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In vitro characterization of β-cyclodextrin liposomal formulation of cisplatin

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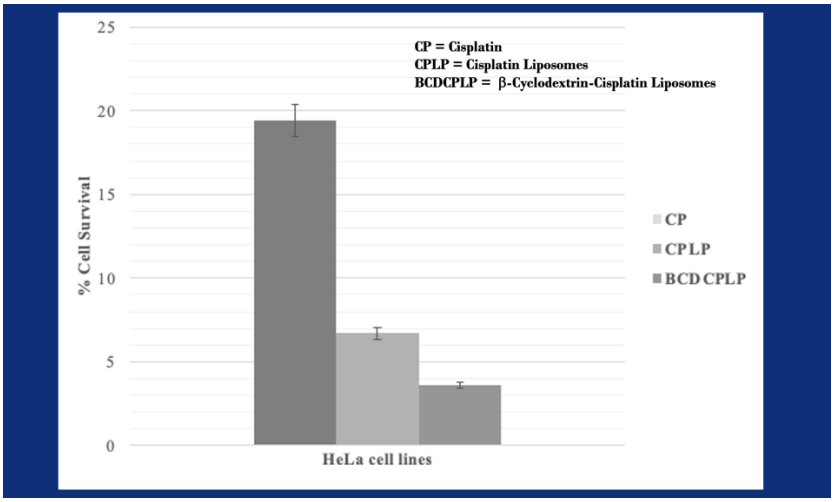
Graphical Abstract



Time-dependent inhibition of cell growth was observed in HeLa cells exposed to IC_{90} levels of cisplatin from various formulations. CPLP and BCDCPLP treatments led to a 2.90-fold and 5.39-fold reduction in IC_{90}, respectively, compared to CP alone. Maintaining liposome integrity enhanced uptake efficiency.

Abstract

This study aimed to develop a nanomedicine-based approach to protect healthy tissue during cancer treatment, improve patient compliance, reduce adverse drug reactions, and enhance the quality of life. By utilizing hydroxypropyl-β-cyclodextrin (BPCP) to increase drug hydrophilicity and liposomes for controlled release of cisplatin, the hydroxypropyl-β-cyclodextrin-cisplatin liposomal formulation (BCDCPLP) demonstrated improved stability, targeted delivery, and reduced toxicity. The study confirmed that the above formulation had the highest cytotoxicity against HeLa cells, with significantly lower IC_{50} and IC_{90} values than cisplatin alone. This is attributed to improved drug stability and cellular uptake. Caspase-3 and cleaved PARP assays confirmed that BCDCPLP and CP-loaded liposomes induce substantial apoptosis, with BCDCPLP showing the highest increase in caspase-3 activity and PARP cleavage at 85 kDa. The ability of BCDCPLP to enhance apoptosis is further demonstrated through Western blot analysis, where the density of PARP cleavage bands is the highest in BCDCPLP-treated cells, followed by CPLP and CP-treated ones. The study highlights that the cumulative release of PARP from CP-nanocarrier complexes leads to more efficient apoptosis than free CP. These findings align with the synergistic effects observed when combining CP with PARP inhibitors, which induce mitochondrial membrane permeabilization and cytochrome C release, activating the intrinsic apoptosis pathway. The potential of BCDCPLP in reducing the required dosage while maintaining therapeutic efficacy offers promising advances in cancer therapy.

Keywords: Liposome, cisplatin, cyclodextrin, apoptosis, HeLa cells, PARP, caspase-3, cancer

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Purpose, Rationale, and Limitations

This study will investigate a nanomedicine in the in vitro pathways of free cisplatin (CP), CP-loaded liposomes (CPLP), hydroxypropyl-β-cyclodextrin&cisplatin (BCDCP)-loaded liposomes (BCDCPLP), and about apoptosis. Additionally, the study will assess the efficacy of cytotoxic effects and identify specific molecular signatures in HeLa cell lines treated with CPLP and BCDCPLP. The research employs caspase-3 assays and measures the activity of poly(ADP-ribose) polymerases (PARP) to evaluate cytotoxicity in HeLa cell lines and identify potential therapeutic agents and applications.

Before clinical applications, further research is needed to explore the role of nanocarrier components and their impact on drug release, dosage, and cancer-targeting mechanisms. This will pave the way for optimized treatment outcomes with reduced side effects.

Introduction

Cancer remains a significant global health issue, especially in less-developed regions. According to the World Health Organization, in 2022, the three most prevalent cancer types were lung, breast, and colorectal cancers. Lung cancer was the most common, with 2.5 million new cases, representing 12.4% of all new cancer cases. Female breast cancer was second (2.3 million cases, 11.6%), followed by colorectal cancer (1.9 million cases, 9.6%), prostate cancer (1.5 million cases, 7.3%), and stomach cancer (970,000 cases, 4.9%) [1]. Cervical cancer is the eighth most prevalent cancer and the ninth leading cause of cancer death globally, with 661,044 new cases and 348,186 deaths. Expanded implementation of the WHO Cervical Cancer Elimination Initiative can eradicate it as a public health issue [1].

Chemotherapy, especially cisplatin regimens, is often limited by low penetrative cell membrane, low efficacy, and significant toxicity, underscoring the need for novel anticancer agents [2]. Modifying existing prodrugs is a promising, cost-effective strategy to enhance therapeutic potential, reduce toxicity, and improve apoptotic efficacy in nanomedicine applications involving cyclodextrins (CD). Apoptosis, a programmed cell death, plays a crucial role in growth and development, marked by chromatin condensation, cytoplasmic shrinkage, and DNA fragmentation [3].

