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Original Article

Nanoliposomal curcumin as a potential pro-apoptotic agent for cervical cancer: Evidence from Bcl-2 and Bax modulation in HeLa cells

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Abstract

Cervical cancer remains a major cause of cancer-related mortality, and treatment effectiveness is often limited by drug resistance and systemic toxicity. Curcumin has shown anticancer activity, but its clinical translation is constrained by poor solubility and low bioavailability. Nanoliposomal curcumin (Cur-NLPs) may improve intracellular delivery; however, its dose-dependent effect on apoptosis-related proteins in cervical cancer cells remains insufficiently defined. This study evaluated the dose-dependent modulation of Bcl-2 and Bax protein expression by Cur-NLPs in HeLa cells. HeLa cells were assigned to five groups: negative control, cisplatin 5 µg/mL as positive control, and Cur-NLPs at 100, 150, and 200 µg/mL. After 24 hours of treatment, Bcl-2 and Bax expression were assessed by immunofluorescence and quantified as corrected total cell fluorescence (CTCF). Data were analyzed using one-way ANOVA, Tukey HSD post hoc test, Pearson correlation, and linear regression. Significant between-group differences were observed for both Bcl-2 ($F=8.99$; $p=0.002$) and Bax ($F=132.38$; $p<0.001$). Cur-NLPs progressively reduced Bcl-2 expression and increased Bax expression, with the most pronounced effect observed at 200 µg/mL. Bcl-2 expression at 200 µg/mL and Bax expression at 150–200 µg/mL were significantly altered compared with the negative control, while the 200 µg/mL dose showed no significant difference from cisplatin for either marker. Cur-NLPs concentration showed a strong negative correlation with Bcl-2 expression ($r=-0.847$; $p<0.001$) and a strong positive correlation with Bax expression ($r=0.942$; $p<0.001$). Linear regression further confirmed dose-dependent effects, with Cur-NLPs concentration explaining 79.6% and 88.8% of the variance in Bcl-2 and Bax expression, respectively. These findings indicate that Cur-NLPs can shift the Bcl-2/Bax balance toward a pro-apoptotic profile in HeLa cells, supporting its potential as a curcumin-based nanodelivery strategy for cervical cancer. Further studies using functional apoptosis assays, additional cervical cancer cell lines, and in vivo models are needed to confirm its translational relevance.

Keywords: Cervical cancer, apoptosis, Bcl-2, Bax, nanoliposomal curcumin

Introduction

Cervical cancer remains a significant global health burden, with more than 600.00 new cases and more than 340.00 deaths recorded worldwide in 2020, disproportionately affecting low- and middle-income countries including Indonesia, where the age-standardized incidence rate reaches 24.4 per 100.000 women [1]. Persistent infection with high-risk human papillomavirus (HPV) genotypes 16 and 18 drives cervical carcinogenesis through constitutive expression of oncoproteins E6 and E7, which abrogate p53-mediated transcriptional regulation, resulting in

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overexpression of the anti-apoptotic protein Bcl-2 and concurrent suppression of the pro-apoptotic effector Bax [2,3]. This dysregulation impairs the intrinsic mitochondrial apoptotic pathway, sustaining malignant cell survival and contributing to resistance against cytotoxic agents such as cisplatin, the current chemotherapeutic standard of care, whose clinical utility is further limited by systemic toxicities including nephrotoxicity and myelosuppression [4].

Curcumin, the principal bioactive polyphenol derived from *Curcuma longa* rhizome, has been extensively investigated as a phytochemical anticancer agent with demonstrated capacity to downregulate Bcl-2 and upregulate Bax expression across multiple cancer cell lines [5]. However, its translation into clinical application is critically hindered by poor aqueous solubility, rapid systemic metabolism, and consequent low oral bioavailability, biopharmaceutical limitations that cannot be resolved through dose escalation alone [6]. Nanoliposomal curcumin represents a validated pharmaceutical technology strategy to overcome these constraints: phospholipid bilayer vesicles (50–200 nm) protect curcumin from enzymatic degradation, prolong systemic circulation, and enhance intracellular delivery via membrane fusion and endocytic pathways, thereby substantially improving pharmacokinetic performance relative to free curcumin [7].

