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Publication Date

2026-06-25

Peer reviewed|Thesis/dissertation

Systematic Evaluation of Pharmacological Synergy Between Autophagy inhibitor and
Chemotherapy in Neuroblastoma

By

ZEXI LIU
THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Biomedical Engineering

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

Jinhwan Kim, Chair

Erin G Brown

Yuanpei Li

Committee in Charge

2026

Abstract

High-risk neuroblastoma (NB) remains one of the most lethal pediatric malignancies, with treatment resistance representing a critical barrier to improved clinical outcomes. Autophagy-mediated drug resistance, wherein cancer cells exploit lysosomal machinery to sustain metabolic homeostasis under therapeutic stress, is a primary driver of this resistance. Bisaminoquinoline 13 (Baq13) is a self-assembling one-component new-chemical-entity nanomedicine (ONN) that induces lysosomal dysfunction and autophagy blockade with 30-fold greater antiproliferative potency than hydroxychloroquine (HCQ). To systematically evaluate Baq13's potential as a chemosensitizer in NB, this study investigated its combination with standard-of-care agents Vincristine and Cisplatin across multiple treatment schedules in SK-N-SH and BE(2)-C cell lines. Combination efficacy was assessed via MTT-based checkerboard titration matrices analyzed with the Highest Single Agent (HSA) synergy model. Additionally, qPCR was performed to assess transcriptional modulation of autophagy markers such as p62, BAX, and BAK1 to elucidate mechanistic underpinnings of the observed synergy patterns. Results demonstrated that drug synergy is highly schedule- and drug-dependent: Baq13 exhibited robust synergy with Vincristine under simultaneous co-treatment and Baq-priming paradigms, attributable to Baq13's neutralization of lysosomal acidity preventing ion-trapping sequestration of Vincristine. In contrast, minimal synergy was observed with Cisplatin, consistent with its nucleus-targeting, lysosome-independent mechanism. qPCR confirmed dose-proportional

upregulation of p62, corroborating autophagy blockade as the primary sensitization mechanism. Overall, Baq13 represents a promising schedule-optimized chemosensitizer in NB, with lysosomal pH modulation as a targetable axis to overcome drug sequestration-mediated resistance.

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1.Introduction

1.1 Neuroblastoma: Epidemiology and Clinical Features

Neuroblastoma (NB) is the most common extracranial solid tumor in children, especially under 5 years old (1). It accounts for ~8–10% of pediatric cancers and ~15% of childhood cancer deaths (1). NB arises from the sympathetic nervous system (most often the adrenal medulla), and patient age and tumor biology critically influence prognosis. Risk stratification by the INRG (International Neuroblastoma Risk Group) and INSS (International Neuroblastoma Staging System) uses stage, age, histology, and genetic factors (notably MYCN amplification in ~20–25% of cases) to categorize low-, intermediate-, or high-risk disease (2).

Current therapies are risk-adapted. Low- and intermediate-risk NB typically require surgery and minimal chemotherapy, yielding excellent outcomes (5-year survival >90%) (2). Meanwhile, high-risk neuroblastoma (HRNB) constitutes approximately 50-60% of all neuroblastoma cases and is associated with poor prognostic indicators such as MYCN amplification, metastatic disease, and unfavorable histology (3). Despite significant advancements in treatment, outcomes for children with HRNB remain poor, with a 5-year overall survival rate ranging from 51-62% (3). Standard front-line therapy includes rapid multiagent induction chemotherapy (e.g. Cisplatin, Vincristine, Etoposide, etc.) to reduce tumor bulk, followed by surgical resection of the primary mass (2).

Even with modern protocols, only ~50% of high-risk patients survive long-term (2).

Relapse is common: over half of high-risk patients ultimately experience recurrent disease (often metastatic) (2). Relapsed neuroblastoma responds poorly to therapy, and long-term survival post-relapse is low. These facts underscore an unmet need for new therapies that can overcome tumor resistance and improve survival.

1.2 Autophagy in Cancer

Autophagy (specifically macroautophagy) is a conserved cellular recycling process. Under stress (e.g. starvation, hypoxia), cells form double-membrane vesicles (autophagosomes) around intracellular cargo; these fuse with lysosomes to degrade and recycle macromolecules (5). This process provides nutrients and clears damaged organelles. In normal cells, basal autophagy maintains homeostasis; in cancer cells, autophagy is often upregulated as a survival mechanism. Indeed, many tumors rely on autophagy to tolerate metabolic stress, resist apoptosis, and resist therapies (5)(6). For example, RAS-driven cancers (e.g. some pancreatic or lung cancers) require autophagy to maintain mitochondrial function and nucleotide pools (6). Inhibiting autophagy (genetically or pharmacologically) can limit tumor growth in such models (6). Furthermore, cancer therapies often induce cytoprotective autophagy; preclinical data show that blocking autophagy (e.g. with chloroquine) can sensitize tumors to chemotherapy or targeted drugs (5).

Accumulating evidence demonstrates that intercepting autophagy can potentate the antitumor efficacy of traditional chemotherapies, targeted inhibitors, or immunotherapies (5). Chloroquine (CQ) and hydroxychloroquine (HCQ) raise

lysosomal pH and block autophagosome degradation; they have proven safety in trials but modest single-agent efficacy (5). More potent approaches include dimeric quinoline/quinacrine compounds: for example, *Lys05* (a dimeric chloroquine analogue) and *DQ661* (a dimeric quinacrine). These compounds inhibit the lysosomal enzyme palmitoyl-protein thioesterase 1 (PPT1), leading to robust autophagy blockade (5). Indeed, dimeric CQ/quinacrines have been engineered to be 100–1000× more potent than CQ (5). There are additional strategies targeting upstream regulators (e.g. VPS34 inhibitors like SAR405) or downstream effectors (e.g. TFEB/MITF pathways) (7).

1.3 Autophagy-Mediated Chemoresistance in NB

Autophagy contributes to chemoresistance in neuroblastoma by enabling tumor cells to survive chemotherapy-induced stress through multiple mechanisms (8)(9). It acts as a cytoprotective process that degrades damaged organelles and proteins to maintain energy balance, thereby dampening drug-induced apoptotic signaling (8)(10).

Consequently, chemotherapeutic agents induce autophagy in NB cells, which inhibits apoptosis and provides metabolic substrates for survival (9)(11). Autophagy can also promote drug efflux and enhance DNA repair (reported in other cancers) to further reduce drug efficacy (8)(12). Experimental studies confirm this protective role: in NB models, inhibiting autophagy (via ATG5 knockdown or chloroquine) sensitizes cells to chemotherapy and increases apoptosis (9)(10).

1.4 Evidence for Autophagy Inhibitor Synergy

A growing number of preclinical studies demonstrate that inhibiting autophagy can overcome NB chemoresistance (9-13). When NB cells are exposed to drugs like doxorubicin, vincristine, cisplatin, etoposide or TMZ, autophagy is induced as a survival response. Belounis *et al.* (2016) showed that NB cells treated with chemotherapy increase LC3-II levels (autophagosome marker) and that blocking this autophagy (HCQ or ATG5 siRNA) remarkably increases chemotherapy-induced cell death (9). Treating NB xenografts with HCQ + vincristine significantly slowed tumor growth compared to vincristine alone (9).

Autophagy can delay or inhibit apoptosis by degrading pro-apoptotic factors or maintaining energy supply. Wear *et al.* (2023) reported that adding HCQ or 3-Methyladenine (autophagy inhibitor) to cisplatin or TMZ treatment in NB cells enhanced caspase activation and apoptotic fraction, indicating that autophagy was restraining drug-induced apoptosis (10).

Autophagy recycles intracellular nutrients to fuel metabolism under stress. By maintaining ATP and amino acid pools, autophagy helps NB cells survive cytotoxic stress. Pathania *et al.* (2024) found a novel regulator (SAP30) that upregulates autophagy in NB; SAP30-driven autophagy promoted cell growth and protected cells from chemotherapy, consistent with a metabolic-support role (11).

Beyond these, Horwacik *et al.* (2015) found that 3-MA potentiates the cytotoxicity of sulforaphane in BE(2)-C neuroblastoma cells (12). Liu *et al.* (2024) showed that

genipin induces protective autophagy in SK-N-SH cells, and autophagy inhibition renders these cells more sensitive to genipin (13). Collectively, these studies support that blocking autophagy sensitizes NB cells to various agents by allowing full activation of apoptotic pathways.