Păduraru et al. [4] conducted a comprehensive review of anticancer drug complexation-nanocarriers, focusing on including complex-based nanocarriers, their delivered cargoes, and the specific cancer types targeted. The study particularly highlights cyclodextrin (CD)-based drug delivery systems, examining their association with various cancer types and anticancer drugs. However, it is noteworthy that the study does not explore CP within these systems, pointing to a significant gap in research for this widely utilized chemotherapeutic agent. Similarly, Singh et al. [5] reviewed emerging trends in CD-based drug delivery systems, also noting the absence of CP in their evaluations.

In current trends (2020-present), advancements in nanoscale technology have facilitated the creation of nanovesicles (NV) with customizable properties for precise drug delivery. NV can penetrate tissues, overcome biological barriers, and interact with cells, making them ideal drug carriers. In cancer therapy, nanotechnology integration enhances drug efficacy, reduces toxicity, and improves patient compliance. Panyosak (2023) studied the behavior of nanovesicles of physicochemical profiles composed of B-Cyclodextrin (BCD) and CP for anti-tumor activity and decreased toxicity affected normal surrounding tumor tissues. NV encapsulating CP complexes within hydroxypropyl-B-cyclodextrin, showing sustained release from liposomes, which may reduce dosage, minimize toxicity, and improve tumor-targeted delivery [6]. These results highlight the significance of combination therapies in addressing the complexities of cancer, suggesting that this model, hydroxypropyl-beta-cyclodextrin-cisplatin liposomes (BCDCPLP) and CD-CP-loaded liposomes (CPLP), multi-targeted strategies can synergistically target tumor cells and enhance patient outcomes [7, 8]. Furthermore, Ponder and Boise (2019) conducted a thorough review of the role of caspase-3 in apoptotic cell death, emphasizing its activation in response to DNA damage and the subsequent cleavage of proteins such as poly(ADP-ribose) polymerase (PARP), which is essential for DNA repair. The cleavage of PARP by caspase-3 disrupts cellular functions and facilitates apoptosis [9].

Materials and methods

Inclusion complex preparation

A 1:1 molar ratio of CP and BCD was prepared using freeze-drying. The process commenced with dissolving CP in deionized water. Subsequently, the BCD solution was added to the CP mixture and stirred for 10 minutes to ensure homogeneous dispersion. Finally, the mixture was subjected to freeze-drying using a Labconco freeze-dryer.

Preparation of drug-loaded NV (Liposomes)

Liposomes were prepared using the Bangham method with sonication. The lipids, DPPC, and cholesterol (CHL) were mixed in a 6:4 molar ratio at a final concentration of 40 mM. The lipids were dissolved in an organic phase (e.g., chloroform: ethanol), and the organic solvent was removed by rotary evaporation. The remaining lipid film was dried under a vacuum for 24 hours. Subsequently, deionized water was added to the lipid film to hydrate the phospholipids and form vesicles. The vesicles were then loaded with CP and BCDCP (20 mM concentration, equal to CP by mechanical disturbance at 60–70°C for 45 minutes. The resulting dispersion was then sonicated at 20–30 kHz for 5 minutes using an ultrasonic probe (Vibra Cell, Newtown, CT, USA) to achieve a uniform size distribution of liposomes.

Dissolution Study: CP Release Profile in Various Formulations

The release profiles of BCDCPLP, CPLP, and free CP were evaluated using a flow-through cell dissolution tester, following the guidelines outlined in USP <711> DISSOLUTION, Apparatus 4 (Sotax® Flow-Through Cell dissolution tester) [10]. This apparatus is widely recommended for formulations with poor solubility and modified-release and extended-release dosage forms [6]. As shown in Figure 1, the cumulative release profiles of CP (time course of CP release) for free drug, CPLP, and BCDCPLP formulations, when using Apparatus 4, more closely aligned with the theoretical release pattern compared to those obtained with Apparatus 2 [6, 10, 11].

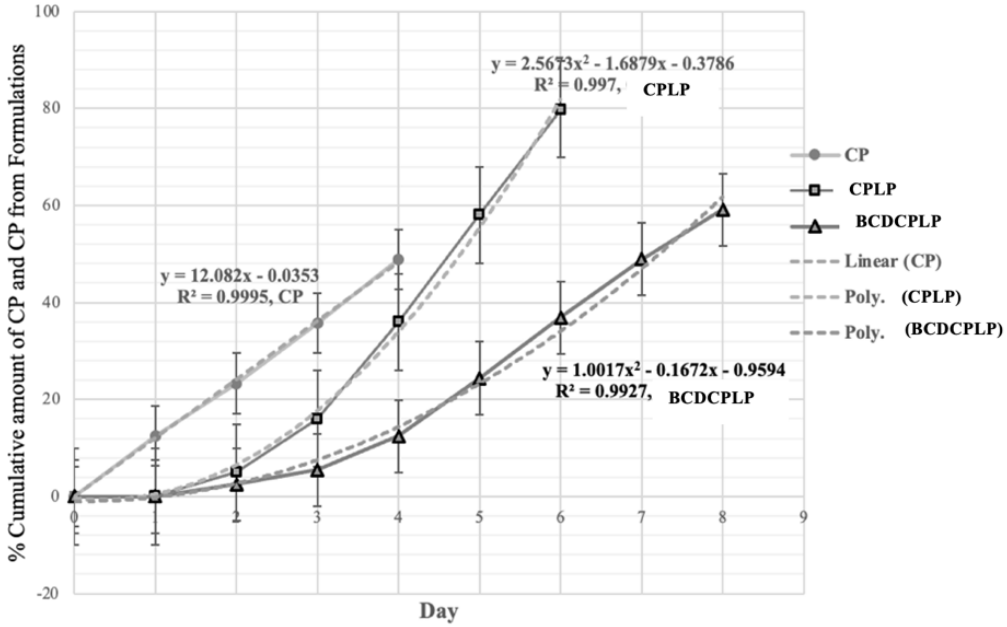


Figure 1. % Cumulative amounts of CP in various formulations (μM) for 10 days.