Despite the recognized potential of nanoliposomal curcumin (Cur-NLPs), critical evidence gaps persist. Studies evaluating curcumin in HeLa cells have predominantly employed free curcumin formulations [8] or examined Cur-NLPs exclusively in combination with cisplatin [9,10], precluding mechanistic attribution to the nanoliposomal formulation as a monotherapy. Furthermore, no study has established a quantitative dose-response relationship for Cur-NLPs on Bcl-2 and Bax protein expression through immunofluorescence-based fluorescence intensity quantification in HeLa cell culture, a prerequisite for informed dose selection in downstream functional apoptosis studies. The aim of this study was therefore to evaluate the dose-dependent modulation of Bcl-2 and Bax expression by Cur-NLPs as monotherapy in HeLa cells, benchmarked against cisplatin as a positive control, to provide foundational preclinical evidence supporting nanoliposomal encapsulation as a phytochemical delivery strategy in cervical cancer pharmacotherapy.

Methods

Study design

This experimental in vitro study was conducted between January and March 2026 at the Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia. HeLa cells were assigned to five independent treatment groups: negative control, positive control (cisplatin 5 µg/mL), and Cur-NLPs at 100, 150, 200 µg/mL. Following 24-hour treatment, Bcl-2 and Bax protein expression were assessed by immunofluorescence and quantified as corrected total cell fluorescence (CTCF) to evaluate dose-dependent pro-apoptotic effects.

Cell culture

HeLa cells (ATCC CCL-2, Manassas, VA, USA) were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Gibco) at 37°C in 5% CO_2. Upon reaching 60–80% confluence, cells were detached with 0.25% trypsin-EDTA (Gibco), and viability was confirmed by trypan blue exclusion before seeding.

Nanoliposomal curcumin preparation

Curcumin was extracted from *C. longa* rhizomes by Soxhlet extraction using 96% ethanol at 60°C for 8 hours, followed by rotary evaporation and n-hexane purification. Cur-NLPs were prepared by thin-film hydration of soy phosphatidylcholine (S-100), cholesterol, curcumin, and Tween 80 in chloroform, hydrated with phosphate-buffered saline (PBS) at 60°C for 30 minutes, and probe-sonicated at 80 Watts (W) for 3 minutes. Particle size was measured using a particle size analyzer (Shimadzu SALD-7500nano, Kyoto, Japan); a single peak was observed with a Z-average diameter of 130.6 nm and a polydispersity index (PDI) of 0.163, indicating a homogeneous monodisperse nanoparticle population.

Treatment groups and protocol

HeLa cells were seeded at a density 2000 cells/well in 96-well plates in total volume of 100 µL per well (1.5 µL suspension containing 2000 cells in 98.5 µL complete medium) and allowed to adhere for 72 hours until 60-80% confluence was reached. The culture medium was then replaced with 100 µL of treatment solution per well. Cur-NLPs treatment solutions were prepared by diluting the stock solution (50 mg/mL) in complete medium to achieve target concentrations of 100, 150, and 200 µg/mL. Treatments were assigned to five groups: negative control (complete medium), positive control (cisplatin 5 µg/mL; Kalbe, Jakarta, Indonesia), and Cur-NLPs at 100, 150, and 200 µg/mL. Following addition of treatment solutions, plates were subjected to gentle tilting to ensure homogeneous distribution without disturbing the cell. Cells were incubated for 24 hours at 37°C in 5% CO_2 before analysis. The cisplatin dose was selected based on its established capacity to induce apoptosis in HeLa cells near the reported median lethal dose (LD_{50}) of 10.5 µg/mL.

Immunofluorescence and image quantification

Following 24-hour treatment, the culture medium was aspirated from each well and cells were washed with Dulbecco's phosphate-buffered saline (DPBS). Cells were fixed with 4% paraformaldehyde (PFA) in DPBS (±50 µL per well) for 10 minutes at room temperature, followed by washing with DPBS. For permeabilization, wells were washed five times with 0.1% Triton X-100 in PBS (1 minute each), then washed three times with PBS (2 minutes each) and air-dried. Blocking was performed by incubating wells with 1% bovine serum albumin (BSA) for 30 minutes at room temperature. Wells were then incubated overnight at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti-Bcl-2 (Bcl-2 Ser87 polyclonal antibody; Bioss Antibodies, Woburn, MA, USA) and phycoerythrin (PE)-conjugated anti-Bax polyclonal antibody (Bioss Antibodies, Woburn, MA, USA), both diluted in 1% BSA. Following overnight incubation, wells were washed three times with PBS (5 minutes each), then incubated with 4',6-diamidino-2-phenylindole (DAPI) (1:1000) for 5 minutes for nuclear counterstaining. Wells were washed again three times with PBS (5 minutes each), mounted with mounting medium, and cover slipped. For each treatment group, two fields per replicate were imaged, with fields selected randomly by an independent observer to minimise selection bias. Five cells per fields were selected for CTCF quantification, yielding 30 cells per treatment group. Fluorescence intensity was quantified as CTCF using Fiji software (NIH, Bethesda, MD, USA) using formula: CTCF = integrated density – (area of selected cell × mean fluorescence of background readings).

