

Enhancing the Cytotoxic Activity of Encorafenib Using Lipid-Polymer Hybrid Nanoparticles: An Integrated *In Silico* and *In Vitro* Evaluation

Divya Zambre¹, Shweta Jaiswal¹, Ujban Md Hussain¹, Dr. Veena Belgamwar^{1*}

¹Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, Maharashtra, India.

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For Correspondence:

Veena Belgamwar, Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, Maharashtra, India.

Abstract

Glioblastoma multiforme (GBM) is one of the most aggressive cancers of brain that remains highly therapy resistant due to the presence of the blood-brain barrier and the complexity of cancer biology. Encorafenib, a strong inhibitor of BRAF, could be considered a good drug candidate for repositioning into GBM, but the poor brain permeability and efflux due to P-glycoprotein could limit its use in the clinical setting. This study aimed to evaluate the potential of encorafenib through an integrated *in silico* approach and enhance its *in vitro* cytotoxic activity using lipid-polymer hybrid nanoparticles (LPHNPs). The interaction of the compound with selected GBM targets (EPHA2, PTPRZ1, LSD1, and ALDH1A3) was analysed via molecular docking, followed by drug-likeness, ADMET, and toxicity prediction. The HTCC-LPHNPs, containing lipid core and quaternized chitosan (HTCC) shell, were developed via the nanoprecipitation technique. The cytotoxicity profile of the encorafenib-loaded nanoparticles was determined in the U87 glioblastoma cells using MTT assay, flow cytometry-based Annexin V-FITC/PI apoptosis test, and cell-cycle analysis. Encorafenib showed promising binding capabilities with the GBM-associated targets, and the strongest docking affinities were shown by LSD1 (−9.8 kcal/mol) and ALDH1A3 (−9.4 kcal/mol) targets. *In silico* prediction studies confirmed poor BBB penetration and P-glycoprotein substrate properties, which further emphasised the importance of a suitable drug delivery method. The optimised HTCC-LPHNPs formulation had an average particle size of 141.4 ± 2.3 nm, PDI of 0.445 ± 0.02 , positive zeta potential, and $73.6 \pm 1.8\%$ entrapment efficiency with enhanced drug release in comparison with free encorafenib. More importantly, the IC_{50} value reduced significantly from 45.71 ± 1.51 µg/mL for free encorafenib to 9.63 ± 1.27 µg/mL for HTCC-LPHNPs ($p < 0.0001$). Moreover, nanoparticle treatment induced apoptosis by increasing the apoptotic cells and the Sub-G1 cell population. Thus, the combined results reveal the significant increase in *in vitro* cytotoxic effect of encorafenib when used in combination with HTCC-LPHNPs.

Keywords: Glioblastoma; Encorafenib; Lipid-polymer hybrid nanoparticles; Quaternized chitosan; Molecular docking; Apoptosis; Cell-cycle analysis

Introduction

Glioblastoma multiforme (GBM) is the most frequent and deadly type of primary brain cancer that exhibits an extremely aggressive nature along with an unavoidable recurrence. Regardless of multimodal therapy that includes surgery, radiation, and temozolomide, the results of the treatment are still disappointing¹. Numerous clinical trials have indicated a median survival period of 12–18 months for patients suffering from glioblastoma, and survival rates for 5 years are lower than 10%². A number of factors pose challenges to the delivery of successful systemic treatments for GBM. The first is the blood-brain barrier (BBB). Due to the blood-brain barrier, it is difficult for chemotherapeutic agents to pass into the brain. This problem is complicated by the fact that even in cases when the BBB does not prevent the penetration of the drug, due to GBM's invasive nature and biological heterogeneity, there remain some cancer cells resistant to conventional therapies. The heterogeneity of GBM cells plays an important role in therapy failure^{3,4}.

Given this situation, the strategy of exploiting dysregulated signalling pathways in GBM would be a promising therapeutic strategy⁵. More specifically, abnormal activation of the MAPK signalling pathway through BRAF is a promising target. BRAF V600E mutations (a mutation that leads to constitutive activation of the signalling pathway) have been identified in a small subset of GBM (approximately 1–2%, in certain subtypes)^{6,7}. Encorafenib (Braftovi®) is a powerful FDA-approved small molecule inhibitor of mutated BRAF (used for BRAF V600E/K melanoma and colorectal cancer patients). It has been shown that BRAF inhibitors lead to tumour control in BRAF V600E mutant gliomas^{8,9}. The drawback of systemically administered encorafenib lies in the poor brain uptake. Encorafenib is a substrate of P-glycoprotein and other efflux pumps¹⁰.

Nanoparticles offer a strategy to overcome these delivery barriers. In this study, lipid-polymer hybrid nanoparticles (LPHNPs) were employed as the delivery platform, integrating the drug-loading capacity of a

lipidic core with the stabilising and surface-functional properties of a polymeric coating ^[11]. In addition to the development of a nanocarrier, we also conducted in silico studies to support the use of encorafenib as a drug repurposed for the treatment of GBM. In order to predict the binding of encorafenib to various glioma targets, molecular docking has been done, while the ADME/T prediction has assessed its drug-likeness and brain barrier permeability. This provides the basis for formulating the nanoparticles. Overall, the main objective of this research work is a comprehensive investigation of encorafenib in the context of GBM. Using docking and prediction modelling, we are trying to find out its strengths and weaknesses as a compound and formulate HTCC-LPHNPs to improve its efficacy.

2. Materials and methods

2.1 Materials

The Encorafenib hydrochloride was gifted to the laboratory by Hetero Drugs Ltd., Hyderabad, India. Poloxamer 407 was procured from Sigma-Aldrich (St. Louis, MO, USA). N-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (HTCC), which was synthesised in-house according to the laboratory procedure, was used as the polymer to prepare nanoparticles. Phospholipon® 90G was a gift from Lipoid GmbH (Ludwigshafen, Germany). Methanol, acetic acid, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO) and all other chemicals of analytical grade were used as is. Distilled water was used for all experiments.

