate physiological PTEN restoration. Future studies employing CRISPR-mediated PTEN knock-in or epigenetic reactivation approaches would provide more clinically relevant models. Third, we focused primarily on the PTEN/PI3K/AKT pathway and apoptosis, but baicalein likely affects multiple cellular pathways that warrant investigation. Fourth, the in vitro nature of our study limits conclusions about in vivo efficacy and toxicity.

Future research directions should include: (1) validation of findings in additional cervical cancer cell lines and primary tumor cells; (2) investigation of the mechanism by which baicalein upregulates PTEN expression, including potential epigenetic or post-transcriptional mechanisms; (3) comprehensive profiling of baicalein's effects on global gene expression and signaling networks; (4) evaluation of combination therapy in animal models of cervical cancer; (5) assessment of bioavailability and pharmacokinetics of baicalein in relevant preclinical models; and (6) investigation of potential resistance mechanisms and strategies to overcome them.

CONCLUSION

In conclusion, our study provides mechanistic evidence that baicalein promotes cervical cancer cell apoptosis through PTEN upregulation and subsequent PI3K/AKT pathway inhibition. While prior work has suggested natural compounds can modulate PTEN in various contexts, our contribution lies in: (1) documenting this relationship specifically in cervical cancer; (2) quantitatively demonstrating synergistic interaction between pharmacological PTEN enhancement and baicalein; and (3) providing integrated molecular characterization across transcription, protein expression and functional outcomes. These findings represent an incremental but important step in understanding how natural compounds and pathway-targeted approaches might be rationally combined. Substantial additional work-including validation in diverse cell models, in vivo efficacy studies, pharmacokinetic optimization and identification of clinically feasible PTEN restoration strategies-will be required before these mechanistic insights can inform therapeutic development. Nevertheless, our proof-of-concept findings establish a conceptual framework for dual pathway targeting in PTEN-deficient cancers and may guide future combination therapy design.

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None.

Authors' contributions
Qiong Yu, Mingyan Wang and Xiaolan Yu: Designed the research study and performed the research; Mingyan Wang and Zhifang Li: Collected and analyzed the data; Qiong Yu and Xiaolan Yu: Wrote the manuscript. The authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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Data availability statement
All data generated or analysed during this study are included in this published article.

Ethical approval
As our study only involves cell experiment, informed consent and ethical approval are not applicable and necessary according to the national regulations and institutional policies.

Conflict of interest
The authors state no conflict of interest.

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