The release profile of free CP exhibited a linear trend, indicating a direct correlation between cumulative CP release and time (days). In contrast, the CPLP and BCDCPLP formulations demonstrated sigmoidal or polynomial release curves, indicating more complex release kinetics [11]. Apparatus 4 provided a more accurate representation of the rate-limiting step, significantly impacting the observed release

patterns for CPLP and BCDCPLP compared to Apparatus 2. The dissolution apparatus and medium were prepared and maintained at 37 ± 0.5°C, with the medium being filtered and degassed to ensure consistent experimental conditions. The liposomal formulations were placed in the flow-through cell, which was filled. The dissolution tester was then activated, and the pump initiated the circulation of the dissolution medium at a flow rate of 10 mL/min. Samples were collected at predetermined time intervals, and 20 µL aliquots of the released samples were filtered and analyzed using ultraviolet (UV) spectroscopy at a wavelength of 301 nm with a Perkin Elmer Lambda Bio UV/Vis Spectrometer [8]. All experiments were performed in triplicate to ensure reproducibility and statistical accuracy.

Cell Lines And Cell Culture

HeLa (JCRB9004) cells were purchased from ANH SCIENTIFIC, JAPAN, and authenticated for cell cultures. This cell line is derived from cervical adenocarcinoma. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal calf serum. Dimethyl sulfoxide from Sigma-Aldrich (St. Louis, Missouri, USA). L-glutamine was from Gibco BRL (Grand Island, New York, USA). Penicillin-streptomycin was from Gibco BRL, Gaithersburg, Maryland, USA. Trypsin was from Gibco Invitrogen Corp. (Carlsbad, California, USA). HeLa cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for optimal growth.

MTT Cell Proliferation Assay

The MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was utilized to assess the proliferation and survival of HeLa cells by measuring their metabolic activity. This colorimetric assay is based on living cells reducing the MTT substrate, producing dark blue formazan crystals. The color change indicates active cell proliferation and survival.

Once adherent, HeLa cells were trypsinized and seeded into 96-well plates. After 24 hours, the cells were treated with CP, CPLP, and BCDCPLP for 72 hours, with CP as a positive control. All experiments were conducted in triplicate to ensure reproducibility. Following the manufacturer's instructions (Sigma-Aldrich Company, St. Louis, MO), the MTT assay was used to evaluate the antiproliferative activity of CPLP and BCDCPLP. After the 72-hour incubation period, the culture medium was removed, and 20 µL of a 5 mg/mL MTT solution in phosphate-buffered saline (PBS) was added to each well, followed by incubation for 4 hours. The resulting formazan crystals were then dissolved in 120 µL of isopropanol, and the optical density was measured at 570 nm using a microplate reader.

The amount of formazan produced is inversely proportional to the number of viable cells. Each treatment's mean values and standard error were calculated and expressed as a percentage of the control group (empty liposomes). The concentration of CP required to inhibit cell growth by 50% (IC50) was calculated using linear interpolation based on the following formula:

IC_{50}=(concentration at which absorbance is 50% of the control)–([50% of control absorbance–absorbance at zero concentration]/[absorbance at highest concentration–absorbance at zero concentration])×(highest concentration-concentration at which absorbance is 50% of control).

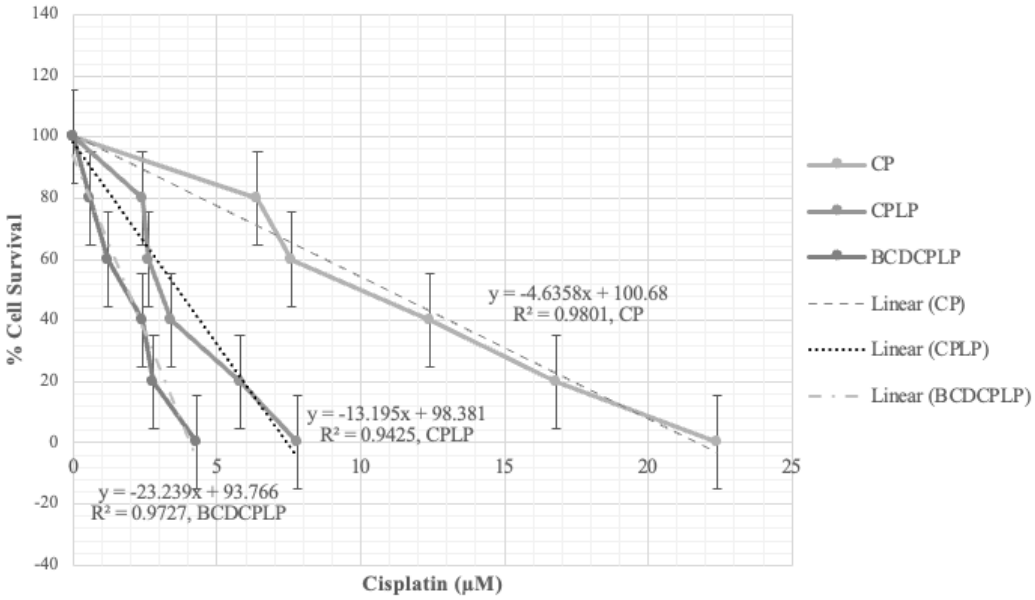
The IC_{90} was calculated similarly, with 90% absorbance substituting for 50% in the above formula. The IC_{50} and IC_{90} values represent the concentrations needed to achieve 50% and 90% cytotoxicity, respectively, see Table 1.