In-vitro experiments required Dulbecco's Modified Eagle Medium (DMEM, high glucose), which was purchased from Merck Ltd., Mumbai, India. The fetal bovine serum (FBS) and antibiotic-antimycotic solutions were obtained from Gibco, Thermo Fisher Scientific (Waltham, MA, USA), and MTT was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Selection and Preparation of Ligands

The structural representations of encorafenib (CID: 50922675) and temozolomide (CID: 5394), reference compound, were acquired from the PubChem online resource (<https://pubchem.ncbi.nlm.nih.gov>) in the structure data file (SDF) ^[12]. The ligands were then loaded into PyRx 0.8, where an energy minimization process was conducted through the use of the Open Babel

component with the MMFF94 force field to get the most stable conformations of the molecules. These minimized molecules were then exported into the Protein Data Bank, Partial charge (Q), Atom type (T) (PDBQT) format ^[13].

2.3 Selection and preparation of target proteins

The three-dimensional structure of the therapeutic target for glioblastoma-associated proteins, including Eph receptor A2 (EPHA2, PDB code: 3CZU), protein tyrosine phosphatase receptor type Z1 catalytic domain (PTPRZ1, PDB code: 5AWX), lysine-specific demethylase 1 (LSD1, PDB code: 2DW4), and aldehyde dehydrogenase 1A3 (ALDH1A3, PDB code: 7QK9), have been taken from the Protein Data Bank (PDB) (<https://www.rcsb.org>). The process of protein preparation has been done manually in Discovery Studio Visualizer ^[14]. All non-essential interactions, such as co-crystallised ligand, water molecules, and heteroatoms, have been removed. Polar hydrogen atoms were added, and the prepared protein structures were saved in PDB format before conversion into PDBQT format for docking studies ^[15].

2.4 Molecular docking studies

Docking simulation experiments were conducted through the use of PyRx 0.8 with the aid of the AutoDock Vina docking tool to determine the binding affinity of encorafenib with respect to the chosen glioblastoma-related molecular targets. The developed ligand and protein structures were then uploaded into PyRx, and a receptor grid was created through the definition of the active site based on the structure of the co-crystallised ligand or the active site residues of the protein. The docking experiment was conducted with the default search algorithm in AutoDock Vina, while the pose with the minimum binding free energy (kcal/mol) was used for analysis. In addition, temozolomide was used as a benchmark ligand to conduct a comparative analysis of the binding affinity of encorafenib with the target proteins. The docking results were then analysed using Discovery Studio Visualizer to generate both 2D interaction maps and 3D docking poses. The nature of the molecular interactions, such as hydrogen bonds, carbon-hydrogen bonds, π -cation interactions, π -alkyl interactions, halogen interactions, and others, was analysed ^[16].

Table 1: Molecular targets selected for docking studies ^[17-20]

Target Protein	PDB ID	Biological Function	Role in Glioblastoma	Rationale for Selection
EPHA2	3CZU	Receptor tyrosine kinase involved in cell signaling	Promotes tumor cell proliferation, migration, invasion, angiogenesis, and poor prognosis.	Highly overexpressed in GBM and considered a promising therapeutic target.
PTPRZ1	5AWX	Regulates cell growth, differentiation and signaling	Maintains glioma stem cells, enhances migration and invasion.	Frequently overexpressed in GBM and associated with tumor aggressiveness.

LSD1	2DW4	Histone demethylase regulating gene expression	Controls epigenetic regulation, stemness, proliferation and therapy resistance	Important epigenetic target implicated in GBM progression
ALDH1A3	7QK9	Detoxification enzyme and cancer stem cell marker	Associated with mesenchymal phenotype, stemness, invasion and chemoresistance	Biomarker of aggressive GBM and therapeutic resistance

2.5 Drug-likeness prediction

The physicochemical properties and drug likeness behaviour of encorafenib were determined via the online web tool SwissADME (<http://www.swissadme.ch>). The canonical SMILES structure of encorafenib generated via PubChem was used as input into the web server. The physicochemical parameters, which include molecular weight, number of hydrogen bonds as donors and acceptors, topological polar surface area, lipophilicity, solubility, gastrointestinal absorption, blood-brain barrier penetration, prediction as P-glycoprotein substrate, rule of five, and medicinal chemistry parameters, were analysed²¹.

2.6 ADMET prediction

The pharmacokinetics of encorafenib were predicted with the help of the pkCSM web server (<https://biosig.lab.uq.edu.au/pkcsml>). The canonical SMILES structure of encorafenib was provided to predict its ADMET properties. Various parameters such as solubility in water, intestinal absorption, blood-brain barrier permeability, central nervous system (CNS) permeability, P-glycoprotein substrate, CYP450 interaction, total clearance, AMES toxicity, hERG inhibition, hepatotoxicity, and skin sensitisation were studied²².

2.7 In silico toxicity prediction

Encorafenib toxicity profile was analysed using the ProTox-3.0 web server (<https://tox.charite.de/protox3>). The canonical SMILES representation of encorafenib was provided to predict the following toxicities: acute oral toxicity, toxicity class, organ toxicity, carcinogenicity, mutagenicity, immunotoxicity, cytotoxicity, and possible toxicity targets. Prediction of the oral LD₅₀ value and toxicity classification were considered in order to determine the general safety profile of the drug candidate²³.

Taking into account the molecular docking and in silico pharmacokinetic analysis results, encorafenib was chosen for further formulation in HTCC-lipid-polymer hybrid nanoparticles.

2.8 Preparation of Encorafenib-Loaded HTCC-Lipid-Polymer Hybrid Nanoparticles

The encorafenib-loaded HTCC-lipid-polymer hybrid nanoparticles (HTCC-LPHNPs) were formulated via the nanoprecipitation (solvent injection) technique with little modifications. In brief, the required amount of encorafenib and Phospholipon® 90G was dissolved in methanol to obtain the organic phase. The encorafenib solution was sonicated until complete dissolution,

followed by dissolution of the lipid with constant stirring. The aqueous phase was prepared by dissolving HTCC and Poloxamer 407 in distilled water with constant stirring until a clear solution was obtained. The organic phase was subsequently added to the aqueous phase in drops while homogenising continuously using a high-shear homogeniser running at 12,000 rpm for 15 min, which led to the self-assembly of lipid-polymer hybrid nanoparticles. The resultant nanosuspension was stirred further for 30 min at ambient temperature for complete removal of the organic solvent. The developed HTCC-LPHNP suspension was employed for further characterisation, in vitro drug release studies, and biological investigations^{24,25}.