1.5 Rationale for Studying Baq13

Autophagy-mediated drug resistance is a well-established survival mechanism in NB, whereby cancer cells rely on lysosomal degradation to recycle damaged organelles and sustain metabolic homeostasis under therapeutic stress. Inhibition of autophagy has therefore emerged as a promising strategy to sensitize neuroblastoma cells to conventional chemotherapy by disabling this cytoprotective escape route and restoring drug-induced cytotoxicity. The Baq13 (previously developed and generously provided for this study by our collaborator Dr. Yuanpei Li and his group) is a bis-aminoquinoline derivative created by hybridizing a lysosomotropic detergent MSDH with the autophagy inhibitor Lys05 (14). The design leverages a pH-sensitive behavior: at neutral pH (in blood/tissue), Baq forms stable nanoparticles; upon acidification inside lysosomes, the particles dissociate and release active monomers. These monomers exert dual actions: the detergent moiety disrupts lysosomal membranes (causing lysosomal membrane permeabilization and cathepsin release), while the bisaminoquinoline moiety inhibits autophagy flux (via mechanisms similar to Lys05) (14). In effect, Baq enforces lysosomal catastrophe while blocking the usual autophagic cleanup. This dual mechanism is hypothesized to be more lethal to cancer

cells than conventional drugs.

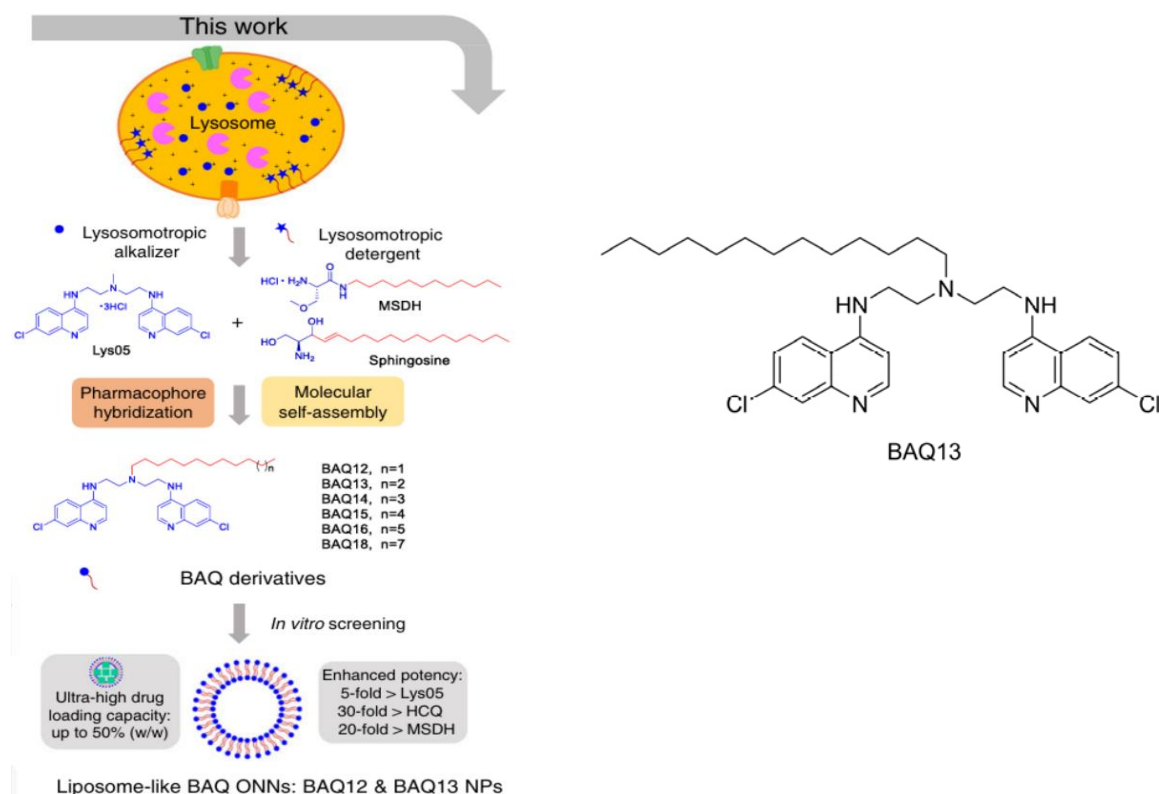


Figure 1. Rationale of design and chemical formula of Baq (bis-aminoquinoline) derivative. Adapted from Ma Z. et al., Nat. Commun., 2020, 11, 4615.

Preliminary data support these advantages (14). Baq12/13 ONNs have shown excellence in vitro and in vivo activity: they induced extensive lysosomal disruption and autophagy inhibition in cancer cells, leading to cell death (14). Notably, they achieved ~30-fold higher antiproliferative activity than HCQ (14). In mouse models, Baq ONNs displayed favorable pharmacokinetics and caused dramatic tumor regressions as single agents (14). By contrast, Lys05 alone (while active) lacks

intrinsic detergent activity, and HCQ is much weaker. In combination studies, Baq particles can co-deliver other drugs (e.g. napabucasin) to tumors, potentially enabling synergistic regimens (14). Hence, Baq's unique chemistry (bisaminoquinoline head + hydrophobic tails) offers mechanistic synergy over existing autophagy inhibitors. Though still early-stage, these findings motivate testing Baq in neuroblastoma, where the vulnerable lysosomal phenotype of NB cells might be exploited.

In this project, I aim to systematically evaluate the schedule-dependent synergy of Baq13 in combination with standard-of-care chemotherapeutic agents Vincristine and Cisplatin in HRNB. Two representative cell lines were selected to capture the genetic heterogeneity of HRNB: BE(2)-C, which harbors MYCN amplification, and SK-N-SH, which is MYCN non-amplified. As MYCN amplification is the predominant oncogenic driver in HRNB and a key determinant of treatment resistance and prognosis, this pairing allows for assessment of whether Baq13-mediated chemosensitization is broadly applicable across tumors with distinct molecular backgrounds.

The objective of this study is to evaluate the anticancer activity and mechanism of Baq13 in high-risk neuroblastoma models. Specifically, I aim to (1) assess Baq13's cytotoxicity as a single agent and in combination with standard-of-care chemotherapeutic agents Vincristine and Cisplatin; (2) define quantitative dosing parameters that maximize therapeutic synergy while minimizing dosage; (3) measure Baq13's impact on autophagy and apoptosis at the transcriptional level; and (4)

characterize the drug synergy of schedule-dependent co-treatment by examining how the timing and sequencing of Baq13 relative to chemotherapy modulates combination efficacy.

2. Methods

2.1 Cell Culture: Human derived NB cell lines including mycn amplified SK-N-BE(2)-C and mycn unamplified SK-N-SH were obtained from ATCC (American Type Culture Collection) and cultured following the manufacturer's instructions to ensure phenotypic stability. Culture media, serum, and dissociation reagents were selected based on ATCC's product-specific recommendations. Subculturing was performed when cells reached the suggested confluence (typically 80–90%). All cells were grown in their recommended basal media supplemented with fetal bovine serum (FBS) and maintained in a humidified atmosphere at 37°C with 5% CO₂. All cells were cultured, passaged, and maintained strictly according to the official protocols and guidelines provided by ATCC.

2.2 Viability assays: Cell viability was assessed by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. Briefly, cells were seeded in 96-well plates at a density of 7,000 cells per well and treated as indicated. Following the treatment, cells were incubated with MTT reagents for 4.5 h. The absorbance (OD values) at 570 nm was determined via a microplate reader. Results were expressed as the average cell viability, calculated using the formula: $[(OD_{treat} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\%]$ (14).

2.3 Drug Combination Treatment and Checkerboard Matrix Design:

To investigate the interaction between Baq and two different chemotherapy agents (Vincristine and Cisplatin), three distinct treatment regimens were designed:

2.3.1 Simultaneous Treatment: Both Baq and the chemotherapy agent were added to the cells at the same time and incubated for 24 h.

2.3.2 Sequential Treatment (Baq → Chemo): Cells were initially exposed to various concentrations of Baq. After an incubation period of 24 h, the drug-containing medium was carefully aspirated. The cells were then replenished with fresh culture medium containing the indicated concentrations of chemotherapy agents for a subsequent 24 h of incubation.

2.3.3 Sequential Treatment (Chemo → Baq): Cells were initially exposed to various concentrations of chemotherapy agents. After an incubation period of 24 h, the drug-containing medium was carefully aspirated. The cells were then replenished with fresh culture medium containing the indicated concentrations of Baq for a subsequent 24 h of incubation.

Drug synergy was evaluated by performing a checkerboard titration matrix in 96-well plates. The matrix was constructed with increasing concentrations of the chemotherapy agent along the vertical axis and increasing concentrations of Baq along the horizontal axis.