Statistical analysis

Normality and homogeneity of variance were assessed using the Shapiro-Wilk and Levene tests. Group differences in CTCF values were analyzed by one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) post hoc test. Pearson correlation and linear regression were used to assess the dose-response relationship between Cur-NLPs concentration and Bcl-2/Bax expression. All analyses were performed using IBM SPSS Statistics v29 (IBM Corp., NY, USA).

Results

Bcl-2 and Bax expression in HeLa cells following Cur-NLPs treatment

Immunofluorescence assessment of Bcl-2 and Bax expression in HeLa cells was performed after 24-hour treatment. Bcl-2 was detected using FITC (green), Bax with PE (red), and nuclei with DAPI (blue); qualitative fluorescence changes were observed with increasing Cur-NLPs concentration (**Figure 1**). The negative control exhibited high Bcl-2 and low Bax fluorescence, reflecting an anti-apoptotic protein profile. Increasing Cur-NLPs concentrations (100–200 µg/mL) were associated with progressive reduction in Bcl-2 and elevation in Bax fluorescence. The positive control (cisplatin 5 µg/mL) similarly reduced Bcl-2 and elevated Bax, consistent with apoptosis induction (**Figure 1**).

Quantitative validation was performed via CTCF analysis and the mean CTCF values are presented in **Table 1**. One-way ANOVA demonstrated statistically significant between-group

differences in both Bcl-2 expression (F=8.99; p=0.002) and Bax expression (F=132.38; p<0.001) (**Table 1**). All parametric assumptions were satisfied: Shapiro–Wilk tests confirmed normality across all groups (Bcl-2: p=0.223–0.993; Bax: p=0.178–0.734), and Levene's test confirmed homogeneity of variance (Bcl-2: p=0.326; Bax: p=0.705).