2.9 Physicochemical Characterisation of HTCC-LPHNPs

2.9.1 Particle Size, Polydispersity Index, and Zeta Potential

The average particle size, polydispersity index (PDI), and zeta potential of the optimised encorafenib-loaded HTCC-lipid polymer hybrid nanoparticles (HTCC-LPHNPs) were measured by dynamic light scattering (DLS) with Horiba SZ-100 Zeta Analyser (Horiba Ltd, Kyoto, Japan). The nanoparticle dispersion was first diluted 100 times with distilled water to reduce multiple scattering before measuring. All experiments were conducted at 25 ± 0.5 °C in triplicate²⁶.

2.9.2 Entrapment Efficiency

Entrapment Efficiency of optimised HTCC-LPHNPs was estimated using the indirect centrifugation technique. In brief, HTCC-LPHNPs were centrifuged at 15,000 rpm for 60 minutes at 4 °C to separate the free (non-entrapped) drug from nanoparticles. The supernatant containing the free drug was collected, and 1 mL of supernatant was diluted to 10 mL with methanol to dissolve encorafenib completely before the analysis. The dilute sample was measured using the UV spectrophotometer at 262.5 nm²⁷.

2.9.3 Transmission Electron Microscopy (TEM)

The morphology of the optimised HTCC-LPHNPs encapsulating encorafenib was studied by using Transmission Electron Microscopy (TEM) (Tecnai G² 12, FEI Company, Eindhoven, The Netherlands). In short, a few drops of the suitably diluted dispersion of the nanoparticles were deposited on the carbon-coated copper grid. The excess of the sample was wiped off using filter paper, after which the grid was left to dry before observation. The analysis was carried out with an accelerating voltage of 120 kV²⁸.

2.10 In Vitro Drug Release Study

In vitro drug release study of encorafenib-loaded hybrid nanoparticles of HTCC-lipid-polymer (HTCC-LPHNPs) was performed using a Franz diffusion cell as per a previous study with some modifications. A dialysis membrane having a molecular weight cut-off of 12–14 kDa was used and was equilibrated in distilled water. A known quantity of nanoparticle dispersion was added to the donor side of the Franz cell, and the receptor side was filled with phosphate buffer solution having a pH of 6.4, maintained at 37 ± 0.5 °C with continuous magnetic stirring. Samples were withdrawn from the receptor side at specified time intervals (0.5–48 h) and replaced with the same volume of fresh phosphate buffer to keep the system in sink condition. Encorafenib concentration released from nanoparticles was determined using a UV-visible spectrophotometer at a wavelength of 262.5 nm²⁹.

2.11 Cell Culture

Human glioblastoma cell line (U87) was obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in Dulbecco's Modified Eagle Medium (high glucose DMEM) along with 10% v/v Fetal Bovine Serum (FBS) and 1% v/v antibiotic–antimycotic mixture. The cells were maintained in a humidified CO₂ incubator maintained at 37 ± 0.5 °C with 5% CO₂, and the medium was changed every other day. Subculturing of the cells was done every two days to ensure the growth of the cells in an exponential phase.

2.12 Cell Viability Assay (MTT Assay)

The cytotoxic activity of free encorafenib and encorafenib-loaded HTCC-lipid-polymer hybrid nanoparticles (HTCC-LPHNPs) was determined by MTT assay. U87 cells were seeded in 96-well plates at a seeding density of 2×10^4 cells/100 µL of complete DMEM per well and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO₂ to allow cell attachment. Upon incubation, the medium was removed, and fresh medium supplemented with the respective formulation concentrations was added, and cells were incubated for 24 h. After the incubation time, the medium was removed, and fresh medium with MTT reagent at a concentration of 0.5 mg/mL was added and incubated for 3 h to allow for the formation of intracellular formazan crystals. Then the MTT-containing medium was removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well for dissolving the formazan crystals. The absorbance was measured at a wavelength of 570 nm in the microplate reader. The IC₅₀ values of free encorafenib and encorafenib-loaded HTCC-LPHNPs were analyzed to measure the improvement in their antiproliferative effect³⁰.

2.13 Cellular Apoptosis Analysis by Flow Cytometry

Apoptosis in the cells post-treatment with free encorafenib and encorafenib loaded into HTCC-lipid polymer hybrid nanoparticles (HTCC-LPHNPs) was carried out using the Annexin V-FITC/propidium iodide (PI) dual staining method. The U87 cells were exposed

to the relevant formulations at predetermined IC₅₀ values for 24 h. The cells that were not exposed to any of the treatment methods were the controls. The treated cells were then harvested and washed twice in cold PBS and suspended at a concentration of 1×10^6 cells/mL in 1× binding buffer. The cell suspension was divided into tubes based on appropriate labelling for unstained cells, control cells, Annexin V-FITC alone, PI alone, and treatment groups. Annexin V-FITC and PI (Elabscience, E-CK-A111) were used as per the manufacturer's protocol in their respective samples. Then, the samples were vortexed and kept at room temperature for 15 minutes in the absence of light. Subsequently, 1× binding buffer was added to the samples, and the cells were analyzed using a BD FACS Lyric™ flow cytometer within 1 hour of incubation. The collected data were analyzed based on the fluorescence of Annexin V-FITC and PI³¹.

2.14 Cell Cycle Analysis by Flow Cytometry

The impact of the use of free encorafenib and encorafenib-loaded HTCC-lipid-polymer hybrid nanoparticles (HTCC-LPHNPs) on the cell cycle distribution of the U87 cells was analyzed through PI staining and flow cytometry. Cells were cultured and exposed to the respective compounds at predetermined concentrations for 24 h. The untreated cells served as the control group. The cells were detached from the surface of the flask through trypsinization and harvested through centrifugation. The cells were washed once with chilled PBS, and about 1×10^6 cells were suspended in 100 µL PBS. Fixation was performed by suspending the cells in a mixture of 900 µL of 70% ethanol in centrifuge tubes and incubating at 4 °C for more than 2 h. Upon fixation, the cells were centrifuged, and the obtained pellet was resuspended in PI staining buffer, which comprised 0.1% (v/v) Triton X-100, 10 µg/mL propidium iodide, and 100 µg/mL DNase-free RNase A in PBS. Incubation of the samples was done in the absence of light for 30 min at room temperature. After incubation, the stained cells were analyzed through flow cytometry. Cell cycle distribution was analyzed according to DNA content, and the percentage of cells at sub-G1, G0/G1, S, and G2/M stages was determined³².

