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Development of TRPM2-Targeted Lipid Nanoparticle System for Delivery of Trametinib in Pancreatic Cancer

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Abstract: *Pancreatic cancer is a highly malignant tumour with an inferior prognosis. Current treatment methods have significant limitations of drug permeability and side effects. Recent studies have found that TRPM2 is highly expressed in pancreatic cancer cells. Based on this molecular characteristic, this study will aim to develop a targeted nano delivery system (TNP-TRPM2-Ab) that specifically targets TRPM2 for the delivery of the hydrophobic drug Trametinib. Through the covalent coupling of nanoparticles and antibodies, it is anticipated that precise recognition of cancer cells and controlled drug release will be achieved.*

Keywords: *TRPM2, trametinib, the MEK pathway, lipid nanoparticles, targeted drug delivery, pancreatic cancer*

1. Introduction

Pancreatic cancer (PC) is a fatal disease with a poor prognosis and a constantly increasing incidence rate[1]. According to statistics, its 5-year survival rate is less than 4%[2]. In some countries, it is even as low as 2%[1]. Although there are currently treatment methods for PC, due to the desmoplastic stroma of the tumour, traditional therapeutic drugs (such as gemcitabine) have difficulty spreading in the tumour tissue[3]. At the same time, the drugs also have serious side effects on the human body. Therefore, it is important to explore new drug targets and efficient, low-toxicity administration methods.

The nanoparticle-based drug delivery system (NP-DDS) presents a novel solution to the challenges associated with traditional drug treatments. The structure of tumour cells differs from that of healthy cells. The rapid proliferation of the former leads to incomplete vascular structures, loose arrangement of endothelial cells, and underdeveloped lymphatic systems. Nanoparticles (NPs) can enter the tumour tissue through the gaps between endothelial cells. The poorly developed lymphatic system in tumours limits immune cell access, making it challenging to eliminate tumour tissue. It provides a physiological basis for NPs to enter and remain in the tumour tissue, known as the Enhanced Permeability and Retention (EPR) effect. Moreover, the editable surface of the NPs makes them highly selective, allowing for the specific delivery of drugs and avoiding a range of side effects caused by traditional medicines that harm healthy cells[4]. Recent studies have found that *TRPM2* is overexpressed in PC cells, which exerts a significant regulatory role in tumour growth[2]. *TRPM2* is a calcium ion channel located on the cell surface. Calcium ions are crucial second messengers in various signalling pathways that regulate many critical cellular processes[2. It can directly or indirectly activate multiple subunits of the protein kinase C (PKC) family (PKCα, PKCε, or PKCδ) by controlling calcium ion influx[2]. These proteins activate the MAPK/MEK pathway in tumour cells. Among them, the activation signal of the upstream RAF(Rapidly Accelerated Fibrosarcoma) is mainly transmitted to the downstream ERK through MEK (MAPK/ERK kinase) [2]. The activated MEK catalyses the phosphorylation of ERK, resulting in the formation of p-ERK. Subsequently, p-ERK enters the cell nucleus and promotes the expression of various genes related to cell proliferation and migration[2]. Trametinib is an MEK inhibitor that can block the MEK pathway. However, its extreme hydrophobicity and side effects of administration have limited its clinical application[5].

Based on the above background, *TRPM2* overexpressed on the surface of PC cells will be used as the therapeutic target. A surface-modified anti-TRPM2 antibody and a nano-delivery system encapsulating

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the hydrophobic drug Trametinib (TNP-TRPM2-Ab) will be constructed. This nanoparticle binds explicitly to *TRPM2* on the surface of cancer cells and enters the cancer cells through endocytosis. The nanoparticle gradually degrades in the acidic environment of the cell, exposing the internal Trametinib. Then, Trametinib kills cancer cells by blocking the MEK pathway, achieving the purpose of precisely targeting cancer cells while minimizing side effects. I predict that increasing concentrations and treatment durations of TNP-*TRPM2*-Ab in PANC-1 pancreatic cancer cells targeting *TRPM2* will decrease viability, tumour size, ERK phosphorylation (p-ERK), and cytotoxicity to normal cells more effectively than Trametinib alone.