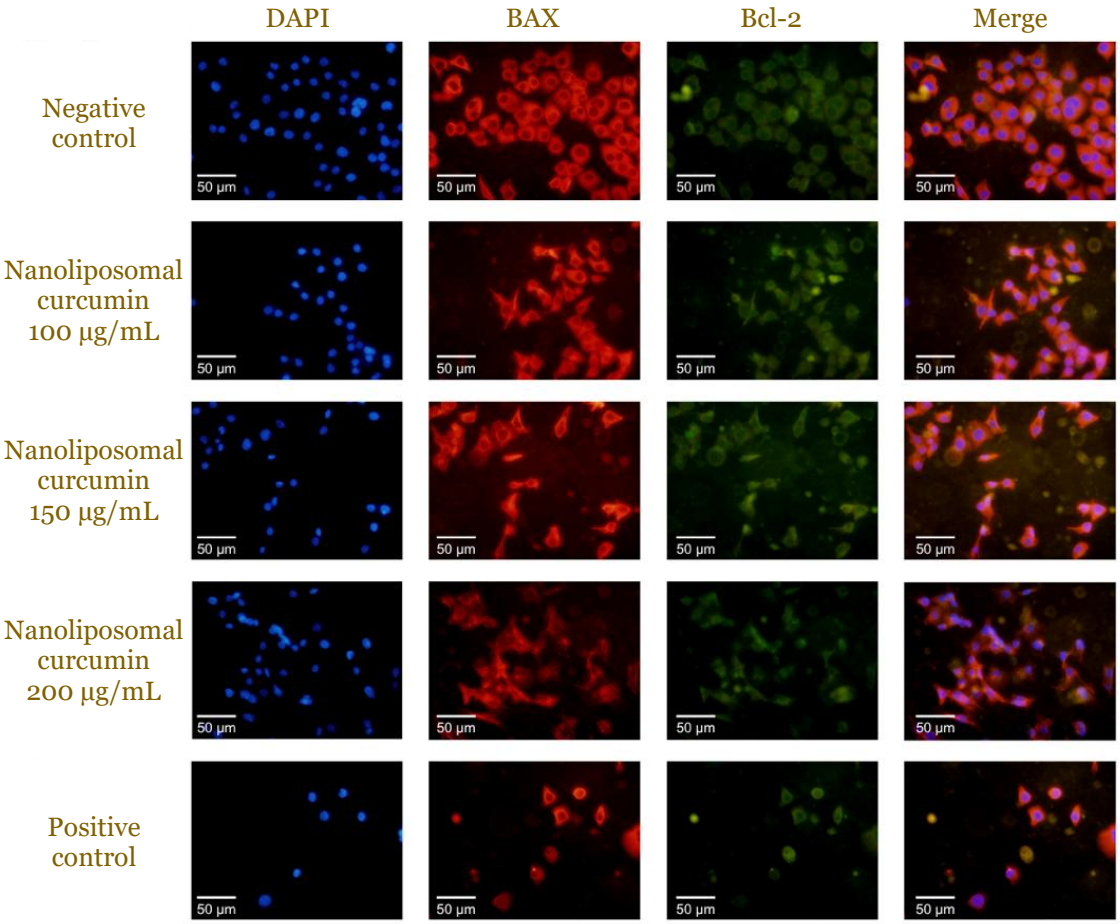

Figure 1. Immunofluorescence images of Bax and Bcl-2 in HeLa cells following 24-hour treatment with Cur-NLPs at various concentrations, viewed under Olympus IX-71 at 40× magnification. Nuclei were stained with DAPI (blue); Bax is shown in red (PE), Bcl-2 in green (FITC). The merged image displays all channels. Rows represent treatment groups: negative control (untreated), Cur-NLPs 100, 150, and 200 µg/mL, and positive control (cisplatin 5 µg/mL). For each treatment group, two randomly selected fields were imaged per replicate (n=3 replicates per group); five cells per field were selected for quantification, yielding 30 cells per treatment group for fluorescence quantification.

Table 1. Mean corrected total cell fluorescence (CTCF) values of Bcl-2 and Bax expression across treatment groups

Group	Mean corrected total cell fluorescence (CTCF) values	
	Bcl-2 (mean±SD)	BAX (mean±SD)
Negative control	74,162.0±4,053.9	154,127.0±4,110.4
Cur-NLPs 100 µg/mL	73,827.9±4,794.1	154,425.9±4,332.5
Cur-NLPs 150 µg/mL	67,868.9±1,923.8	201,133.6±4,531.8
Cur-NLPs 200 µg/mL	61,083.6±2,193.0	215,147.5±6,463.0
Positive Control	65,207.5±2,288.5	217,866.7±4,176.5
F	8.99	132.38
p-value*	0.002	<0.001

*Analyzed using one-way ANOVA test

Tukey HSD post hoc analysis revealed that statistically significant Bcl-2 downregulation relative to the negative control was achieved exclusively at 200 µg/mL (p<0.004), whereas doses of 100 (p=1.000) and 150 µg/mL (p=0.200) did not produce significant reductions (**Figure 2**).

Bcl-2 expression at 200 µg/mL was not statistically significant to cisplatin positive control (p=0.554), while the positive control differed significantly from negative control (p<0.044). For Bax, significant upregulation was evident at 150 µg/mL and 200 µg/mL compared to both the negative control and Cur-NLPs 100 (both p<0.0001), while the negative control and Cur-NLPs 100 µg/mL did not differ from each other (both p=1.000) (**Figure 2**). Bax expression at 200 µg/mL was not statistically significant to cisplatin positive control (p=0.953), while Cur-NLPs at dose 150 µg/mL showed significant differences from both 200 µg/mL (p=0.032) and positive control (p=0.011) (**Figure 2**). These comparisons are limited to protein expression only and do not imply functional apoptotic equivalence.