To ensure statistical power and reproducibility, six replicates ($n = 6$) were performed for each treatment combination. Following the specified incubation period, cell viability was measured via the MTT assay as described above. The resulting dose-response data were then integrated into Combenefit 2 for the calculation of synergy

scores based on the Highest Single Agent (HSA) model.

2.4 Synergy Analysis: To quantify the synergistic effect, dose-response data of combined drug treatment were processed using Combenefit 2 software (15). The degree of interaction was assessed based on the results of HSA (Highest Single Agent) model analysis. Interaction analysis was performed using the HSA model, where the expected additive response is defined by the maximum efficacy of the individual components. Any surplus effect beyond this single-agent maximum is quantified as HSA synergy. (16). Synergy was visualized using 3D surface mapping and 2D heatmaps, where Deep Blue regions indicated synergy and warm colors represented antagonistic effects.

2.5 RT-qPCR analysis: Total RNA was extracted from treated cells using TRIzol reagent (Invitrogen) followed by the standard phenol-chloroform extraction method. The concentration and purity of the isolated RNA were assessed via Nanodrop. For each sample, 2 µg of total RNA was reverse-transcribed into cDNA in a 20 µL reaction volume using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. qPCR amplification was performed in a 20 µL reaction containing 2 µL of diluted cDNA, Power SYBR Green PCR Master Mix (Thermo Fisher), and specific primers. The reaction was conducted on a CFX96 Real-Time PCR Detection System (Bio-Rad).

To ensure the absence of genomic DNA contamination, a non-template control (NTC) was included in every run. All reactions were performed in triplicate. The relative

mRNA expression levels of target genes were normalized to the internal control GAPDH using the $2^{-\Delta\Delta C_t}$ method. All procedures and analyses were conducted according to the MIQE guidelines standard (17). The specific primer sequences used in this study are summarized below.

Primer Sequence:

(1). MAP1 LC3B

forward 5'-AGCAGCATCCAACCAAAATC-3',

reverse 5'-CTGTGTCCGTTACCAACAG-3' ;

(2). p62(SQSTM1)

forward 5'-ATCGGAGGATCCGAGTGT-3',

reverse 5'-TGGCTGTGAGCTGCTCTT-3'

(3). BAX

Forward 5'-CCCGAGAGGTCTTTTCCGAG-3'

Reverse 5'-CCAGCCCATGATGGTTCTGAT-3'

(4). CLCN7

Forward 5'-CCACGTTACCCCTGAATTTTGT-3'

Reverse 5'-AAACCTTCCGAAGTTGATGAGG-3'

(5). BAK1

Forward 5'-GAGAGCCTGCCCTGCCCTCT-3'

Reverse 5'-CCACCCAGCCACCCCTCTGT-3'

2.6 Statistics: Statistical analyses were performed using GraphPad Prism version 10.6.1. Data are presented as mean $\pm$ SD. One-way ANOVA was performed after assessing variance equality with the Brown-Forsythe test. When variances were equal ($P > 0.05$), ordinary one-way ANOVA followed by Tukey's or Dunnett's multiple comparisons test was used. When variances were unequal ($P < 0.05$), Welch's or Brown-Forsythe ANOVA followed by Dunnett's T3 test was applied. Two-way mixed-effects model ANOVA followed by Tukey's multiple comparisons test was used. P values are indicated in the figure legends: ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3. Results

3.1 Dose-Dependent Cytotoxicity and IC50 Determination of Single Agent Baq13

Prior to combination studies, the cytotoxic potency of Baq13 as a single agent was characterized in both SK-N-SH and BE(2)-C HRNB cell lines to establish appropriate dose ranges for subsequent combinatorial analysis. As aforementioned, these two lines were selected to represent the spectrum of MYCN status in HRNB, with BE(2)-C harboring MYCN amplification and SK-N-SH being MYCN non-amplified, thereby allowing assessment of Baq13's efficacy across distinct oncogenic backgrounds. Cell viability was assessed via MTT assay following 24-hour treatment with increasing concentrations of Baq13.

In BE(2)-C cells, Baq13 treatment produced a clear dose-dependent reduction in viability (Fig. 4). Viability remained near baseline at lower concentrations but declined sharply at doses of 4 μ M and above, with near-complete loss of viability observed at 8 μ M. Statistical analysis confirmed significant differences between the control and higher dose groups, indicating potent cytotoxic activity in this MYCN-amplified background.

In SK-N-SH cells, a similar dose-dependent pattern was observed, with significant reductions in viability emerging at 4 μ M and becoming pronounced at 6 and 8 μ M (Fig. 7). Notably, statistically significant differences were detected between the control and doses of 4 μ M and above ($p < 0.001$), while lower concentrations of 1 and

2 μ M did not reach statistical significance, suggesting a cytotoxic threshold consistent with an IC₅₀ in the 2–4 μ M range.

Taken together, both cell lines yielded IC₅₀ values in the range of 2–4 μ M, with no substantial discrepancy in Baq13 sensitivity across the two distinct genetic backgrounds. These findings confirm Baq13's potency as a single agent at low micromolar concentrations, consistent with previously reported antiproliferative activity in other cancer models. The IC₅₀ values derived from this characterization were subsequently used to define the dose matrix for checkerboard combination experiments, ensuring that combination doses spanned both sub- and supra-IC₅₀ ranges to allow comprehensive mapping of synergistic and antagonistic regions.

Next, the cytotoxic potency of Cisplatin as a single agent was similarly characterized in both cell lines to establish appropriate dose ranges for combination experiments.

Following 24-hour treatment with increasing concentrations of Cisplatin, both SK-N-SH and BE(2)-C cells demonstrated dose-dependent reductions in viability (Fig. 3&6). In BE(2)-C cells, viability declined progressively with increasing Cisplatin concentrations, with statistically significant reductions observed at doses of 5 μ M and above, yielding an estimated IC₅₀ in the range of 5–10 μ M. A similar pattern was observed in SK-N-SH cells, where viability dropped markedly at comparable concentrations, with significant differences confirmed between the control and higher dose groups, consistent with an IC₅₀ within the same range.

The cytotoxic potency of Vincristine as a single agent was also characterized in both

SK-N-SH and BE(2)-C cell lines via MTT assay to establish dose ranges for subsequent combination studies. In BE(2)-C cells, Vincristine treatment produced a dose-dependent reduction in viability across the tested concentration range of 0–50 nM (Fig. 5). Viability remained near baseline at lower concentrations of 1–15 nM, with no statistically significant differences detected relative to the untreated control. Significant reductions in viability emerged at 25 nM and above, with statistically significant differences confirmed at 25, 35, and 50 nM ($p < 0.01$ or $p < 0.05$), indicating a cytotoxic threshold in the low tens of nanomolar range. In SK-N-SH cells, a similar dose-dependent pattern was observed, with viability remaining largely unchanged at concentrations up to 15 nM (Fig. 8). Statistically significant reductions were detected at 25 nM and above, with increasing significance at higher concentrations reaching $p < 0.001$ and $p < 0.0001$ at 35 and 50 nM respectively. At the highest tested concentration of 50 nM, viability in both cell lines was reduced to approximately 40–50% of control, suggesting an IC₅₀ in the range of 25–50 nM for both lines under the current treatment conditions.

These single-agent characterization data for both Baq13 and chemotherapies established the dose boundaries used to construct the combinatorial checkerboard matrix, ensuring adequate coverage of sub- and supra-IC₅₀ concentrations for subsequent synergy analysis.