2. Materials and Methods

2.1. Materials

Nanoparticle components: PEG–PLGA–COOH copolymer; Trametinib (MEK inhibitor); DiR (1,1′-dioctadecyl-3,3,3’,3’-tetramethyl-indotricarbocyanine iodide) for NIR tracking.

Conjugation/chemistry reagents: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC); N-hydroxysuccinimide (NHS); ethanolamine; MES buffer; PBS/HEPES (amine-free); 3.5 kDa dialysis tubing; 100 kDa centrifugal filters; sterile syringes and 0.2 μm filters.

Assay reagents and kits: BCA Protein Assay Kit; CCK-8 cell viability kit; SDS-PAGE gels and running/transfer buffers; protein stain (e.g., Coomassie/BlueSafe); RIPA lysis buffer with protease and phosphatase inhibitors; Alexa Fluor 488 dye (for NP fluorescence labeling); Matrigel (for invasion); Transwell inserts (8 μm pores).

Antibodies: Rabbit polyclonal anti-TRPM2 (for NP conjugation); anti-p-ERK (Thr202/Tyr204), total ERK and β-actin primary antibodies; HRP-conjugated secondary antibodies; optional isotype IgG control.

Cell lines and culture media: PANC-1 human pancreatic cancer cells; DMEM supplemented with 10% FBS and penicillin/streptomycin; DPBS; trypsin-EDTA.

***In vivo* materials**: female mice (CD1-Foxn1nu, 6 weeks, 20–35 g); soflurane for anesthesia; CO_2 for euthanasia; sterile PBS for injections.

2.2. Instruments and Software

Dynamic light scattering (DLS)/zeta analyzer (e.g., Malvern Zetasizer); HPLC system; transmission electron microscope (e.g., FEI Tecnai); confocal laser scanning microscope; flow cytometer; microplate reader (e.g., BioTek Cytation); near-infrared imaging system for DiR biodistribution; refrigerated centrifuge; digital calipers. Data analysis with ImageJ, FlowJo, Image Lab, and GraphPad Prism.

2.3. Nanoparticle Synthesis

Trametinib-loaded nanoparticles were prepared by nanoprecipitation[6]. PEG-PLGA-COOH and trametinib were dissolved in ethyl acetate (organic phase) and dropwise added into an equal volume of deionised water under stirring to induce self-assembly[7]. The dispersion was gently stirred for 30 min to stabilise particles, dialysed to remove residual solvent and free drug, and stored at 4 °C[7].

2.4. Nanoparticle Characterisation

Nanoparticle size and homogeneity were assessed by DLS at room temperature using an ionic dispersion medium (not DI water) and reported as Z-avg diameter and PDI (mean ± SD, ≥3 independent preps)[8]. Surface charge was determined as zeta potential in the same medium after gentle filtration to remove aggregates/bubbles; $|\zeta| > \sim 20$ mV was taken to indicate acceptable colloidal stability[8]. Morphology and shell formation were visualised by TEM on carbon-coated grids[9] particle diameters from representative micrographs were quantified in ImageJ to generate a size distribution for comparison with DLS. Encapsulation efficiency (EE%) and drug loading (DL%) for Trametinib were quantified by separating free drug via ultrafiltration and measuring Trametinib in the filtrate and in disrupted nanoparticle fractions by HPLC[10]. EE% = (Total dosage - Free dosage) / Total dosage × 100%; DL% = (Encapsulated drug amount) / Total nanoparticle mass × 100%. *In vitro* drug release is evaluated by dialysis in pH 7.4 PBS and pH 5.5 acetate buffer with HPLC quantification at predefined timepoints

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(37 °C, 0.5/1/2/4/8/24/48 hours) to generate cumulative release profiles representing physiological and tumour-like acidic conditions[11]. Then, NPs are placed in PBS with pH 7.4 and 10% FBS separately for continuous monitoring of particle size and PDI from 0 to 72 hours, and whether precipitation/aggregation occurred is recorded.