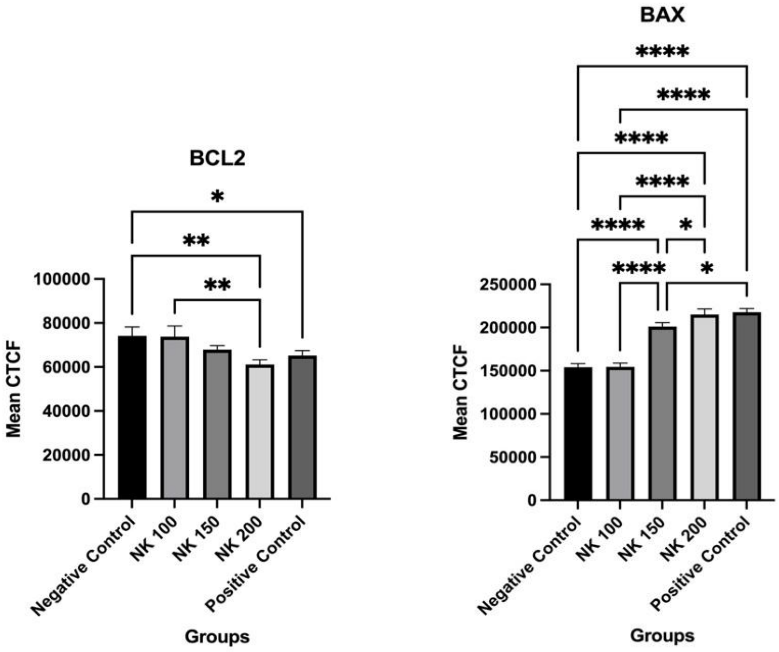

Figure 2. The graph shows the average values of corrected total cell fluorescence (CTCF) for Bcl-2 and Bax expression with ± standard deviation in each treatment group. Fluorescence quantification was based on 30 cells per treatment group (five cells per fields, two fields per replicate, n=3 replicates). The notation * indicates a significant difference between groups (p<0.05), ** indicates a very significant difference between groups (p<0.01) and **** indicates a highly significant difference between groups (p<0.0001) based on the Tukey HSD test.

Correlation between Cur-NLPs concentration to Bcl-2 and Bax expression

Pearson correlation analysis revealed a strong negative correlation between Cur-NLPs dose and Bcl-2 expression (r=−0.84; p<0.001) and a strong positive correlation with Bax expression (r=0.94; p<0.001) (**Table 2**), indicating that increasing Cur-NLPs doses progressively downregulated Bcl-2 while upregulating Bax in HeLa cells.

Table 2. Pearson correlation and linear regression analysis between Cur-NLPs concentration and Bcl-2/Bax expression

Variable	Correlation coefficient (r)	Constant (a)	B coefficient (dose)	R^2	Regression equation	F	p-value
Cur-NLPs → Bcl-2	-0.847	86,709.9	-127.44	0.79	Bcl-2=86,709.9−127.4×dose	27.25	<0.001
Cur-NLPs → BAX	0.942	99,153.2	607.21	0.88	BAX=99,153.3+607.2×dose	55.57	<0.001

Dose expressed in µg/mL; CTCF values used as expression units.

Linear regression confirmed a significant dose-response relationship for both proteins (**Table 2**). Cur-NLPs concentration explained 79.6% of the variance in Bcl-2 expression (R^2=0.796; F=27.251; p=0.001), with each 1 µg/mL increase in dose associated with a 127,443 CTCF unit reduction (regression equation: Bcl-2=86709.991−127.443×dose). For Bax expression, Cur-NLPs concentration accounted for 88.8% of variance (R^2=0.888; F=55.576;

p<0.001), with each 1 µg/mL increase associated with a 607,216 CTCF unit increase (regression equation: Bax=99153.295+607.216×dose) (**Table 2**).

Discussion

The present study demonstrated that Cur-NLPs progressively suppressed Bcl-2 and upregulated Bax in HeLa cells in a concentration-dependent manner, with the most pronounced effects at 200 µg/mL. The dose-dependent Bcl-2 reduction reaching levels statistically equivalent to cisplatin at the protein expression level is consistent with the possibility that the nanoliposomal formulation delivers sufficient intracellular curcumin to counteract the anti-apoptotic signaling sustained by E6 in HeLa cells [11,12]. In this cellular context, E6 promotes p53 degradation via the ubiquitin-proteasome pathway, thereby sustaining Bcl-2 transcription and enabling apoptosis evasion [3,13].