2.15 Statistical Analysis

Experiments were conducted in triplicate, and data are shown as mean $\pm$ standard deviation (SD). Data analyses were carried out using GraphPad Prism software version 10.1 (GraphPad Software Inc., San Diego, CA, USA). Data from in vitro drug release and MTT assay were analysed using two-way ANOVA followed by Šídák's multiple comparisons test. IC₅₀ values were compared using an unpaired two-tailed Student's t-test. Two-way ANOVA followed by Dunnett's multiple comparisons test was performed for the analysis of data from apoptosis and cell cycle analyses, with the untreated control being taken as the reference.

3. Results and Discussion

3.1 Molecular Docking Analysis

Binding affinity studies using molecular docking were conducted on encorafenib toward four glioblastoma-related target molecules, such as Eph receptor A2 (EPHA2, PDB ID: 3CZU), protein tyrosine phosphatase receptor type Z1 (PTPRZ1, PDB ID: 5AWX), lysine-specific demethylase 1 (LSD1, PDB ID: 2DW4), and aldehyde dehydrogenase 1A3 (ALDH1A3, PDB ID: 7QK9). The choice of these proteins was due to their proven involvement in the progression of glioblastoma, invasion of tumours, maintenance of glioma stem cells, epigenetic regulation, and drug resistance. Temozolomide, which is currently used as the chemotherapeutic agent for glioblastoma treatment,

was chosen as the reference molecule. Binding structures and interaction studies are shown in Figure 1. Results of the docking study are shown in Table 2. Based on comparative docking analysis, it was found that encorafenib showed equal or better binding affinity towards three out of four examined targets. The strongest binding affinity was recorded towards LSD1 (−9.8 kcal/mol), ALDH1A3 (−9.4 kcal/mol), and EPHA2 (−6.9 kcal/mol); however, temozolomide was more effectively bound to PTPRZ1 (−6.5 kcal/mol) in comparison with encorafenib (−6.0 kcal/mol). Along with favourable docking values, it is important to note that encorafenib formed a number of hydrogen bonds and interactions of different types, namely, π -cation, π -anion, π -alkyl, hydrophobic, and halogen interactions^{33,34}.

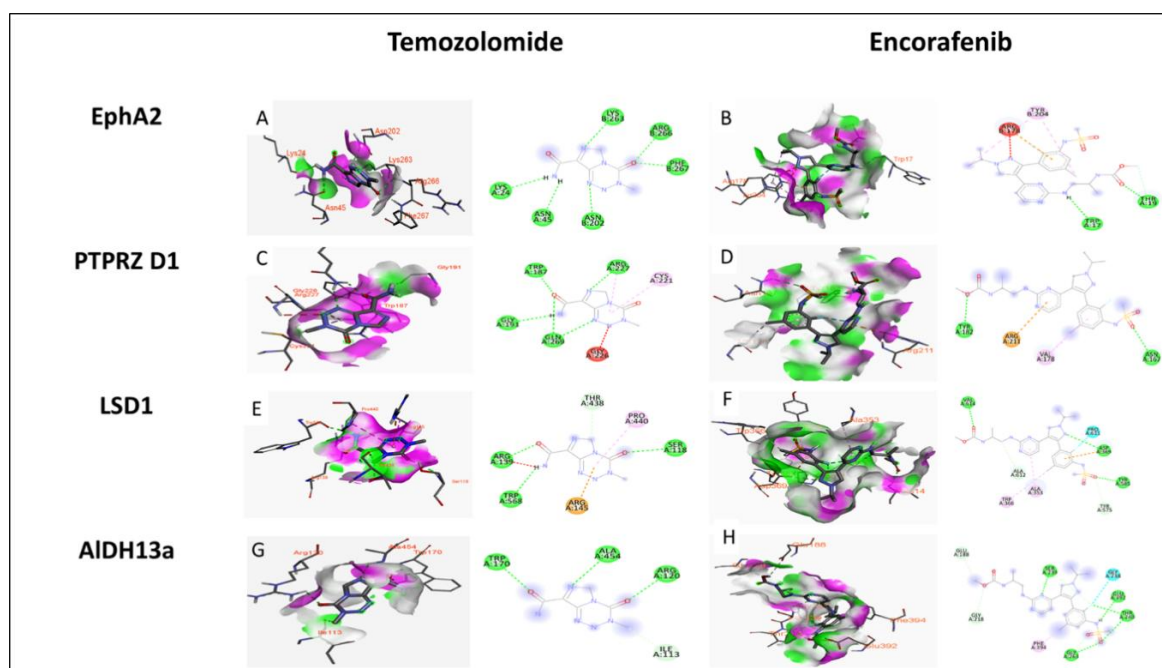


Figure 1. Comparative molecular docking analysis of temozolomide and encorafenib against selected glioblastoma-associated molecular targets. Three-dimensional binding conformations and corresponding two-dimensional interaction maps are shown for EPHA2 (PDB ID: 3CZU), PTPRZ1 (PDB ID: 5AWX), LSD1 (PDB ID: 2DW4), and ALDH1A3 (PDB ID: 7QK9).

The EPHA2 and PTPRZ1 proteins are receptors associated with proliferation, migration, invasion, and stem cell phenotype in glioblastomas. Encorafenib showed slightly higher binding affinity to the EPHA2 protein compared with temozolomide and made extra hydrophobic and electrostatic contacts, which might stabilise the complex of the ligand and protein. While temozolomide was marginally better bound to the PTPRZ1 protein, encorafenib established a broad array of hydrogen-bonding, π -cation, π -alkyl, and halogen interactions, implying a stable binding mode inside the receptor binding pocket. It might be concluded that encorafenib could affect receptor-mediated signalling in glioblastoma cells. Out of all the targets, LSD1 demonstrated the highest binding affinity for encorafenib (−9.8 kcal/mol), which significantly exceeds the binding affinity to temozolomide (−8.0 kcal/mol).