2.5. Covalent Conjugation and Characterisation of Anti-TRPM2 Antibody-Functionalized Nanoparticles

Anti-TRPM2 antibodies are covalently conjugated to carboxylated PEG-PLGA nanoparticles using the EDC/NHS coupling method[12]. Briefly, nanoparticles are activated in MES buffer with EDC and NHS[13], followed by reaction with anti-TRPM2 antibodies (pre-treated in amine-free buffer) under near-neutral pH at room temperature and then incubated at 4°C in the dark to form stable amide bonds. Unbound antibodies are removed by centrifugation or ultrafiltration, and the conjugated nanoparticles are resuspended in PBS. For *in vivo* imaging, DiR is co-loaded into the lipid phase to enable NIR tracking of NP biodistribution[14]. Antibody loading efficiency is quantified using the BCA assay, and stability is assessed by monitoring antibody release after 1/3/5 days at 37 °C[15]. Successful conjugation is confirmed by SDS-PAGE showing characteristic IgG heavy and light chain bands (~50/25 kDa) [16]. A slight increase in hydrodynamic diameter (~5–20 nm) with minimal change in PDI further indicated effective conjugation and good colloidal stability. To further understand the physical properties of nanoparticles coupled with antibodies, we employ DLS and Zeta potential analysis. The experimental procedures are consistent with those in 2.2. Replicate the experiment three times and take the final average value.

2.6. TRPM2 Targeting and Cellular Uptake Assay

Select PANC-1 pancreatic cancer cells with high TRPM2 expression as a model. Cells are seeded in 24-well plates at a rate of 1×10^5 cells per well and incubated at 37 °C and 5% CO_2 for 24 hours. Subsequently, Alexa Fluor 488 labelled TNP-TRPM2-Ab (75/300 μg mL^{-1}) is added and incubated for 0.5 h and 2 h, respectively[17]. A confocal microscope is used to observe the spatial localization image of the fluorescence. Flow cytometry is used to quantitatively analyse cellular uptake efficiency[18]. The cells are washed twice with PBS, trypsinized, centrifuged and resuspended in PBS. The mean fluorescence intensity (MFI) is recorded to compare the uptake levels at different concentrations and treatment times[19].

2.7. In Vitro Functional Studies

2.7.1. Evaluation of Cytotoxicity and Dose–Response Relationship of TNP-TRPM2-Ab

The PANC-1 cell line is used for *in vitro* toxicity testing. Gemcitabine is set as the positive control, PBS as the negative control. Cells are seeded in 96-well plates and incubated overnight at 37 °C with 5% CO_2. Subsequently, they are treated with 10-1000 nM drugs for 12-72 hours separately. After adding CCK-8 reagent and incubating for 1-4 hours, the absorbance is measured at 450 nm. Each group is replicated three times. The drug concentration and cytotoxicity curves were plotted based on the experimental results.

2.7.2. Assessment of Anti-Invasion and Anti-Migration Activities via Transwell Assay

The cell migration and invasion abilities are evaluated using the Transwell Assay[20]. After pre-culturing the cells in serum-free medium for 6 hours, they were inoculated into the upper chamber with an 8 μm pore size membrane. The lower chamber contains 10% FBS medium. After incubation for 24 hours, the cells were fixed and stained with crystal violet, and the number of migratory/invasive cells was counted under a microscope.