The finding that statistically significant Bcl-2 reduction was achieved exclusively at 200 µg/mL, whereas Bax upregulation reached significance as early as 150 µg/mL, indicates that these two proteins exhibit differential concentration thresholds for Cur-NLPs responsiveness. This dissociation suggests that pro-apoptotic signaling via Bax may be initiated at concentrations that have not yet overcome the anti-apoptotic barrier imposed by residual Bcl-2 activity. A critical minimum intracellular curcumin concentration must therefore be attained before Bcl-2 suppression becomes measurable, a phenomenon consistent with Bcl-2 overexpression enabling cancer cells to sustain survival signaling despite partial pro-apoptotic stimulation [14,15]. From a translational standpoint, this threshold behavior underscores the need to determine in vivo pharmacokinetic parameters capable of sustaining tumor-level concentrations equivalent to 200 µg/mL, as in vitro concentrations do not scale directly to clinical dosing [16].

The statistical equivalence between Cur-NLPs at 200 µg/mL and cisplatin at 5 µg/mL in modulating both Bcl-2 and Bax is a central finding of this study. These two agents achieve comparable protein-level outcomes through mechanistically distinct routes: cisplatin reduces Bcl-2 via DNA crosslinking-induced p53 activation [17], whereas Cur-NLPs may engage multiple upstream pathways concurrently, including E6 suppression, NF-kB inhibition, and direct Bcl-2 phosphorylation blockade (pathway not directly assessed in the present study). This mechanistic breadth is particularly relevant in cisplatin-resistant settings, where Bcl-2 overexpression, enhanced DNA repair capacity, and drug efflux via ATP7A collectively limit platinum efficacy [17,18]. The capacity of Cur-NLPs to suppress Bcl-2 through DNA damage independent mechanisms thus positions it as a candidate therapeutic in cisplatin refractory cervical cancer, where alternative pro-apoptotic strategies are urgently needed.

Bax expression increased significantly at 150 µg/mL Cur-NLPs, with further elevation at 200 µg/mL, reaching levels comparable to cisplatin at both concentrations. The earlier responsiveness of Bax relative to Bcl-2 indicates that pro-apoptotic transcriptional activation operates at a lower curcumin concentration threshold than anti-apoptotic suppression. This dissociation is mechanistically informative: Bax and Bcl-2 are not simply reciprocal pairs but are regulated through distinct upstream signaling inputs, and their independent concentration sensitivities reflect the divergent complexity of each protein's regulatory circuitry [14,19]. The mechanistic basis for Bax upregulation in this model likely involves Cur-NLPs-mediated restoration of functional p53 activity through suppression of HPV-18 E6 oncoprotein expression, thereby enabling p53 driven transactivation of the Bax gene promoter [20,21]. It is further hypothesized that Cur-NLPs induced selective reactive oxygen species (ROS) generation in cancer cells may promote direct Bax conformational activation and translocation to the outer mitochondrial membrane, independent of transcriptional changes [5,12].

When activated, Bax undergoes homo-oligomerization and inserts into the mitochondrial outer membrane, forming pores that facilitate cytochrome c and Smac/DIABLO release [19,22]. The convergence of transcriptional Bax upregulation via p53 restoration and post translational Bax activation via ROS mediated stress may explain why Bax responsiveness preceded Bcl-2 suppression across the concentration range studied. This has a clinically meaningful implication: in tumor microenvironments where Bcl-2 suppression remains incomplete, such as at sub-maximal doses, independently elevated Bax may still be sufficient to initiate mitochondrial outer

membrane permeabilization (MOMP), cytochrome c release, apoptosome assembly, and downstream activation of caspase-9 and caspase-3 [15,23,24].

The strong dose-response relationships confirmed by linear regression indicate that Cur-NLPs concentration is a reliable predictor of apoptotic protein expression within the dose range examined. The higher coefficient of determination for Bax further supports the proposition that Bax transcription is more tightly coupled to curcumin dose, likely reflecting its more direct dependence on p53 transactivation compared to the multilayered regulatory network governing Bcl-2. These regression parameters also provide a quantitative basis for extrapolating effective concentration ranges in future in vivo or ex vivo experimental designs, subject to appropriate pharmacokinetic modelling. Nanocurcumin formulations have been shown to shift the Bax/Bcl-2 ratio more effectively than free curcumin, attributed to improved cellular internalization and prolonged intracellular retention [25]. Similarly, OA400-based nanocurcumin achieved pronounced apoptotic induction in HeLa cells, consistent with the magnitude of Bcl-2 suppression and Bax upregulation observed here [21]. These converging findings across nanoformulation platforms substantiate the 150–200 µg/mL range as the most therapeutically relevant concentration window for curcumin-based nanocarriers in cervical cancer.