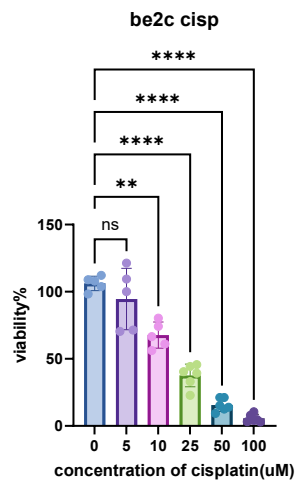


Figure 2: Single agent cytotoxicity of Cisplatin upon BE(2)-C

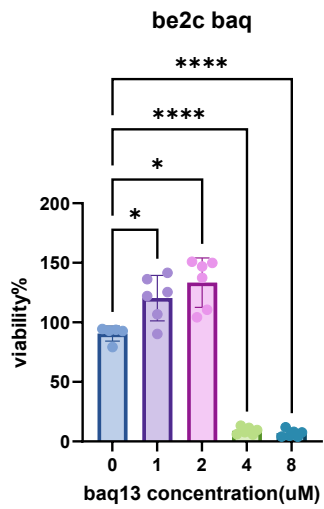


Figure 3. Single agent cytotoxicity of Baq13 upon BE(2)-C

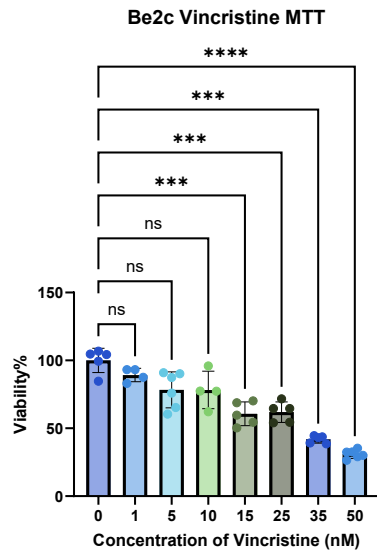


Figure 4. Single agent cytotoxicity of Vincristine upon BE(2)-C

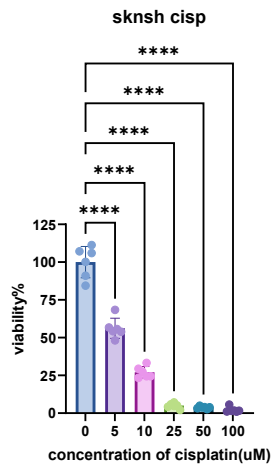


Figure 5. Single agent cytotoxicity of Cisplatin upon SK-N-SH

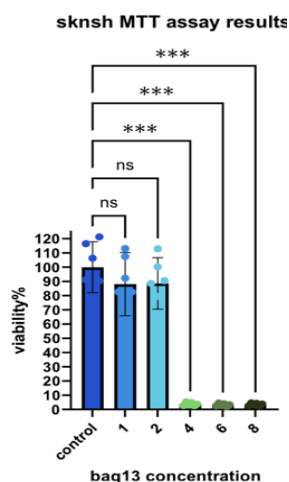


Figure 6. Single agent cytotoxicity of Baq13 upon SK-N-SH

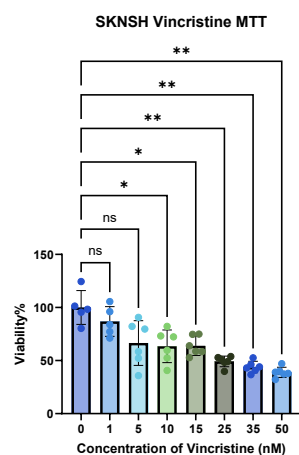


Figure 7. Single agent cytotoxicity of Vincristine upon SK-N-SH

3.2 Serum Facilitated Efficacy of Baq13

Given Baq13's physicochemical properties as a self-assembling nanoparticle, its cytotoxic efficacy may be influenced by the solvent used for drug dilution, as different aqueous environments can affect nanoparticle stability, aggregation state, and effective drug concentration. To address this, Baq13 was prepared in four different diluents — FBS only, PBS, complete media (FBS + EMEM + P/S), and

EMEM alone — and cytotoxicity was compared across the same concentration range.

A preliminary comparison between FBS-containing medium and PBS was first conducted in both SK-N-SH and BE(2)-C cells. In SK-N-SH cells, Baq13 diluted in FBS-containing medium consistently produced greater cytotoxicity compared to PBS across multiple dose groups, with statistically significant differences observed at intermediate concentrations at 3–4 μM (Fig. 9). At higher doses, both conditions converged toward near-complete loss of viability, suggesting that solvent effects are most pronounced within the sub-IC₅₀ range. A similar trend was observed in BE(2)-C cells, where FBS-diluted Baq13 again demonstrated superior cytotoxic potency compared to PBS-diluted Baq13, particularly at doses of 2–3 μM (Fig. 10).

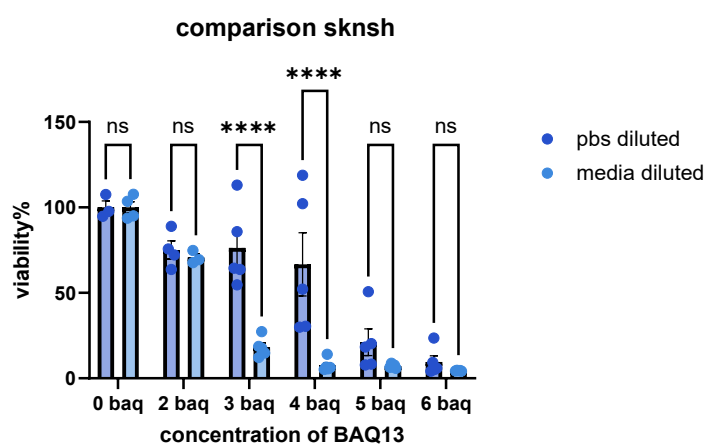


Figure 8. Serum facilitated cytotoxicity of Baq13 in SK-N-SH cells.

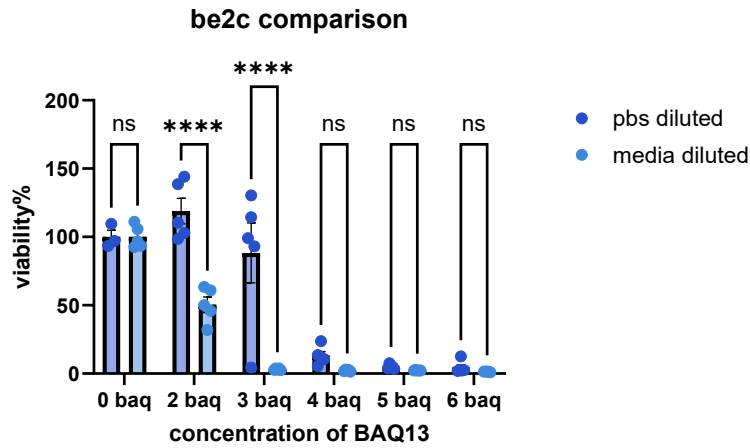


Figure 9. Serum facilitated cytotoxicity of Baq13 in BE(2)-C cells.

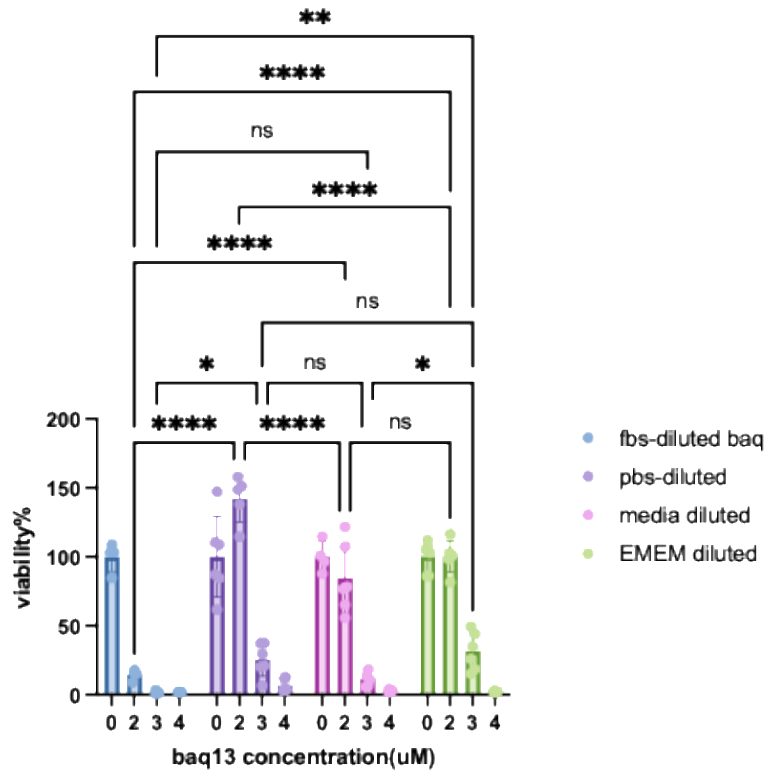


Figure 10. A four-way comparison of serum facilitated efficacy on Baq13 cytotoxicity in neuroblastoma cell line.

Beyond that, a four-way comparison was subsequently performed in order to more comprehensively characterize serum facilitated efficacy (Fig. 11). Baq13 diluted in FBS only exhibited the greatest cytotoxic potency, followed by complete media, PBS,

and EMEM, which produced the weakest response. This gradient suggests that serum components in FBS play a primary role in enhancing Baq13's bioavailability, potentially by stabilizing nanoparticle assembly or facilitating membrane interactions and cellular uptake. In contrast, EMEM alone appeared to compromise Baq13's efficacy.

To ensure consistency and maximize reproducibility across all subsequent combination experiments, FBS-containing medium was adopted as the standard diluent for Baq13 preparation throughout this study.