Table 1. Inhibitory Concentration of Liposomal CP on HeLa Cell Line

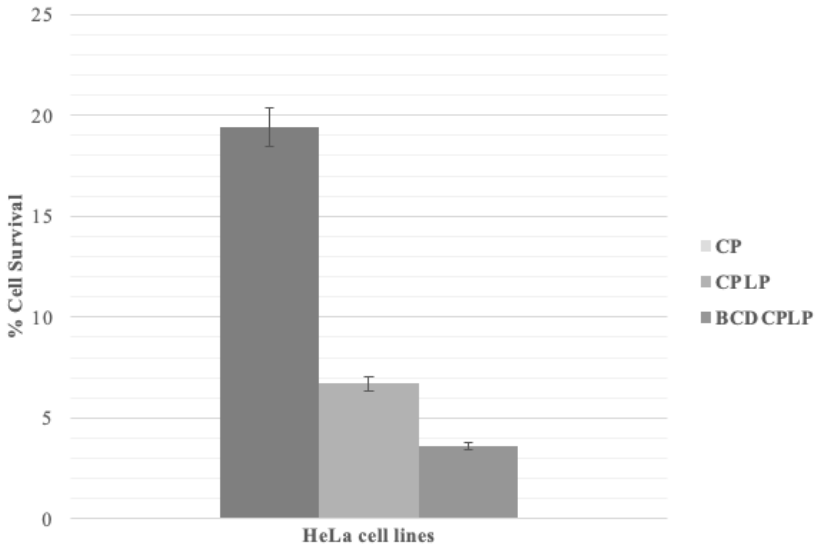
Inhibitory Concentration	Various Formulations (µM)		
	CP Alone	CPLP	BCDCPLP
IC_{90}[a]	19.41	6.70	3.60
IC_{50}[a]	10.93	3.67	1.88

[a]Mean of triplicate experiments (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide [MTT] assay after 72 hours of exposure.

The IC_{90} values revealed that the inhibitory effect of liposomal CP increased over time at this concentration (n = 3, ± SD). Results are shown in Figure 2A, with the CP, CPLP, and BCDCPLP concentrations tested in the range of 0–25.0 µM.



(A)



(B)

Figure 2. Effect of CP alone, CPLP, and BCDCPLP on proliferation/survival as assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) assay. CPLP and BCDCPLP inhibited the growth of the HeLa cell lines tested. Values represent triplicate experiments' mean and standard error after 72 hours of CP alone, CPLP, and BCDCPLP exposure.

*(A) Dose-dependent inhibition indicated the relationship between cell survival (%) and CP concentration from CP alone, CPLP, and BCDCPLP after incubating for 72 hours. (n = 3, ±SD). **

(B) Time-dependent inhibition of HeLa cell growth was observed in cells exposed to IC_{90} levels of CP from various formulations. The IC_{90} is the concentration of growth inhibition at which cell growth is inhibited by 90%. This demonstrates that the inhibitory effect of liposomal CP increased over time at the IC_{90} concentration. (n = 3, ±SD).

Apoptosis Detection: Caspase-3 Assay

HeLa cells were seeded into 4-well plates at a density of 50,000 cells per well and incubated overnight. The following day, cells were treated with five times the IC_{50} concentrations (µM) of CP (54.65 µM), CPLP (18.35 µM), and BCDCPLP (9.40 µM) for 72 hours [9]. The IC10 (10% inhibition concentration) was also utilized for each drug-loaded liposomal formulation.

After 72 hours of incubation, the culture medium was removed, and the cells were trypsinized and resuspended in a fresh medium. The cells were centrifuged at 250 g for 10 minutes, and the supernatant was carefully discarded. Subsequently, 25 µL of cold lysis buffer was added to the cell pellet.

Assay buffer and dithiothreitol (DTT) stock solution were added per the manufacturer's protocol (Abcam, Cambridge, UK). Then, 5 µL of the caspase-3 colorimetric substrate Ac-DEVD-pNA was added to each well. Caspase-3 cleaves this substrate, releasing the chromophore pNA (p-nitroaniline), whose quantity is proportional to caspase-3 activity.

pNA absorbs light at a wavelength of 405 nm, allowing its concentration to be quantified using a spectrophotometer. Caspase-3 activity was calculated based on the absorbance readings at 405 nm using a Tecan Infinite Pro microplate reader (Tecan, Männedorf, Switzerland). As per the assay protocol, the extent of apoptosis was measured relative to positive controls (without substrate) and negative controls (untreated cells) [9, 12]. All experiments were conducted in triplicate to ensure reproducibility and statistical reliability.

Caspase-3 activity was calculated using the following formula:

Caspase-3 activity = pNA released (nmol) / (time of incubation in hours) / (volume of sample in mL)

Apoptosis Detection

Caspase-3 Assay

Cells were seeded into 4-well plates at a density of 50,000 cells per well and incubated overnight. Subsequently, the cells were treated with 5 times their respective IC_{50} concentrations (µM) of CP alone (54.65 µM), CPLP (18.35 µM), and BCDCPLP (9.40 µM) for 72 hours [9]. The IC_{10}:10% inhibition concentration was employed for each drug-loaded liposome formulation.

Following the 72-hour incubation period, the culture medium was removed, and the cells were trypsinized and resuspended in fresh media. The suspended cells were centrifuged at 250 g for 10 minutes. The supernatant was carefully discarded, and 25 µL of cold lysis buffer was added to the cell pellet.