This protein is known to be an essential epigenetic regulator playing a role in chromatin remodelling, preservation of glioma stem cells, and therapeutic resistance and can be considered an effective target for glioblastoma treatment^{35–39}. Similarly, the binding affinity of encorafenib to ALDH1A3 (−9.4 kcal/mol) was shown to be significantly higher compared to the binding affinity of temozolomide (−6.1 kcal/mol). ALDH1A3 is linked to the mesenchymal glioblastoma subtype, metabolic remodelling, cancer stem cell maintenance, and chemoresistance. It is worth highlighting that both the proteins mentioned demonstrate high binding affinity since they are responsible for the increased aggressiveness of glioblastoma and relapse. Conclusively, from the findings of this study on molecular docking of encorafenib, it was seen that the drug is able to interact

stably with different therapeutically important proteins, which are found in glioblastomas and which participate in RTK signalling, epigenetics, and cancer stem cell biology. Despite the fact that molecular docking is a purely theoretical prediction of ligand-protein interaction, the potential for multi-target interaction of

encorafenib makes it worth studying further as a repurposed treatment option for glioblastoma. The findings formed the basis for *in silico* assessment of its drug-likeness, pharmacokinetic characteristics, and toxicity, leading to the development of a brain-targeting HTCC-lipid-polymer hybrid nanoparticle platform^{3,36,40}.

Table 2. Key Molecular Interactions between Ligands and Target Proteins

Protein	Ligand	Docking Score (kcal/mol)	Hydrogen Bonding Residues	Other Non-covalent Interactions
EPAH2	Encorafenib	-6.9	Trp17, Thr19	π -Cation (Arg178); π -Alkyl (Tyr204)
	Temozolomide	-6.4	Lys24, Asn45, Asn202, Lys263, Arg266, Phe267	Predominantly hydrogen bonding
PTPRZ1	Encorafenib	-6.0	Tyr182, Asn167	π -Cation (Arg211); π -Alkyl (Val178); Halogen interaction (Pro611)
	Temozolomide	-6.5	Trp187, Gly191, Gln269, Arg227	π -Alkyl (Cys221); Unfavorable donor-donor interaction (Gly226)
LSD1	Encorafenib	-9.8	Val614, Asp369, Tyr585	π -Anion (Asp369); π -Alkyl (Trp366, Ala353); Halogen interaction
	Temozolomide	-8.0	Arg139, Trp568, Ser118	π -Cation (Arg145); π -Alkyl (Pro440); Unfavorable donor-donor interaction
ALDH1A3	Encorafenib	-9.4	Ser239, Glu392, Thr240, Gly263	π -Alkyl (Phe394); Halogen interaction (Gly238)
	Temozolomide	-6.1	Trp170, Ala454, Arg120	Carbon hydrogen bond (Ile113)

3.2 Drug-Likeness Prediction Using SwissADME

The drug-likeness and physicochemical properties of encorafenib were predicted using the SwissADME web server, and the results are summarized in Table 3. Encorafenib exhibited a molecular weight of 540.01 g/mol, a consensus LogP of 2.49, and a topological polar surface area (TPSA) of 148.51 Å², indicating moderate lipophilicity but relatively high molecular polarity. The compound possessed 11 rotatable bonds, 8 hydrogen bond acceptors, and 3 hydrogen bond donors, reflecting adequate molecular flexibility and hydrogen-bonding capability. SwissADME further predicted moderate aqueous solubility, low gastrointestinal absorption, lack of blood-brain barrier permeation, and identified encorafenib as a substrate of P-glycoprotein. Furthermore, the compound exhibited a bioavailability score of 0.17, two Lipinski rule violations, and no PAINS alerts, suggesting acceptable drug-likeness without major structural liabilities. The relatively high TPSA together with poor predicted BBB permeability suggests that passive diffusion of encorafenib into the brain is likely to be limited following conventional administration. In addition, P-glycoprotein-mediated efflux may further restrict drug accumulation within the central nervous system. These predicted pharmacokinetic limitations provide a strong rationale for the development of a targeted nanocarrier system capable of enhancing brain delivery while bypassing the blood-brain barrier. Therefore, HTCC-based lipid-polymer hybrid nanoparticles were developed to

improve the nose-to-brain delivery and therapeutic efficacy of encorafenib.^{10,21,41}

Table 3. Drug-Likeness and Pharmacokinetic Properties of Encorafenib Predicted Using SwissADME

Parameter	Predicted Value
Molecular formula	C ₂₂ H ₂₇ ClFN ₇ O ₄ S
Molecular weight (g/mol)	540.01
Hydrogen bond acceptors	8
Hydrogen bond donors	3
Rotatable bonds	11
Topological polar surface area (TPSA, Å ²)	148.51
Consensus LogP	2.49
Water solubility (ESOL)	Moderately soluble
Gastrointestinal (GI) absorption	Low
BBB permeant	No
P-glycoprotein substrate	Yes
Bioavailability score	0.17
Lipinski rule violations	2
PAINS alerts	0
Synthetic accessibility	4.35

3.3 ADMET Prediction Using pkCSM

Pharmacokinetics and toxicity of encorafenib were additionally assessed through the use of the pkCSM web-server, and the results are provided in Table 4 below. Encorafenib had a very low aqueous solubility ($-3.657 \log \text{ mol/L}$) as well as low Caco-2 permeability and moderate intestinal absorption (76.90%). The drug was predicted to be a substrate for P-glycoprotein, thus implying efflux-mediated transport. Distribution study predicted poor blood-brain barrier permeability ($\log \text{BB} = -2.211$) and low central nervous system permeability ($\log \text{PS} = -3.532$). This is consistent with the low brain

penetration predicted in the SwissADME tool. Encorafenib was found to be both a substrate and an inhibitor of the CYP3A4 enzyme. In terms of toxicity, a negative AMES test was combined with non-inhibition of hERG I, while inhibition of hERG II and hepatotoxicity were predicted. Collectively, the pkCSM predictions validate the SwissADME prediction, as low aqueous solubility, poor BBB permeability, and P-glycoprotein efflux are the main factors limiting the brain penetration of the drug. It provides additional justification for developing a lipid-polymer nanoparticle system for improving drug transport to the brain and treatment of glioblastoma¹⁰.