3.3 Transcriptional Modulation of Autophagy and Apoptosis Genes

To mechanistically validate Baq13's capacity to inhibit autophagy at the transcriptional level, qPCR was performed to assess the expression of p62 (SQSTM1), a well-established hallmark of autophagic blockade that accumulates when autophagic flux is impaired. Baq13 treatment was applied at two dose levels — a low dose and a high dose bracketing the IC₅₀ — in both BE(2)-C and SK-N-SH cells, with fold change in p62 expression calculated relative to untreated controls.

In BE(2)-C cells, p62 expression showed a dose-dependent increase following Baq13 treatment, with the low dose group exhibiting a modest but detectable upregulation compared to control (Fig. 12). The high dose group produced a markedly greater elevation in p62 transcript levels, demonstrating a clear dose-proportional relationship between Baq13 concentration and autophagy inhibition. Statistical analysis confirmed significant differences between the control and high dose groups ($p < 0.0001$), as well

as between the low and high dose conditions.

A consistent pattern was observed in SK-N-SH cells, where p62 expression again increased in a dose-dependent manner upon Baq13 treatment (Fig. 13). The high dose group elicited a substantial upregulation of p62, significantly exceeding both the untreated control and the low dose group ($p < 0.0001$).

Collectively, these results confirm that Baq13 suppresses autophagic flux in both neuroblastoma cell lines in a dose-dependent manner, as evidenced by transcriptional accumulation of p62, providing mechanistic support for Baq13's role as an autophagy inhibitor.

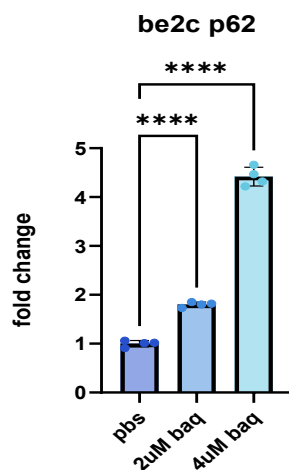


Figure 11. Relative mRNA expression of p62 in BE(2)-C treated with Baq13

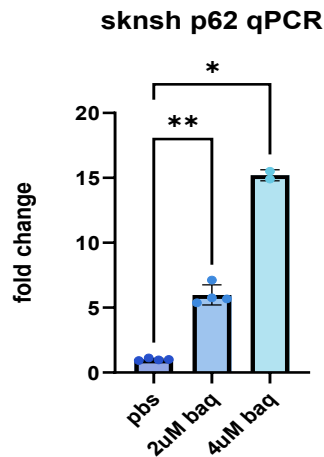


Figure 12. Relative mRNA expression of p62 in SK-N-SH treated with Baq13

In addition to the classic autophagic marker p62, the relative mRNA expression of pro-apoptotic genes BAX and BAK1 was measured in both cell lines to assess whether Baq13 treatment modulates apoptotic pathway activation at the transcriptional level, following treatment with 2 µM and 4 µM Baq13.

In SK-N-SH cells, BAX expression showed a modest but statistically significant decrease at 2 µM Baq13 compared to control ($p < 0.05$), with expression returning toward baseline at 4 µM, a difference that did not reach statistical significance (Fig. 14). BAK1 expression was similarly reduced at 2 µM ($p < 0.01$), with partial recovery observed at 4 µM that again did not reach significance relative to control (Fig. 15). The overall magnitude of change in both genes was modest, with fold changes remaining within approximately 0.2–0.3 of baseline levels.

In BE(2)-C cells, both BAX and BAK1 displayed dose-dependent downregulation upon Baq13 treatment, with statistically significant reductions observed at both 2 µM and 4 µM for each gene (BAX: $p < 0.001$ and $p < 0.0001$ respectively; BAK1: $p <$

0.0001 and $p < 0.01$ respectively) (Fig. 16&17). Expression levels declined to approximately 50% of control at 2 μM and remained similarly suppressed at 4 μM , representing statistically significant but modest reductions in pro-apoptotic gene expression.

SKNSH relative BAX mRNA expression

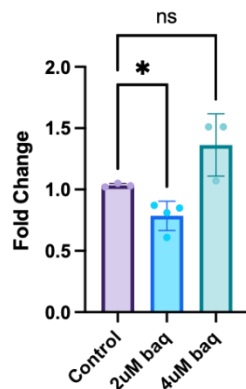


Figure 13. Relative mRNA expression of BAX in SK-N-SH treated with Baq13

SKNSH relative BAK1 mRNA expression

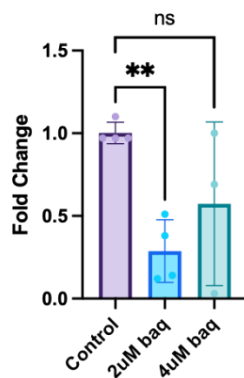


Figure 14. Relative mRNA expression of BAK1 in SK-N-SH treated with Baq13

Be2c relative BAX mRNA expression

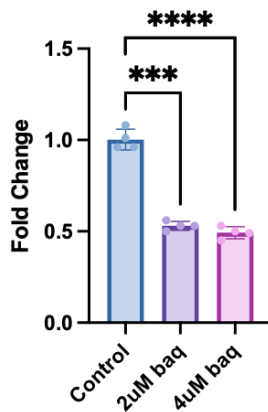


Figure 15. Relative mRNA expression of BAX in BE(2)-C treated with Baq13

Be2c relative BAK1 mRNA expression

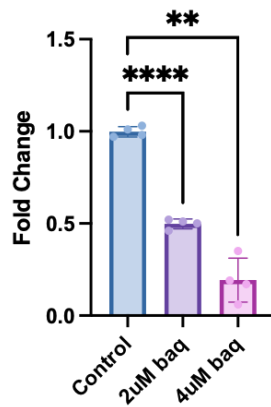


Figure 16. Relative mRNA expression of BAK1 in BE(2)-C treated with Baq13

Taken together, Baq13 treatment produced statistically significant but modest changes in BAX and BAK1 transcript levels across both cell lines, with similar patterns of direction and dose-dependence of these changes across SK-N-SH and BE(2)-C cells.

3.4 Comparison of Combinatorial Treatment: Baq13 Combined with Cisplatin and Vincristine

3.4.1 Simultaneous co-treatment in SK-N-SH and BE(2)-C

To evaluate the combinatorial potential of Baq13 with standard-of-care

chemotherapeutic agents, simultaneous co-treatment experiments were first conducted in both SK-N-SH and BE(2)-C cell lines using a checkerboard titration design. Baq13 was combined with either Vincristine or Cisplatin simultaneously across a matrix of dose pairs with 24-hour incubation, and synergy was quantified.

Combination treatment of Baq13 and Vincristine demonstrated robust synergy in SK-N-SH cells, with synergistic regions spanning broadly across the tested dose matrix, particularly at mid-to-high concentrations of both agents (Fig. 18). A similarly consistent synergistic landscape was observed in BE(2)-C cells, where the HSA synergy plot revealed extensive blue regions across multiple dose pairs, indicating that the combination produces greater cytotoxicity than either agent alone regardless of MYCN amplification status (Fig. 19).

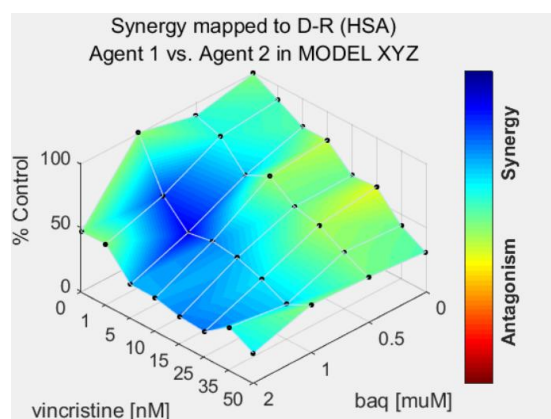


Figure 17. Representative synergy analysis of simultaneous Baq13 with Vincristine upon SK-N-SH

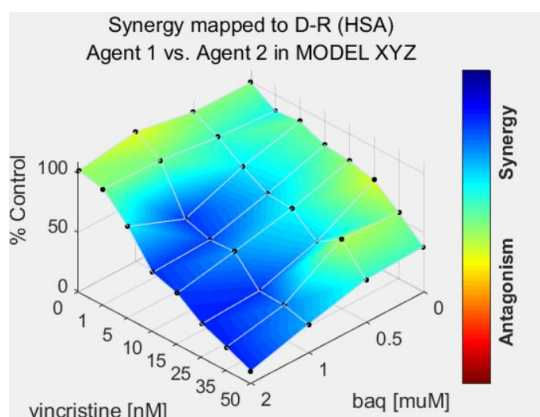


Figure 18. Representative synergy analysis of simultaneous Baq13 with Vincristine upon BE(2)-C

In contrast, the combination of Baq13 and Cisplatin produced a markedly different synergy profile. In SK-N-SH cells, the HSA plot was predominantly green to yellow across the dose matrix, indicative of minimal to no synergy (Fig. 20). In BE(2)-C cells, modest synergy was observed in limited regions of the dose matrix, though the overall landscape remained substantially less synergistic compared to the Baq13-Vincristine combination (Fig. 21).