The assay buffer and dithiothreitol (DTT) stock solution were added to the cells according to the manufacturer's instructions (Abcam, Cambridge, UK). Caspase-3 colorimetric substrate (Ac-DEVD-pNA) (5 µL) was added to the cells. Caspase-3 cleaves the substrate, releasing the chromophore pNA (p-nitroaniline). The amount of pNA released is proportional to the caspase-3 activity in the sample.

pNA absorbs light at a wavelength of 405 nm, enabling the measurement of the sample's absorbance using a spectrophotometer. The caspase-3 activity in the sample is then calculated based on the measured absorbance. After incubation, the absorbance of the wells was read at 405 nm using a Tecan Infinite Pro microplate reader (Tecan, Männedorf, Switzerland). Per the assay protocol, the extent of apoptosis was measured relative to positive and negative controls (without substrate as positive and cells as negative). Experiments were conducted in triplicate to ensure reproducibility and statistical reliability [9, 12].

The calculation of the amount of caspase-3 activity using the following formula:

Caspase-3 activity = pNA released (nmol) / (time of incubation in hours) / (volume of sample in mL)

The Determining Role of Poly ADP-Ribose Synthase (PARP)

Cells were seeded into 4-well plates (1×10^6 cells/well) and incubated for 48 hours. The next day, the cells were treated with five times their IC_{50} concentration of CP alone (per PARP detection protocol) and CPLP and BCDCPLP at the IC_{10}. Then, the media was removed, and the cells were trypsinized and resuspended in fresh media. The suspended cell suspension was centrifuged for 3 minutes at 750 rpm, at 4°C. The cell pellets were washed with chilled PBS and centrifuged again. The cell pellets were cooled to −80°C. After that, the chilled cell pellets. Then, accumulated and lysed by adding RIPA buffer 100 µL, which composed of 20 mM Tris(hydroxymethyl)aminomethane hydrochloride pH 7.5, 1 mM Disodium ethylenediaminetetraacetate, 150 mM Sodium Chloride, 1 mM Ethylene glycol-bis (2-aminoethyl ether)-N, N, N', N'-tetra acetic acid, 2.5 mM Sodium pyrophosphate, 1% Nonidet P-40, 1% Sodium deoxycholate, 1 $\mu g \cdot mL^{-1}$ leupeptin, 1 mM B-glycerophosphate, 1 mM Sodium orthovanadate and centrifuged the lysed cells at 14,000 rpm at 4°C for 10 minutes; afterward, stored the supernatant at −80°C.

The bicinchoninic acid (BCA) assay was conducted to quantify proteins. A 10 µL cell lysate was mixed with 100 µL of BCA reagent and incubated at 37°C for 30 minutes. The samples' optical density was 570 nm using a Tecan Infinite Pro microplate reader and paralleled with a bovine serum albumin (BSA) standard. The bicinchoninic assay (BCA) was used as a quantitative method to detect the protein concentration for 10 µL of cell lysate was added to each sample. Lysate with 100 µL of BCA reagent, then incubated at 37°C for 30 minutes. The samples were measured absorbance at 570 nm (Thermo Fisher Scientific). BSA was a standard of results.

The samples were each mixed with 5 µL of a prepared buffer containing B-mercaptoethanol and SDS-Nupage (1:20) and boiled at 80°C for 5 minutes before centrifugation gently. The samples were then loaded into a 4–20% Bis-Tris gel. (Fisher Scientific, Loughborough, UK) and electrophoresis was carried out. The gel proteins were then transferred electrically to a PVDF membrane soaked in transfer buffer solution. The blocking process was performed on the PVDF protein membrane using milk solution (5 g of semi-skimmed dry milk in 100 mL of Tris-buffered saline with tween 20 [TBST]) at 25°C for 1 hour. The PVDF protein membrane was incubated with primary antibody (PARP) diluted in 1× TBST at 1:2000 overnight at 25°C. The membranes were washed and incubated with TBST 1×, then with a secondary antibody (anti-rabbit AB) with blocking milk solution in a dilution ratio of 1:2500 for 1 hour at 25°C. The reactive protein bands were then detected using an ECL (Enhanced chemiluminescence) imaging detecting system. Experiments were carried out in triplicate.

Results and Discussion

The released profile of CP in various formulations (Dissolution study)

Polynomial trend lines of CP, CPLP, and BCDCPLP can be employed to validate nanomedicine's controlled release behavior for specific therapeutic applications [6, 10, 11]. These trend lines can be used to evaluate nanomedicines that exhibit controlled drug release rates or responsiveness to specific stimuli. This can lead to more controlled or sustained drug release, potentially enhancing therapeutic efficacy and reducing side effects. Additionally, nanomedicines offer the advantages of enhanced targeting and complex pharmacokinetics, which are not achievable with free drugs. In contrast, free drugs typically exhibit a linear release profile, highlighting the significant advantages of nanomedicines in drug delivery. Polynomial trend lines can further optimize the design of nanomedicines for specific therapeutic applications. [10, 11] CPLP and BCDCPLP exhibited polynomial trend lines, while CP alone followed a linear trend line [6]. The distinct release behavior of each formulation provided valuable insights into their pharmacokinetics and highlighted the potential advantages of modified nano-drugs. CP alone exhibited a linear cumulative amount release, represented by the equation $y = 13.133x + 0.3556$, with a high R^2 value of 0.9931, indicating a strong correlation. This is consistent with previous studies by Hayat et al. (2019). CPLP exhibited polynomial regression, with the equation $y = 6.9556x - 2.3704$ ($R^2 = 0.8984$) and an equation of BCDCPLP is $y = 3.515x - 4.7378$ ($R^2 = 0.898$).

Polynomial trend lines offer more versatility when modeling complex release profiles than

linear trend lines. Modified nano-drugs with polynomial release profiles are more suitable for sustained release, prolonged half-life, heightened stability, targeted delivery, reduced side effects, and distribution patterns. Hayat et al. [8] and Real et al. [11] have all shown that modified nano-drugs can exhibit these advantages. Although it was initially concluded that BCDCPLP and CPLP would release the drug in a controlled manner, further studies were needed. This is because the drug release behavior is complex, depending on how CP was entrapped in BCD and then in NVs with different components.