2.7.3. Inhibition of MEK/ERK Pathway by Nanoparticles Evaluated through Western Blot

ERK and p-ERK expression is detected by Western blot to evaluate the inhibitory effect of TNP-TRPM2-Ab on the MAPK/MEK/ERK signalling pathway. U0126 serves as a positive control for MEK1/2-specific inhibitors[21]. After treatment for 12, 24, and 48 hours, cells are lysed, proteins are extracted and quantified. After separation by SDS-PAGE, they are transferred to membranes, incubated with anti-pERK/anti-ERK/β-actin antibodies, respectively[22]. Chemiluminescence development and the p-ERK/ERK ratio are analysed using Image Lab.

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2.8. In Vivo Studies

Prepare 9 female mice. Anesthetize the mice with 3% isoflurane, and then subcutaneously inject PANC-1 cells into the backs of the mice [23]. Ten days later, the mice are randomly divided into four groups: 1) TNP-TRPM2-Ab; 2) Trametinib; 3) Gemcitabine (positive control); 4) PBS (negative control). The dosage is set at 1/5/10/15 mg/kg. The length (L) and width (W) of the tumour are measured with vernier calipers every day, and the volume $V = (L \times W^2)/2$ is calculated. Changes in body weight and food intake are also recorded. CO_2 euthanasia is performed at preset time points (3/6/10 days). The tumour and major organs (liver, kidney, heart, and spleen) are removed for subsequent analysis. To evaluate the *in vivo* distribution, TNP-TRPM2-Ab is co-encapsulated with DiR during the formulation stage (see 2.3), and near-infrared fluorescence in vivo imaging is performed at 0.5/2/4/8/24 h after the first dose to compare the fluorescence signal and tissue distribution at different time points. To evaluate systemic toxicity and histological changes, HE staining is performed on major organs. Tumour cell apoptosis is assessed using TUNEL method[24], and tumour tissue proteins are extracted for Western Blot, p-ERK and total ERK levels (p-ERK/ERK ratio) are detected to determine the inhibition of the MEK/ERK pathway.

2.9. Statistical Analysis

All experiments are replicated in triplicate, and data are expressed as mean ± SD. Unless otherwise stated, statistical differences between groups are analysed by one-way ANOVA followed by Tukey's post hoc test. A p value < 0.05 is considered statistically significant.

3. Results

Table 1. The combination of possible results.

Combination Results # (CR#)	TNP-*TRPM2*-Ab decreased viability by colony formation assay compared to Trametinib alone.	TNP-*TRPM2*-Ab decreased tumour size by weight compared to Trametinib alone.	TNP-*TRPM2*-Ab decreased ERK phosphorylation (p-ERK) by WB compared to Trametinib alone.	Support of the hypothesis
CR1	+	+	+	Full
CR2	+	+	-	Partial
CR3	+	-	+	Partial
CR4	+	-	-	Partial
CR5	-	+	+	Partial
CR6	-	+	-	Partial
CR7	-	-	+	Partial
CR8	-	-	-	Fully Contradicts

Table legend: The "+" indicates that the observed result in the experiment is the same as that of the positive control group (gemcitabine / U0126) and is opposite to that of the negative control group (PBS) and has statistical significance. It is worth noting that the positive control may vary in different experiments. In the Western blot, the positive control is U0126. The other positive controls are all gemcitabine. The "-" means that the phenomenon observed in the experiment is different from that of the positive control group, and is the same as, or different from, both the negative control group and the positive control group or has no statistical significance.

As can be seen in table 1:

CR1: If I observe that, compared to Trametinib alone, TNP-TRPM2-Ab decreases colony-formation viability, decreases tumour size by weight and decreases p-ERK by Western blot. This situation fully supports my hypothesis.

CR2: If I observe that, compared to Trametinib alone, TNP-TRPM2-Ab decreases colony-formation viability and decreases tumour size by weight. However, p-ERK does not decrease. This situation partially supports my hypothesis.

CR3: If I observe that, compared to Trametinib alone, TNP-TRPM2-Ab decreases colony-formation viability and decreases p-ERK, however tumour size by weight does not decrease, this situation partially

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supports my hypothesis.