These contrasting results highlight that Baq13's chemosensitizing effect under simultaneous co-treatment is selectively operative against Vincristine but not Cisplatin, suggesting that the mechanism underlying this synergy is specific to drug properties that render certain agents vulnerable to Baq13-mediated lysosomal disruption.

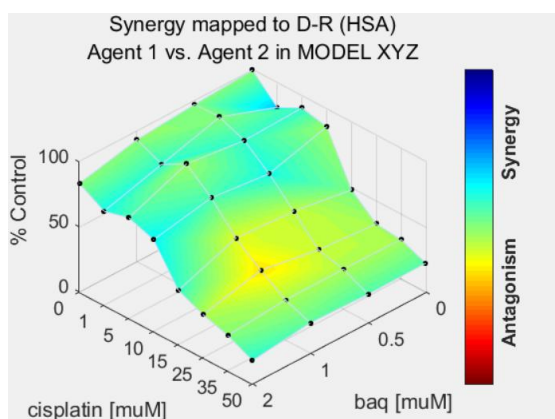


Figure 19. Representative synergy analysis of simultaneous Baq13 with Cisplatin upon SK-N-SH

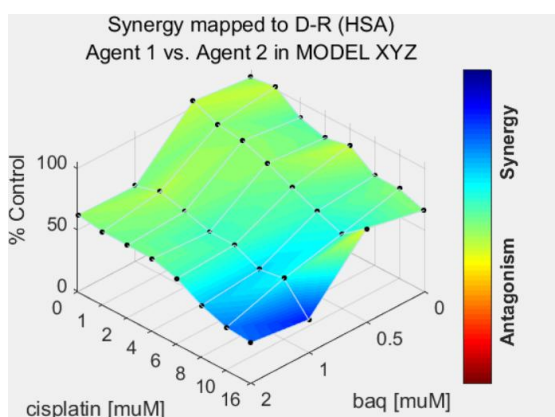


Figure 20. Representative synergy analysis of simultaneous Baq13 with Cisplatin upon BE(2)-C

Given that Baq13's mechanism of action centers on lysosomal disruption and autophagy inhibition — both of which are dynamic, time-dependent processes — the timing of Baq13 administration relative to chemotherapy exposure may critically influence the degree of chemosensitization achieved. Specifically, whether lysosomal pH neutralization is established prior to, concurrent with, or following chemotherapy administration could determine the extent to which drug sequestration is prevented or reversed. To investigate this, two sequential treatment schedules were examined: early Baq13 priming followed by chemotherapy, and chemotherapy pretreatment followed by late Baq13 addition.

3.4.2 Early Baq13 priming → chemotherapy

To determine whether pre-establishing lysosomal disruption prior to chemotherapy exposure could further modulate combination efficacy, an early Baq13 priming schedule was investigated, in which Baq13 was administered first, followed by the addition of Vincristine or Cisplatin.

In BE(2)-C cells, early Baq13 priming with Cisplatin resulted in a modest synergy profile, with yellow-green regions predominating across the dose matrix and limited synergistic areas at select dose combinations (Fig. 22). While this represents a marginal improvement over the minimal synergy observed under simultaneous co-treatment in BE(2)-C, the overall response remained substantially weaker than that achieved with Vincristine under the same priming schedule.

In BE(2)-C cells, early Baq13 priming followed by Vincristine yielded a markedly different outcome, with robust and broadly distributed synergy observed across the dose matrix (Fig. 23). The extensive blue regions indicate that Baq13 priming effectively sensitized BE(2)-C cells to subsequent Vincristine treatment, producing a synergistic profile comparable to or exceeding that observed under simultaneous co-treatment.

In SK-N-SH cells, early Baq13 priming followed by Cisplatin produced a predominantly antagonistic response, with red and orange regions dominating the HSA synergy plot across the majority of dose combinations tested (Fig. 24). This stands in stark contrast to the minimal but non-antagonistic profile observed under

simultaneous co-treatment, indicating that sequential Baq13 priming actively interferes with Cisplatin's cytotoxic activity in this cell line.

In SK-N-SH cells, early Baq13 priming followed by Vincristine produced a modest synergistic landscape, with synergistic regions largely confined to higher dose combinations and a predominantly green-to-yellow profile across the remainder of the dose matrix (Fig. 25). This represents a reduction in synergy compared to the simultaneous co-treatment condition, suggesting that pre-establishing lysosomal disruption before Vincristine exposure does not enhance, and may partially diminish, the sensitization effect in this cell line.

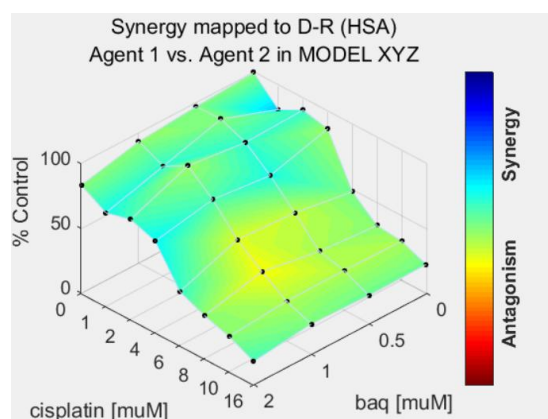


Figure 21. Representative synergy analysis of early Baq13 priming with Cisplatin upon BE(2)-C

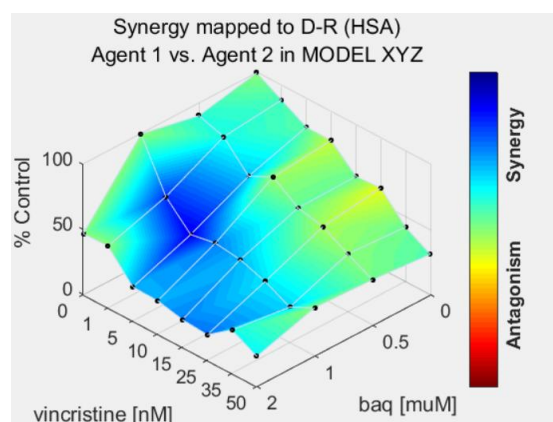


Figure 22. Representative synergy analysis of early Baq13 priming with Vincristine upon BE(2)-C

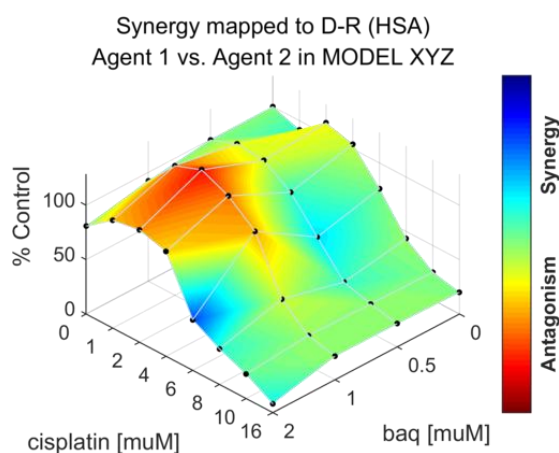


Figure 23. Representative synergy analysis of early Baq13 priming with Cisplatin upon SK-N-SH

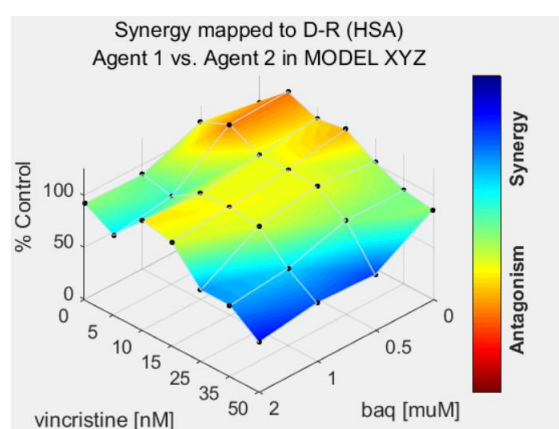


Figure 24. Representative synergy analysis of early Baq13 priming with Vincristine upon SK-N-SH

3.4.3 Chemotherapy pretreatment → Baq13

To evaluate whether chemotherapy pretreatment prior to Baq13 administration could modulate combination efficacy, a late Baq13 schedule was investigated, in which Vincristine or Cisplatin was administered first, followed by Baq13 treatment.