Additionally, BCDCPLP demonstrated a higher prolonged duration and lower dosage than CPLP and CP alone when used in metronomic chemotherapy (MTC) [13]. MTC involves regularly administering lower doses of cancer drugs at minimal intervals to maintain an extended and more therapeutically effective range of plasma drug concentration while minimizing adverse effects. The results of this study suggest that modified nano-drugs with polynomial release profiles offer significant advantages over free drugs for delivering cancer drugs. BCDCPLP and CPLP had a synergistic effect, increasing the drug's action compared to the free drug, related to metronomic chemotherapy, targeting the tumor microenvironment, and activating the immune system to attack tumor cells [13]. It is generally well-tolerated, with fewer side effects than traditional chemotherapy. It is a promising option for patients who cannot tolerate conventional chemotherapy or are at risk for side effects. The advantages of BCDCPLP in MTC are due to its sustained release profile. This profile allows lower drug doses to be administered more frequently, which can help reduce side effects and improve patient compliance. Additionally, the prolonged half-life of BCDCPLP means it can stay in the body longer, providing a more sustained therapeutic effect.

The results of this study suggest that modified nano-drugs can be a valuable tool for improving the delivery of cancer drugs. By providing sustained release, prolonged half-life, and targeted delivery, modified nano-drugs can help improve cancer treatment efficacy, safety, and patient compliance. BCDCPLP exhibits the most desirable physical characteristics, but additional cell line experiments are needed to confirm the preliminary findings and elucidate its mechanism of action against cancer cells before clinical trials can be initiated.

Cytotoxicity of drugs and drug-loaded liposomes (LP)

The proliferation values represent triplicate experiments' mean and standard error after 72 hours of CP alone, CPLP, and BCDCPLP exposure to HeLa cell lines. The results are shown in Figure 2 and Table 1. In Figure 2A, dose-dependent inhibition indicated the relationship between cell survival (%) and CP concentration from CP alone, CPLP, and BCDCPLP after incubating for 72 hours* (n = 3, ±SD). (*significant decrease in IC_{50} compared to single drug treatment ($p < 0.05$)) and Figure 2B, time-dependent inhibition of cell growth in cells exposed to IC_{90} levels of CP from various formulations.

CPLP and BCDCPLP were treated, and a 2.90-fold and 5.39-fold reduction in IC_{90} was observed. These experiments highlight that BCDCPLP exhibited the lowest IC_{50} and IC_{90} values compared to CP alone and CPLP, indicating a significantly improved cytotoxic activity against cancer cells.

This improvement can be attributed to the drug's stability and cellular uptake behavior. Additionally, this study confirmed the integrity of the wall of NV or LP in a culture medium, which aligns with the discovery of Zhang et al. (2011), who reported that regulating the structural integrity of LP in a culture medium enhanced its uptake efficacy in immortalized and transformed cells [14].

The results of these experiments showed that BCDCPLP had the lowest IC_{50} and IC_{90} values, indicating that it is more cytotoxic to cancer cells than CP alone or CPLP. This improvement is likely due to the stability and cellular uptake behavior of BCDCPLP. The integrity of the wall of NV or LP in a culture medium was also confirmed in this study, consistent with the findings of Zhang et al. (2011), who showed that regulating the structural integrity of LP in a culture medium can enhance its uptake efficacy in immortalized and transformed cells [14].

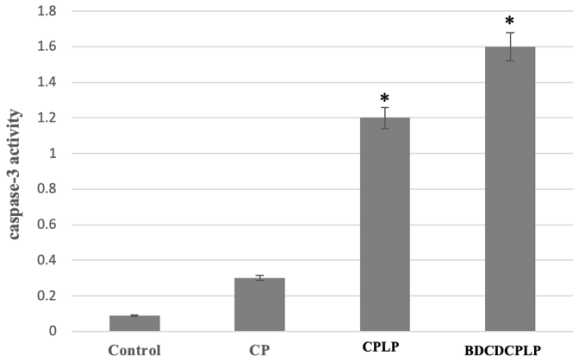
The viability of HeLa cells treated with CPLP and BCDCPLP decreased after 72 hours of incubation. The IC_{90} values (the concentration of

drug required to inhibit cell growth by 90%) for CPLP and BCDCPLP were 2.90 and 5.39 times lower than the IC_{90} value for CP alone, respectively. This suggests that BCDCPLP may be a more effective treatment for cancer than CP alone or CPLP.

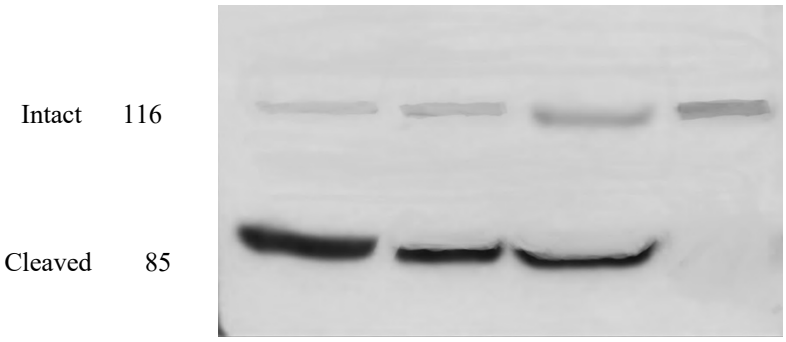
Apoptosis Study

Caspase-3 Assay

The cells were treated with five times their respective IC_{50} concentrations (μM) of CP, CPLP, and BCDCPLP. The caspase-3 assay is a valuable tool for studying apoptosis, a regulated process of programmed cell death essential for maintaining tissue homeostasis and preventing disease. This assay quantifies caspase-3 activation in cells undergoing apoptosis and assesses the efficacy of drugs designed to inhibit this process. Caspase-3, a crucial executioner caspase group member, is a reliable indicator of apoptosis. CP-induced an increase in active caspase-3 expression, signifying cellular apoptosis. In the present study, HeLa cells were treated with CPLP and BCDCPLP at concentrations equivalent to the CP concentration. As shown in Figure 3A, caspase-3 activity was significantly increased in HeLa cells treated with CPLP and BCDCPLP compared to the control (without CP).