Table 4. Pharmacokinetic and Toxicological Properties of Encorafenib Predicted Using pkCSM

Category	Parameter	Predicted Value
Absorption	Water solubility	$-3.657 \log \text{ mol/L}$
	Caco-2 permeability	$-0.073 \log \text{ Papp}$ (10^{-6} cm/s)
	Human intestinal absorption	76.90%
	P-glycoprotein substrate	Yes
Distribution	Blood-brain barrier (BBB) permeability	$-2.211 \log \text{BB}$
	Central nervous system (CNS) permeability	$-3.532 \log \text{PS}$
Metabolism	CYP3A4 substrate	Yes
	CYP3A4 inhibitor	Yes
Excretion	Total clearance	$0.305 \log \text{ mL/min/kg}$
Toxicity	AMES toxicity	No
	hERG I inhibitor	No
	hERG II inhibitor	Yes
	Hepatotoxicity	Yes

3.4 Toxicity Prediction Using ProTox-3.0

Encorafenib toxicity profile prediction was done via the ProTox-3.0 online platform, and the outcomes are shown in Table 5 below. Encorafenib was predicted to have an oral LD_{50} of 1300 mg/kg, and thus, it is in toxicity class IV, meaning that it has relatively low oral toxicity. Encorafenib was predicted to possess hepatotoxicity and immunotoxicity properties, while carcinogenicity, mutagenicity, cytotoxicity, and cardiotoxicity were inactive. While computational toxicity analysis cannot be used as a replacement for experimental toxicity evaluation, the general toxicity prediction indicates that encorafenib is safe to use during further drug development process, but with an emphasis on certain toxicity parameters that have to be experimentally validated. Altogether, the molecular docking analysis, drug-likeness prediction, ADMET analysis, and toxicity prediction give a computational basis to use encorafenib as a promising anticancer drug repurposed for the treatment of glioblastoma patients. At the same time, the predicted low level of brain penetration and pharmacokinetics indicates the necessity of using an efficient targeted drug delivery

system. Hence, encorafenib was loaded into HTCC-lipid-polymer hybrid nanoparticles^[23].

Table 5. Toxicological Profile of Encorafenib Predicted Using ProTox-3.0

Parameter	Prediction
Predicted oral LD_{50} (mg/kg)	1300
Toxicity class	Class IV
Hepatotoxicity	Active
Immunotoxicity	Active
Carcinogenicity	Inactive
Mutagenicity	Inactive
Cytotoxicity	Inactive
Cardiotoxicity	Inactive

3.5 Physicochemical Characterisation of Encorafenib-Loaded HTCC-LPHNPs

The optimised encorafenib-loaded HTCC-lipid-polymer hybrid nanoparticles (HTCC-LPHNPs) were physicochemically characterised for determining their particle size, zeta potential, encapsulation efficiency, and morphological properties.

3.5.1 Particle Size, Polydispersity Index and Zeta Potential

The optimised Enc-HTCC-LPHNPs demonstrated a mean size of 141.4 ± 2.3 nm, a polydispersity index (PDI) of 0.445 ± 0.02 and a zeta potential of $+41.3 \pm 1.2$ mV. The particle diameter is within the range of 200 nm, which is ideal for intranasal administration as the nanoparticles in this size could interact with the nasal epithelium to enhance their passage through the olfactory pathway. The size distribution was acceptable; no secondary peaks were observed, suggesting that nanoparticles have been successfully formed. The highly positive zeta potential revealed that HTCC modification of the particle surface was successful and is expected to increase colloidal stability through electrostatic repulsion. Furthermore, cationic surface charge would help in mucoadhesion through interaction with the negative charge of mucin^{42,43}.

3.5.2 Entrapment Efficiency

An entrapment efficiency of $73.6 \pm 1.8\%$ was achieved in the optimised formulation, demonstrating the effective entrapment of encorafenib in the lipid-polymer hybrid matrix. This higher entrapment efficiency may be

attributed to the hydrophobicity of encorafenib, allowing it to be entrapped in the phospholipid core, and the HTCC polymer in the outer region of the nanoparticles provides stability to their structure⁴⁴.

3.5.3 Morphological Analysis by Transmission Electron Microscopy

TEM analysis of the optimised HTCC-LPHNPs showed that these nanoparticles are mainly spherical in shape, have smooth surfaces, and are dispersed without any sign of aggregation (Figure 2C). The size of these particles analysed through TEM is slightly lower than that analysed via the DLS technique, and this is because the DLS analyses the hydrodynamic diameter. At the same time, the TEM measures the physical diameter of the particles.

3.6 In Vitro Drug Release Study

The in vitro release profile of free encorafenib and encorafenib-loaded HTCC-lipid-polymer hybrid nanoparticles (HTCC-LPHNPs) is depicted in Figure 2D. The free encorafenib had a rapid drug release rate, where $70.17 \pm 1.79\%$ was released in 4 h and attained up to $92.83 \pm 1.99\%$ within 10 h. On the other hand, the HTCC-LPHNPs displayed slower release of the drug with $31.97 \pm 1.08\%$ released in 4 h, $56.74 \pm 1.84\%$ released after 12 h, and $65.12 \pm 1.67\%$ released after 48 h. This slow release can be attributed to the presence of the encorafenib in the lipid-polymer hybrid matrix, where the release of the drug was hindered by the lipid core and the HTCC polymer shell, thus allowing gradual drug release⁴⁵.