CR4: If I observe that, compared to Trametinib alone, TNP-TRPM2-Ab decreases colony-formation viability, however neither tumour size by weight nor p-ERK decreases, this situation partially supports my hypothesis.

CR5: If I observe that, compared to Trametinib alone, TNP-TRPM2-Ab does not decrease colony-formation viability, but decreases tumour size by weight and decreases p-ERK, this situation partially supports my hypothesis.

CR6: If I observe that, compared to Trametinib alone, TNP-TRPM2-Ab does not decrease colony-formation viability and p-ERK does not decrease, but tumour size by weight decreases, this situation partially supports my hypothesis.

CR7: If I observe that, compared to Trametinib alone, TNP-TRPM2-Ab does not decrease colony-formation viability, and tumour size by weight does not decrease. But p-ERK decreases. This situation partially supports my hypothesis.

CR8: If I observe that, compared to Trametinib alone, TNP-TRPM2-Ab does not decrease colony-formation viability, tumour size by weight does not decrease and p-ERK does not decrease. This situation fully contradicts my hypothesis.

4. Discussion

This study aims to verify whether the TNP-TRPM2-Ab has superior anti-tumour efficacy with reduced cytotoxicity compared to Trametinib alone. The results are evaluated through three aspects: colony formation ability, tumour volume and p-ERK phosphorylation. In the results section, I design 8 possible combinations (CR1 – CR8). The following provides a more detailed discussion of these hypotheses.

CR1 fully supports the hypothesis. It indicates that targeted NP delivery of Trametinib not only inhibits the vitality of tumour cells but also reduces tumour size, effectively blocking the MEK/ERK signalling pathway. This suggests that this NP delivery system has high therapeutic efficacy and correct pathway targeting. In subsequent experiments, it is advisable to increase the s ample size of the animals to verify the reproducibility and statistical significance of this result. The treatment duration of the *in vivo* experiments can be extended to test the safety of TNP-TRPM2-Ab in long-term use. A more detailed dose gradient setting can be added to establish PK/PD curves, thereby enabling the analysis of optimal dosage. Additionally, models with low expression or knockout of *TRPM2* can be introduced to verify whether TNP-*TRPM2*-Ab indeed relies on *TRPM2* targeting.

In **CR2**, TNP-TRPM2-Ab decreases colony-formation viability and reduces tumour size by weight, but p-ERK does not decrease, this provides partial support for the hypothesis. Its mechanism of action does not conform to the original assumed pathway. Firstly, it is considered that the drug concentration may be too low to reach the effective threshold required by p-ERK. Secondly, the p-ERK is not fixed and remains unstable. An unreasonable experimental time setting may miss the lowest inhibition point of pERK, resulting in inaccurate results. Although a decrease in p-ERK is observed, the reduction in tumour size and colony formation indicates that other signalling pathways may have intervened in the anti-cancer process[25]. Future experiments can set more detailed concentrations and times for WB detection to observe their dynamic changes.

In **CR3**, TNP-TRPM2-Ab decreases colony-formation viability and p-ERK, but tumour size does not decrease. This also provides partial support, suggesting that this may be due to the drug delivery efficiency or an insufficient drug concentration[26]. In future studies, a concentration gradient (e.g., 5, 10, 20, 30, 40, 50, 100, 200 nM) can be refined for further investigation. The treatment time (12, 24, 48 h) can be extended to draw concentration-effect and time-effect curves, thereby providing a more intuitive exploration of the optimal concentration and time[26]. In addition to the optimization design of nanoparticles, improving the therapeutic specificity of CR6 [27].

In **CR5**, TNP-TRPM2-Ab does not decrease colony-formation viability, yet tumour size and p-ERK both decreases. This provides partial support. This may be related to the unreasonable experimental concentration and time settings. In future research, a more detailed concentration gradient (e.g., 5, 10, 20, 30, 40, 50, 100, 200nM, etc.) and an extended treatment time (12, 24, 48 h) can be used to draw concentration-effect and time-effect curves, to explore the optimal concentration and time intuitively[26].