(A)



(B)

Figure 3 Effect of liposomal CP formulations on apoptosis. Liposomal CP formulations apoptosis of HeLa cells and its effect was equivalent to or better than CP alone at equimolar concentrations. Programmed cell death was assessed by Western blot analysis of poly(ADP-ribose) polymerase (PARP) cleavage after 72 hours of exposure to liposomal CP formulations.

Apoptosis detection via Caspase-3 measurement in HeLa cells incubated with chemotherapies with and without Liposomal CP (n = 3, ±SD). Control refers to cells with no treatment.[1,2] Apoptosis was also assessed through Western blot analysis of poly(ADP-ribose) polymerase (PARP) cleavage after 72 hours of exposure to BCDCPLP, CPLP, CP alone, and LP.

[1] Denotes significance compared to single drug treatment ($p < 0.05$)

[2] Liposomal CP-induced apoptosis in HeLa cells compared to Free CP at equimolar concentrations.

Apoptosis, a controlled process of programmed cell death, is essential for maintaining tissue homeostasis and preventing the development of diseases. It is regulated by the caspase family of proteases, which cleave key proteins involved in cell growth and survival. Poly(ADP-ribose) polymerase (PARP) is a caspase substrate, and its cleavage is a hallmark of apoptosis. Detecting cleaved PARP bands in Western blots is a reliable biomarker of programmed cell death, characterized by a unique pattern of protease activity.

The caspase-3 assay measures caspase-3 activity in cells, offering insights into the extent of apoptosis, described in Pathways in Figure 3A. The ranking of caspase-3 levels was as follows: control (LP) < CP < CPLP < BCDCPLP. CPLP and BCDCPLP treatment resulted in a 4.00-fold and 5.33-fold increase in caspase-3 activity compared to CP alone. These findings were consistent with the cytotoxicity data, indicating that CPLP and BCDCPLP induced apoptosis in HeLa cells.

Poly ADP-Ribose Synthase (PARP) Determines Liposomal CP-Induced Apoptosis in HeLa Carcinoma Cell Lines by Western Blotting for Detection of Cleaved PARP.

Figure 3B shows a Western blot analysis of PARP protein in HeLa carcinoma cells treated with CP and various liposomal formulations. The results indicate that the density of PARP cleavage bands was highest in BCDCPLP-treated cells, followed by CPLP-treated cells and CP-treated cells. These findings suggest BCDCPLP is the most effective formulation for inducing apoptosis in HeLa cells (Figure 4).

The observed PARP cleavage is due to the synergistic effect of the nanoparticle formulation. Shakarchi et al. 12].also reported that hybrid nanoparticles (HNPs) combined with doxorubicin or oxaliplatin for liver cancer therapy-induced PARP cleavage in SK-hep-1 cells, especially when doxorubicin was used alone or in combination therapy [

Western blotting for cleaved PARP is a primary experiment for apoptosis because it is a reliable and widely used method for detecting programmed cell death. Cleaved PARP is a specific apoptotic marker absent in healthy cells. Western blotting is a relatively simple and sensitive technique that can measure cleaved PARP levels in various cell types and tissues. Overall, the findings of this study suggest that BCDCPLP is a promising formulation for inducing apoptosis in HeLa carcinoma cells.

The previously mentioned content indicates that research on CP inclusion complexes encapsulated in nanovesicles or nanoparticles has yet to investigate their apoptosis pathways or mechanisms of action. Additionally, their potential to reduce the required dosage (IC_{90}) for targeting cancer tissue remains unexplored, as noted by PARP cleavage fragments [15,16]. Liposomes act as drug carriers to target cancer cells and enrich lipids for cancer cells. While the CD was a controlled release, the dynamic between CP complexes (CCD) and free CP binds to DNA and forms intra-strand DNA adducts, inhibiting DNA synthesis and cell growth. When CP was imbalanced, CCD released and minimized CP in proper intracellular concentration. Those mentioned above were the major processes, while the minor was the apoptosis pathway. When DNA was damaged, the PARP was released to maintain DNA. Michels et al. (2013) reported that CP and PARP inhibitors synergized in inducing DNA damage foci, mitochondrial membrane permeabilization leading to cytochrome C release, and dissipation of the inner transmembrane potential, caspase activation, plasma membrane rupture and loss of clonogenic potential in non-small cell lung carcinoma cells. This elicits the intrinsic pathway of apoptosis [17]. Thus, PARP was cleaved by caspase-3 at 85 kDa. Thus, the action of CP complexation-nanocarriers activated the release of cumulative PARP in many doses. The cleaved PARP at 85 kDa shows a high-density band. At the same time, free CP action activated the release of non-cumulative PARP by one dose. The cleaved PARP at 85 kDa shows a low-density band.