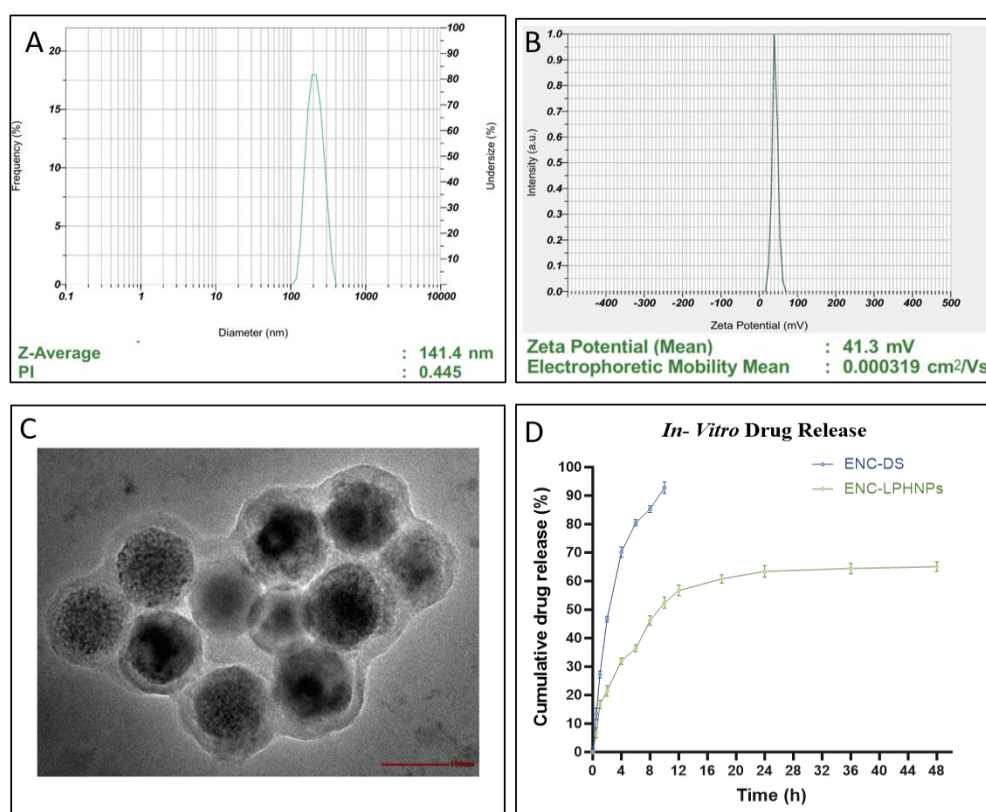


Figure 2. Physicochemical characterization and drug release of ENC-LPHNPs. (A) Particle size distribution, (B) zeta potential, (C) TEM image, and (D) in vitro drug release profile of ENC-DS and ENC-LPHNPs. Data are presented as mean $\pm$ SD ($n = 3$).

3.7 In Vitro Cytotoxicity Evaluation

3.7.1 MTT Cell Viability Assay

The cytotoxic effect of free encorafenib and encorafenib loaded into HTCC-lipid-polymer hybrid nanoparticles (HTCC-LPHNPs) on glioblastoma cells was measured via MTT assay, and the findings are shown in Figure 3A. There is a concentration-dependent decrease in the viability of cells from both formulations; nevertheless, HTCC-LPHNPs showed a significantly higher cytotoxicity than free encorafenib for all concentrations used. Cell viability dropped to $47.14 \pm 2.84\%$ with the application of free encorafenib and to $11.36 \pm 1.61\%$ with HTCC-LPHNPs at $50 \mu\text{g/mL}$, while at the maximum concentration studied ($100 \mu\text{g/mL}$), the viability of cells dropped to $11.45 \pm 1.47\%$ and $3.69 \pm 1.11\%$, respectively. The increased cytotoxicity provided by

HTCC-LPHNPs may be due to better interaction with cells and the sustained release properties of HTCC-LPHNPs^{46,47}.

3.7.2 IC₅₀ Determination

The antiproliferative activity of these formulations was also evaluated using their IC₅₀ values (Figure 3B). The IC₅₀ value of HTCC-LPHNPs ($9.63 \pm 1.27 \mu\text{g/mL}$) was much lower than that of the free encorafenib ($45.71 \pm 1.51 \mu\text{g/mL}$), suggesting a higher cytotoxic effect on glioblastoma cells. This reduction was statistically proven to be significant by performing the statistical analysis using an unpaired two-tailed Student's t-test ($p < 0.0001$). Therefore, these results confirm that encorafenib entrapped in HTCC-lipid-polymer hybrid nanoparticles showed better cytotoxic effects in vitro^{48,49}.