Cur-NLPs at 150–200 µg/mL modulated apoptotic proteins as effectively as cisplatin 5 µg/mL without the systemic toxicities associated with platinum-based chemotherapy, including nephrotoxicity, neurotoxicity, and myelosuppression. This raises the possibility that the therapeutic index of curcumin-based nanoformulations may be superior to that of platinum-based chemotherapy, though this interpretation requires in vivo pharmacological and toxicological evaluation before conclusions can be drawn. Notably, prior studies from our group demonstrated that combining Cur-NLPs with cisplatin synergistically enhances apoptosis in HeLa cells, with significant Bax increases and Bcl-2 decreases at 100 µg/mL Cur-NLPs combined with cisplatin 5 µg/mL [9,10]. The present findings extend this work by establishing that Cur-NLPs as monotherapy, at a higher dose, achieves equivalent pro-apoptotic protein modulation to cisplatin alone, suggesting dual utility as both a standalone agent and a chemosensitizer.

The nanoliposomal carrier is considered integral to the efficacy observed. Free curcumin is constrained by poor aqueous solubility, rapid phase II metabolic conjugation, and low passive diffusion into cells, resulting in limited intracellular accumulation [16]. Encapsulation within a liposomal vehicle protects curcumin from premature degradation, facilitates cellular internalization via endocytic pathways, and sustains intracellular release over an extended period. The resultant enhancement in bioavailability is likely responsible for the magnitude of protein modulation observed at 200 µg/mL, effects that would likely be unattainable at equivalent concentrations of free curcumin. However, the absence of a free curcumin comparator group in this study precludes direct attribution of the observed effects to the nanoliposomal carrier, representing a limitation that should be addressed in subsequent work.

Some methodological considerations should be acknowledged to provide appropriate context for interpreting the present findings. The use of HeLa cells as the sole cell line necessitates caution in generalizing the results; the inclusion of HPV-16 positive cell lines such as CaSki and SiHa in future investigations would enhance the representativeness of findings across a broader spectrum of cervical cancer phenotypes. The absence of free curcumin and empty nanoliposome control groups precludes direct attribution of the observed effects to the nanoliposomal carrier; inclusion of both control arms in future work would allow more precise delineation of formulation-specific contributions. While the protein expression profiles observed in this study are consistent with the induction of apoptosis, definitive confirmation would benefit from direct functional validation through established methods such as Annexin V/PI staining, Sub-G1 cell cycle analysis, or caspase-3/7 activity assays; additionally, the present study employed n=3 biological replicates per group, which is relatively limited, and future studies incorporating a greater number of independent replicates would further strengthen the reliability of conclusions.

Conclusion

Cur-NLPs dose-dependently modulated Bcl-2 and Bax protein expression in HeLa cells, suppressing Bcl-2 and upregulating Bax at 150–200 µg/mL to levels comparable to cisplatin at 5 µg/mL at the protein level. The strong dose-response relationships for both proteins confirmed

by correlation and regression analyses indicates that Cur-NLPs concentration is a reliable predictor of apoptotic protein modulation within the range studied. These findings provide preliminary molecular evidence that Cur-NLPs can shift the Bcl-2 and Bax expression balance toward a pro-apoptotic profile in HeLa cells, though whether this protein level shift translates to functional apoptosis induction remains to be confirmed. Future studies incorporating functional apoptosis validation, expanded cell line models, appropriate formulation controls, and in vivo pharmacokinetic evaluation are needed to support clinical translation.

Ethics approval

This study adhered to ethical guidelines and received approval from the Health Research Ethics Committee, Faculty of Medicine, Universitas Brawijaya. Ethical clearance was granted through exemption letter No. 06/EC/KEPK/01/2026, declaring the research protocol exempt from full ethical review.

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

Declaration of artificial intelligence use

This study used artificial intelligence (AI) tools, ChatGPT and Claude AI, in language refinement to improve grammar, sentence structure, and readability of the manuscript. We confirm that all AI-assisted processes were critically reviewed by the authors to ensure the integrity and reliability of the results. The final decisions and interpretations presented in this article were solely made by the authors.

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