The previous content suggests that research on CP inclusion complexes encapsulated in nanovesicles or nanoparticles has not yet explored their apoptosis pathways or mechanisms of action. Specifically, the potential of these complexes to reduce the required dosage (IC_{90}) for targeting cancer cells remains unexamined, as evidenced by PARP cleavage fragments [15,16]. Liposomes function as drug carriers to specifically target cancer cells and enrich lipids essential for these cells. While cyclodextrin (CD) served as a controlled release system, the dynamics between CP complexes (CCD) and

free CP—binding to DNA and forming intra-strand DNA adducts that inhibit DNA synthesis and cell growth—differ. When CP levels were imbalanced, CCD released minimized CP at appropriate intracellular concentrations. The primary observed process involves DNA damage, leading to PARP release to maintain DNA integrity, with the minor process being the apoptosis pathway. Michels et al. (2013) reported that CP, combined with PARP inhibitors, synergistically induced DNA damage foci, mitochondrial membrane permeabilization, cytochrome C release, dissipation of the inner mitochondrial membrane potential, caspase activation, plasma membrane rupture, and loss of clonogenic potential in non-small cell lung carcinoma cells, thereby triggering the intrinsic pathway of apoptosis [17].

Consequently, PARP is cleaved by caspase-3 at 85 kDa. CP complexation within nanocarriers activates the release of cumulative PARP across multiple doses, resulting in a high-density band of cleaved PARP at 85 kDa. In contrast, free CP activates the release of non-cumulative PARP from a single dose, leading to a low-density band of cleaved PARP at 85 kDa.

Discussion

Hydroxypropyl-beta-cyclodextrin (BCD)-enclosed CP (CPCD) was entrapped in neutral liposomes (LPs) composed of DPPC/CHL (6:4 molar ratio), forming BCDCPLP. This formulation enriches nutrients and non-charged molecules that cancer cells need to convert to energy and expand, which causes cancer cells to multiply more rapidly than healthy tissues. Thus, cancer cells uptake CP more than healthy tissues, keeping healthy tissues safe.

To evaluate their potential as treatment options, CPLP and BCDCPLP were modified to ensure their structural integrity before considering their in vitro sustained release of CP. The cumulative amounts of CP exhibited two trend line patterns: polynomial and linear. Polynomial trend lines (BCDCPLP and CPLP) offer more versatility than linear trend lines (CP alone) and can model more complex release profiles. This versatility makes modified nanodrugs more suitable for sustained release, prolonged half-life, heightened stability, targeted delivery, reduced side effects, and variable absorption and distribution patterns. Additionally, BCDCPLP demonstrated a longer prolonged duration and required a lower dosage than CPLP and CP alone when used in metronomic chemotherapy (MTC). MTC involves regularly administering lower doses of cancer drugs at minimal intervals, aiming to maintain an extended, more therapeutically effective range of plasma drug concentrations.

The cytotoxicity of the formulations was assessed using MTT assays, with BCDCPLP demonstrating superior results against HeLa cells. The MTT assay revealed that BCDCPLP exhibited the lowest IC_{50} values, indicating significantly improved cytotoxic activity against cancer cells. Additionally, the study confirmed the integrity of the nano vesicular (NV) wall in a culture medium, indicating the suitability of the drug delivery system for targeting cells.

The apoptosis pathway was investigated by studying caspase-3 and PARP cleavage fragments. In the caspase-3 apoptosis study, BCDCPLP treatment led to a dose-dependent increase in caspase-3 protein levels, indicating that BCDCPLP induced the highest caspase-3 activity. In the Western blotting for PARP apoptosis study, the density of PARP cleavage bands at 85 kDa, a marker of apoptosis, was highest in BCDCPLP-treated cells, followed by CPLP-treated cells and CP-treated cells. These findings align with the cytotoxicity data, which showed IC_{50} values (dose-dependent inhibition) and caspase-3 activity, suggesting that the synergistic effect between LP and BCD formulation was responsible for the observed PARP cleavage.

Western blotting for cleaved PARP is a reliable and widely used method for detecting programmed cell death. Cleaved PARP is a specific apoptosis marker absent in healthy cells. Western blotting is a relatively simple and sensitive technique that can measure cleaved PARP levels in various cell types and tissues. Additionally, it is a valuable tool for studying apoptosis because it is a sensitive, specific, easy-to-perform, and affordable method for detecting a hallmark of apoptosis.

Although BCDCP encapsulated in nanovesicles (NVs) has demonstrated superior overall performance in numerous studies, the detailed results vary due to the differing components of neutral NVs and the varying ratios of BCDCP within the NVs. Further research is necessary to examine the impact of NV components and

BCD ratios on drug release behavior and to determine how BCD and NV encapsulation can diminish CP concentration, thereby reducing destruction to normal tissue. Creating a comprehensive database from these studies is essential for advancing in vivo and clinical research. Such a database will aid in evaluating and comparing formulations, optimizing their utility, and designing new drugs for therapeutic applications. This will enhance the precision of nanomedicine and consider economic implications.

The potential of BCDCP to reduce the required dosage (IC_{90}) for targeting cancer cells remains largely unexplored, particularly concerning PARP cleavage fragments. Liposomes are effective drug carriers, explicitly targeting cancer cells and enriching essential lipids. While CD has been used as a controlled release system, the dynamics between CCD and free CP differ. Free CP binds to DNA, forming intra-strand adducts that inhibit DNA synthesis and cell growth, whereas CCD releases minimized CP at appropriate intracellular concentrations when imbalances occur..

Conclusion

Combining CP with PARP inhibitors induces mitochondrial permeabilization and cytochrome C release, activating the intrinsic apoptosis pathway. This process involves caspase-3 cleaving PARP at 85 kDa. CP-nanocarrier complexes release cumulative PARP over multiple doses, resulting in high-density bands of cleaved PARP at 85 kDa, whereas free CP releases non-cumulative PARP, producing lower-density bands at the same molecular weight. The primary mechanism observed is DNA damage, leading to the release of PARP to maintain DNA integrity, with apoptosis as a secondary process.

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