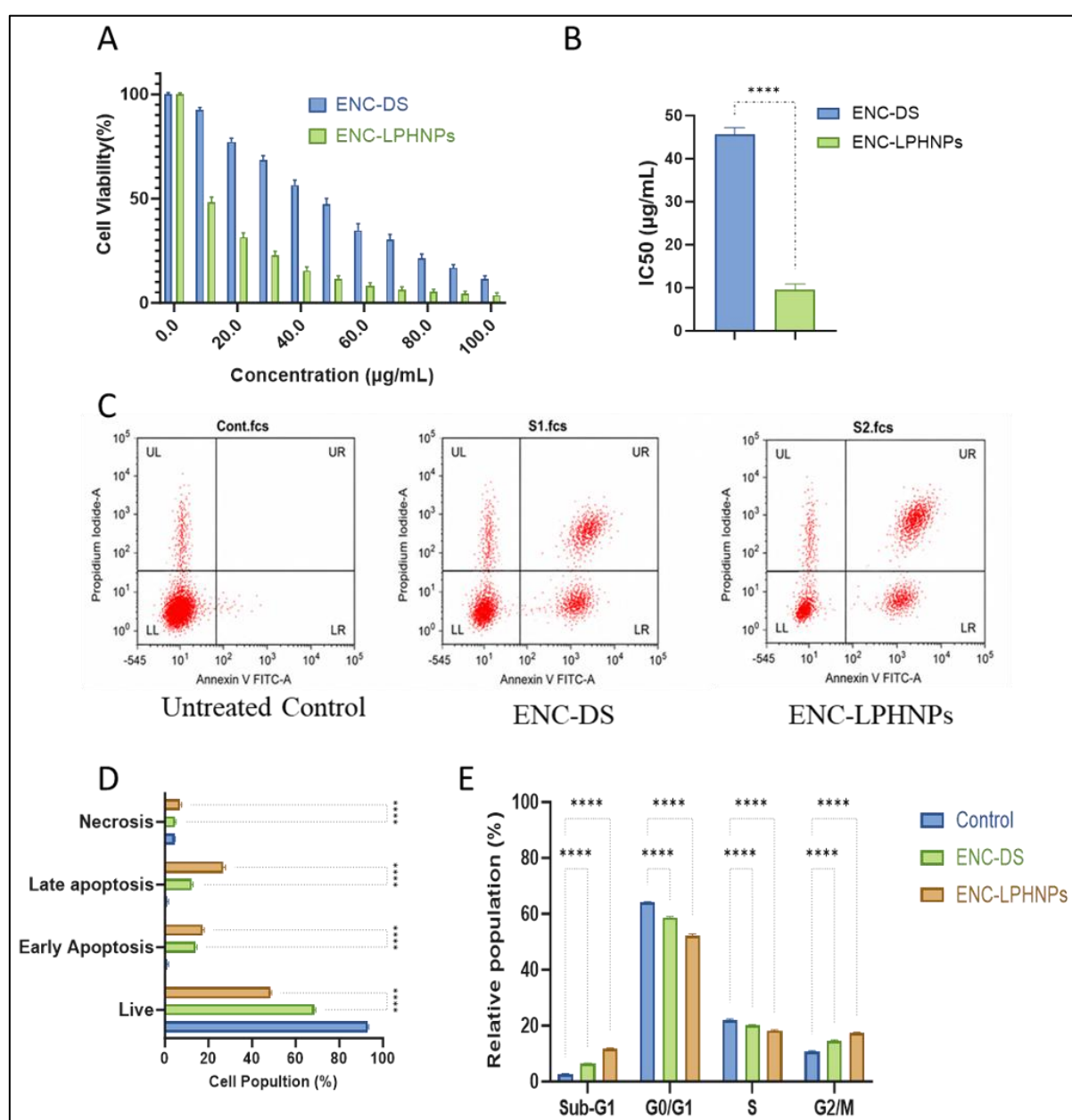


Figure 3. In vitro cytotoxicity, apoptosis, and cell-cycle analysis of ENC-LPHNPs in U87 cells. (A) Cell viability at different concentrations, (B) IC₅₀ values, (C) Annexin V-FITC/PI flow-cytometric analysis of apoptosis, (D) quantitative distribution of viable, early apoptotic, late apoptotic, and necrotic cells, and (E) cell-cycle distribution following treatment with ENC-DS and ENC-LPHNPs. Data are presented as mean $\pm$ SD ($n = 3$).

3.7.3 Cellular Apoptosis Analysis

Flow cytometry-based annexin V-FITC/PI staining assay was used to determine the apoptotic potential of U87 cells exposed to free encorafenib (ENC-DS) and encorafenib-loaded HTCC-lipid-polymer hybrid nanoparticles (ENC-LPHNPs). According to Figure 3C-D, control cells showed a viable population ($93.16 \pm 0.29\%$) with a minor percentage of early ($0.95 \pm 0.67\%$) and late ($1.20 \pm 0.43\%$) apoptosis. The viability of cells decreased by $68.47 \pm 0.72\%$ with an increase in early and late apoptotic cell populations to $14.34 \pm 0.44\%$ and $12.43 \pm 0.56\%$, respectively, on exposure to ENC-DS. In contrast, ENC-LPHNPs showed a significant decrease in viable cells ($48.54 \pm 0.33\%$) with increases in early ($17.46 \pm 0.58\%$) and late ($26.89 \pm 0.91\%$) apoptotic population, respectively^{50,51}.

3.7.4 Cell Cycle Analysis

The effect of free ENC-DS and ENC-LPHNPs on cell cycle distribution in U87 cells was evaluated using PI stain and flow cytometry. As depicted in Figure 3E, untreated control cells exhibited a prominent distribution of G0/G1 cells ($64.2 \pm 0.16\%$), S phase ($22.1 \pm 0.24\%$), and G2/M phase ($10.9 \pm 0.14\%$) with the lower percentage of Sub-G1 cells ($2.8 \pm 0.08\%$). Free ENC-DS treatment caused the decrease in G0/G1 cells to $58.7 \pm 0.31\%$ with an increase in G2/M cells ($14.7 \pm 0.24\%$) and Sub-G1 cells ($6.4 \pm 0.24\%$). ENC-LPHNP treatment showed a marked change in cell cycle distribution with a decrease in G0/G1 and S cells to $52.4 \pm 0.36\%$ and $18.3 \pm 0.21\%$, respectively, and an increase in G2/M and Sub-G1 cells to $17.5 \pm 0.14\%$ and $11.8 \pm 0.22\%$, respectively. The increased Sub-G1 population following ENC-LPHNP treatment further supports the enhanced apoptotic response observed in the Annexin V-FITC/PI assay^{52,53}.

4. Conclusion

This study effectively combined in silico screening, nanoparticle formulation, and biological evaluation to assess the efficacy of encorafenib as a repurposed drug candidate for glioblastoma. In silico investigations revealed positive interaction between encorafenib and several targets associated with glioblastoma, but also indicated that there were certain pharmacokinetic restrictions on its usage, especially in terms of poor brain penetration, necessitating a new method for delivering this drug. The optimal HTCC-LPHNPs possessed suitable physicochemical attributes along with efficient drug loading and controlled release properties. However, the main advantage was the significant improvement in the cytotoxicity of encorafenib against the U87 cells due to nanoparticle encapsulation, as evidenced by the decreased IC_{50} value. Moreover, the increased number of apoptotic cells along with the change in the cell cycle distribution profile, especially the increased Sub-G1 population, was observed from the flow cytometric studies after the treatment of the cells with ENC-LPHNPs. Overall, HTCC-LPHNPs not only enhanced the in vitro cytotoxicity efficiency of encorafenib but also provided a promising tool for future investigations on brain targeting.

Conflict of Interest: The authors declare no potential conflict of interest concerning the contents, authorship, and/or publication of this article.

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