In SK-N-SH cells, Vincristine pretreatment followed by Baq13 produced a predominantly green-to-yellow synergy landscape, with minimal synergistic regions detected across the dose matrix (Fig. 26). This represents a marked reduction compared to both simultaneous co-treatment and early Baq13 priming in the same cell line, indicating that reversing the treatment sequence largely abolishes the

sensitization effect observed when Baq13 precedes or accompanies Vincristine.

In SK-N-SH cells, Cisplatin pretreatment followed by Baq13 produced a modest synergy profile, with limited blue regions emerging at select higher dose combinations against a predominantly green-to-yellow background (Fig. 27). While this represents a marginal improvement over the antagonism observed under early Baq13 priming with Cisplatin in the same cell line, the overall synergy remained limited.

In BE(2)-C cells, Cisplatin pretreatment followed by Baq13 again yielded a modest and largely unremarkable synergy landscape, consistent with the pattern observed across all three treatment schedules for this drug combination in this cell line (Fig. 28).

In BE(2)-C cells, Vincristine pretreatment followed by Baq13 similarly yielded a minimal synergy profile, with the HSA plot dominated by green-to-yellow regions across the tested dose combinations (Fig. 29). Notably, this stands in contrast to the robust synergy observed in BE(2)-C cells under both simultaneous and early Baq13 priming schedules, underscoring that the chemosensitizing benefit of Baq13 in this cell line is contingent upon Baq13 being present before or concurrent with Vincristine exposure.

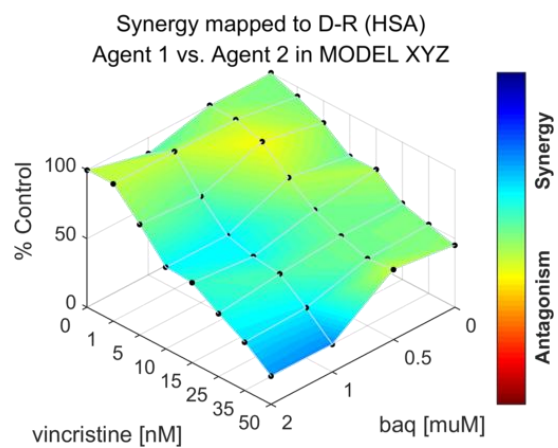


Figure 25. Representative synergy analysis of early Vincristine pretreatment followed by Baq13 upon SK-N-SH

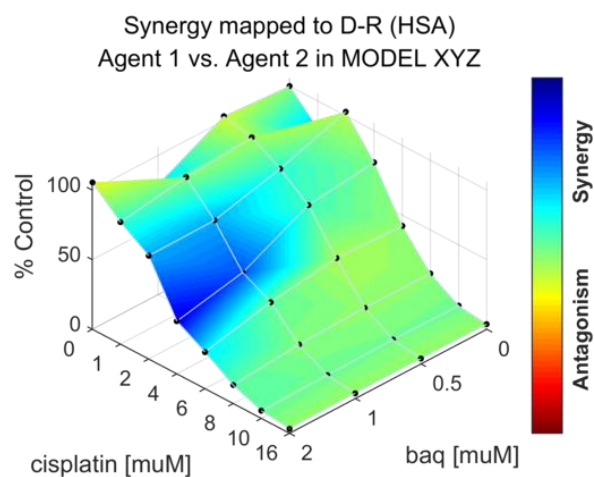


Figure 26. Representative synergy analysis of early Cisplatin pretreatment followed by Baq13 upon SK-N-SH

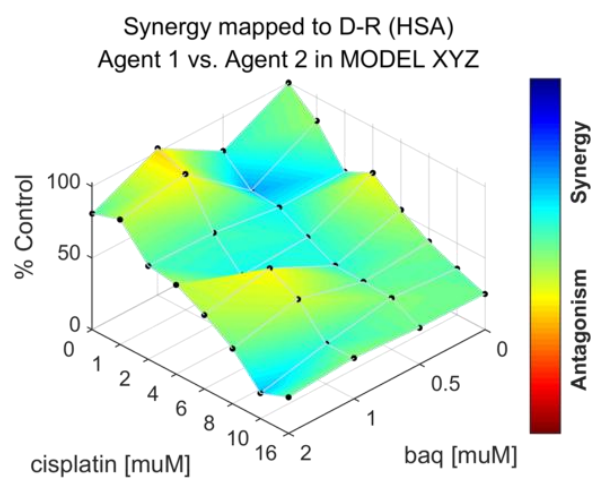


Figure 27. Representative synergy analysis of early Cisplatin pretreatment followed by Baq13 upon

BE(2)-C

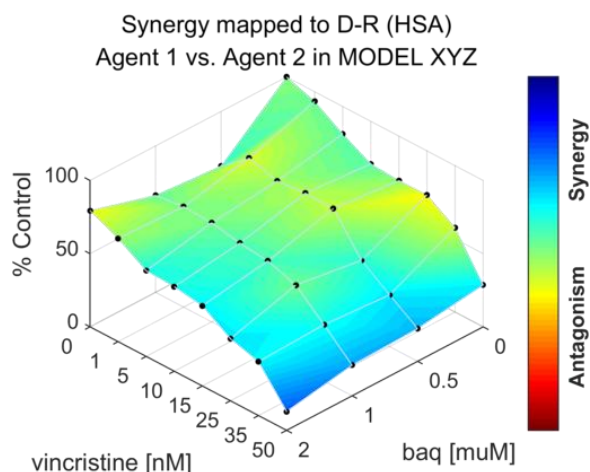


Figure 28. Representative synergy analysis of early Vincristine pretreatment followed by Baq13 upon BE(2)-C

3.5 Summary across all schedules

Taken together, the three treatment schedules reveal a clear pattern of schedule- and drug-dependent synergy in Baq13-based combination therapy. For Vincristine, simultaneous co-treatment and early Baq13 priming consistently produced the most robust synergistic profiles across both cell lines, with BE(2)-C cells demonstrating particularly strong responses under both conditions. In contrast, chemotherapy-first scheduling largely abolished synergy with Vincristine in both cell lines, demonstrating that Baq13's chemosensitizing effect requires its lysosomal disruption to be established prior to or concurrent with Vincristine exposure in order to prevent ion-trapping sequestration and maximize drug availability at its cytosolic target.

Cisplatin, on the other hand, displayed limited synergy with Baq13 across all three schedules and both cell lines, with no paradigm producing a consistently robust synergistic response. This schedule-insensitive pattern is consistent with Cisplatin's

lysosome-independent mechanism of action, whereby its neutral charge and nuclear DNA-targeting activity render it largely unaffected by Baq13-mediated alterations in lysosomal pH. The absence of meaningful schedule dependence for Cisplatin further reinforces that Baq13's chemosensitization operates selectively through a lysosomal sequestration-dependent mechanism.

Collectively, these findings establish that the therapeutic benefit of combining Baq13 with chemotherapy is highly contingent on both the intrinsic properties of the chemotherapeutic agent and the timing of drug administration, with optimal outcomes achieved when Baq13-mediated lysosomal disruption precedes or accompanies exposure to sequestration-susceptible agents such as Vincristine. Notably, this schedule-dependent synergy pattern was conserved across both MYCN-amplified BE(2)-C and MYCN non-amplified SK-N-SH cell lines, suggesting that BAQ13-mediated chemosensitization operates independently of MYCN amplification status and may therefore represent a broadly applicable strategy across neuroblastoma subtypes.

4. Discussion

4.1 Single-agent characterization

The IC₅₀ values of Baq13 in SK-N-SH and BE(2)-C cells were determined to be in the range of 2–4 μ M following 24-hour treatment, consistent with previously reported antiproliferative activity of Baq13 and related Baq derivatives across multiple cancer cell lines at low micromolar concentrations (14). Notably, no substantial difference in Baq13 sensitivity was observed between the MYCN-amplified BE(2)-C and MYCN non-amplified SK-N-SH lines, suggesting that Baq13's cytotoxic mechanism — primarily lysosomal disruption and autophagy blockade — operates independently of MYCN-driven transcriptional programs. This is consistent with Baq13's mechanism of action, which targets the lysosomal compartment rather than oncogene-specific signaling pathways, and suggests broad applicability across neuroblastoma subtypes regardless of MYCN status. The IC₅₀-guided dose matrix design for subsequent combination experiments ensured comprehensive coverage of both sub- and supra-IC₅₀ concentrations, enabling robust mapping of synergistic and antagonistic dose regions via the HSA model.

4.2 Solvent-dependent efficacy: albumin corona hypothesis

Baq13 cytotoxicity was found to be significantly influenced by the solvent used for drug preparation, with FBS-containing medium producing the greatest potency, followed by complete media, PBS, and EMEM alone. This gradient of efficacy is consistent with the physicochemical properties of Baq13 nanoparticles, which self-

assemble into liposome-like structures of approximately 100–140 nm with positively charged surfaces (~ 40 mV) under neutral aqueous conditions (14). Nanoparticles within this size range and bearing hydrophobic surfaces are well-documented to spontaneously adsorb serum proteins upon exposure to biological fluids, forming a protein corona in which albumin is a predominant component (18) (19). Albumin-coated nanoparticles have been shown to exploit albumin receptor-mediated endocytosis pathways, including SPARC- and gp60-mediated transcytosis, to achieve preferential uptake in cancer cells, a principle underlying the clinical success of albumin-bound paclitaxel (nab-paclitaxel, Abraxane) (20) (21). Importantly, Ma et al. demonstrated that Baq13 NPs maintain stable particle size in the presence of 0.5 mM bovine serum albumin, indicating that albumin association does not disrupt nanoparticle integrity but may instead facilitate cellular uptake (14). The near-absence of cytotoxicity in EMEM alone may therefore reflect impaired nanoparticle-cell interactions in the absence of serum-derived corona-forming proteins. These findings underscore the importance of solvent standardization in Baq13 experimental design and suggest that in vivo, the serum environment may actively enhance Baq13 bioavailability through albumin-mediated uptake mechanisms.

4.3 Autophagy and apoptosis markers

The dose-dependent upregulation of p62 (SQSTM1) observed at the mRNA level in both SK-N-SH and BE(2)-C cells provides transcriptional evidence of Baq13-mediated autophagy blockade. p62 is a selective autophagy receptor that is normally

degraded via incorporation into autophagosomes; its accumulation is therefore a well-established hallmark of impaired autophagic flux (22). These findings are consistent with the original characterization of Baq13, in which significant concentration-dependent increases in SQSTM1/p62 protein were observed in pancreatic cancer cells treated with BaqONNs (14). LC3B was also assessed by qPCR in the current study; however, changes in LC3B mRNA expression were minimal and are not reported here. This is consistent with the established understanding that autophagic regulation of LC3B occurs primarily at the post-translational level, through lipidation of cytosolic LC3-I to membrane-bound LC3-II, rather than through transcriptional upregulation of LC3B mRNA. Protein-level detection via western blot is therefore the appropriate and more sensitive method for monitoring LC3B flux, as demonstrated in the original Baq13 characterization study (14), and is proposed as a future direction for mechanistic validation in neuroblastoma models.

Regarding BAX and BAK1, statistically significant but modest changes in mRNA expression were observed upon Baq13 treatment in both cell lines, with the direction and magnitude of change differing between SK-N-SH and BE(2)-C. While the original RNA-seq analysis of Baq13-treated pancreatic cancer cells reported transcriptional upregulation of multiple pro-apoptotic genes including BAX and BAK1 (14), the modest and non-uniform changes observed here at the mRNA level are not unexpected. Apoptotic execution is predominantly governed by post-translational mechanisms, including BCL-2 family protein interactions, mitochondrial

outer membrane permeabilization, and caspase activation cascades, rather than by large-scale transcriptional upregulation of effector genes (23). The modest mRNA-level changes therefore do not preclude meaningful apoptotic activity at the protein level, and future western blot analyses targeting cleaved caspase-3, cleaved PARP, and BAX/BAK1 protein expression are warranted to fully characterize Baq13's pro-apoptotic effects in neuroblastoma.

4.4 Schedule-dependent synergy with Vincristine

The robust synergy observed between Baq13 and Vincristine under simultaneous and early Baq13 priming schedules can be mechanistically attributed to Baq13's capacity to disrupt lysosomal function and neutralize lysosomal pH. Vincristine is a weakly basic, hydrophobic vinca alkaloid that is highly susceptible to lysosomal ion-trapping: upon cellular uptake, Vincristine undergoes protonation within the acidic lysosomal lumen (pH 4.0–5.5), becoming membrane-impermeant and sequestered away from its cytosolic target, the tubulin-microtubule system (24). This sequestration mechanism is a well-documented contributor to chemotherapy resistance in cancer cells with elevated lysosomal activity (25). Baq13, with its strong H⁺ buffering capacity and pH-responsive membrane-disrupting activity, neutralizes lysosomal acidity and induces lysosomal membrane permeabilization (14), thereby either preventing the initial protonation and sequestration of Vincristine or facilitating the release of previously entrapped drug into the cytosol. Under simultaneous co-treatment, this mechanism is directly operative, providing immediate lysosomal deacidification concurrent with

Vincristine exposure. Under early Baq13 priming, pre-established lysosomal disruption may create a permissive intracellular environment that sustains drug availability upon subsequent Vincristine addition, particularly in BE(2)-C cells where the strong synergistic response under priming may reflect the higher baseline autophagic flux associated with MYCN amplification and the correspondingly greater lysosomal burden that Baq13 must overcome.

4.5 Limited synergy with Cisplatin

In contrast, the consistently limited synergy observed between Baq13 and Cisplatin across all three treatment schedules and both cell lines is mechanistically coherent with Cisplatin's distinct pharmacological profile. Cisplatin is a neutral, non-ionizable platinum complex whose primary mechanism of cytotoxicity involves direct nuclear DNA crosslinking, forming intrastrand adducts that activate DNA damage response pathways and ultimately trigger apoptosis (26). Unlike weakly basic drugs susceptible to ion-trapping, Cisplatin's neutral charge renders it insensitive to lysosomal pH gradients, and its intracellular trafficking is not meaningfully dependent on lysosomal routing. Baq13-mediated lysosomal disruption therefore provides no pharmacological advantage for Cisplatin delivery or activity, explaining the absence of schedule-dependent sensitization effects. The marginal synergy observed in BE(2)-C cells under certain conditions may reflect indirect effects of autophagy inhibition on general cellular stress tolerance rather than a specific lysosomal sequestration-release mechanism.

4.6 Timing effects

The schedule-dependent outcomes observed across the three treatment paradigms collectively suggest that the window of Baq13-mediated lysosomal disruption must temporally overlap with or precede the intracellular accumulation of Vincristine in order to maximize chemosensitization. When chemotherapy is administered first, Vincristine likely undergoes rapid sequestration within intact lysosomes before Baq13 can establish sufficient lysosomal disruption, effectively locking the drug in a pharmacologically inactive compartment. The subsequent addition of Baq13 may be insufficient to fully reverse this pre-established sequestration, leading to the attenuated synergy observed under the chemo-first schedule. This temporal dependency underscores the importance of treatment sequencing in lysosomal sequestration-based resistance and has practical implications for clinical scheduling of Baq13-based combination regimens.

4.7 Future directions

The findings of this study establish a proof-of-concept for Baq13-mediated chemosensitization in neuroblastoma in vitro; however, several key questions remain to be addressed. First, the observed synergy should be validated in chemotherapy-resistant neuroblastoma models, including cell lines with acquired resistance to Vincristine or Cisplatin, to determine whether Baq13 can restore sensitivity in a clinically relevant resistance context. Patient-derived neuroblastoma models, including primary tumor-derived cell lines or organoids, would further strengthen the

translational relevance of these findings. Second, protein-level validation of autophagy and apoptosis markers via western blot is necessary to corroborate the transcriptional findings and provide mechanistic depth. Finally, progression to in vivo models is essential to assess whether the schedule-dependent synergy observed in vitro is retained in a more complex tumor microenvironment, and to establish pharmacokinetic and toxicity profiles for Baq13-based combination regimens in neuroblastoma.

5. Conclusion

In summary, Baq13 demonstrated potent single-agent cytotoxicity at low micromolar concentrations in both MYCN-amplified BE(2)-C and MYCN non-amplified SK-N-SH neuroblastoma cell lines, with IC₅₀ values in the 2–4 μ M range independent of MYCN status. Combination studies revealed that Baq13 synergizes robustly with Vincristine in a schedule-dependent manner, with simultaneous co-treatment and early Baq13 priming producing the most consistent synergistic profiles, attributable to Baq13-mediated prevention of lysosomal drug sequestration. In contrast, Cisplatin exhibited limited synergy with Baq13 across all schedules, consistent with its lysosome-independent mechanism of action. Transcriptional upregulation of p62 confirmed Baq13's autophagy-inhibitory activity in both cell lines. Collectively, these findings provide in vitro proof-of-concept for Baq13 as a schedule-optimized chemosensitizer in neuroblastoma and establish a mechanistic and experimental framework for future studies in resistant models and in vivo systems.

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