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For **CR4**, **CR6** and **CR7**, results show significant effects in only one parameter, viability by colony formation assay, tumour size, or p-ERK phosphorylation, which provide limited support for the hypothesis. In CR4, the decrease in cell viability without corresponding reductions in tumour size or p-ERK levels may indicate that TNP-TRPM2-Ab induces cytotoxic effects *in vitro* but fails to achieve sustained *in vivo* target inhibition. Improving tumour accumulation/release kinetics is warranted[28] In CR6, the reduction in tumour size without changes in cell viability or p-ERK phosphorylation may reflect indirect anti-tumour effects unrelated to the MEK/ERK pathway. Multi-pathway Western Blot detection should be used to analyse other possible signalling pathways involved in pancreatic cancer cell formation. In CR7, the reduction in p-ERK without corresponding reductions in tumour size or colony formation. It might be due to the short treatment period (such as only lasting 3-5 days) or the low frequency of administration, which failed to accumulate sufficient cytotoxicity. In future studies, a concentration gradient (e.g., 5, 10, 20, 30, 40, 50, 100, 200 nM) can be refined for further investigation. The treatment time (12, 24, 48 h) can be extended to draw concentration-effect and time-effect curves, thereby providing a more intuitive exploration of the optimal concentration and time[26].

For **CR8**, compared with Trametinib, TNP-*TRPM2*-Ab does not show any reduction in colony formation, tumour size or p-ERK level. The results are completely contradictory to the hypothesis. This suggests that there are significant issues with this nanomedicine system, including unsuccessful drug delivery, failure to release, low targeting binding efficiency, or unreasonable treatment concentration and time settings[26]. Firstly, the concentration setting of the drug may be the most significant issue[26]. Suppose the concentration is too low, even if TNP-*TRPM2*-Ab successfully reaches the target cells[26]. In that case, it will not trigger a signal-level response, nor will it produce an anti-tumour effect at the tissue level. Additionally, since Trametinib is hydrophobic, if the release is incomplete or too slow, the actual effective concentration will also be too low, leading to the non-activation of the signalling pathway. Secondly, the setting of the treatment time may also be a key factor in the failure[26]. For example, if there is a lag effect in drug release, but the detection time point is set too early, the drug efficacy cannot be fully exerted. In future research, a more detailed concentration gradient (e.g., 5, 10, 20, 30, 40, 50, 100, 200 nM, etc.) and an extended treatment time (12, 24, 48 h) can be used to draw concentration-effect and time-effect curves, to explore the optimal concentration and time intuitively. Moreover, this system may also have fundamental problems in terms of targeting ability or structural design. For example, the coupling efficiency of the anti-*TRPM2* antibody is low, resulting in failed targeting binding. Alternatively, there may be insufficient nanoparticle size to enter tumour tissues, among other shortcomings in nanoscale design[29]. A combination of experimental methods, such as SDS-PAGE, the BCA method, and confocal microscopy, is recommended to verify whether the antibody is successfully coupled. Additionally, introducing *TRPM2*-low-expression or knockout cell lines as controls can be used to determine whether this system truly relies on *TRPM2* for its function.

5. Conclusions

This study designed and verified a novel *TRPM2*-targeted nanomedicine delivery system (TNP-TRPM2-Ab) and systematically evaluated its efficacy, safety, and mechanism of action *in vitro* and *in vivo* studies. More importantly, this study is the first to systematically explore the TRPM2-mediated targeted drug delivery mechanism in pancreatic cancer models, thereby filling the research gap in this field and providing new insights for improving the low specificity and high toxicity issues associated with the clinical application of MEK inhibitors. Further work can expand the sample size, optimise the dosage design, and introduce *TRPM2* knockout models to verify its targeted dependence on targeting more deeply. Overall, this study provides a theoretical basis and experimental foundation for future individualised and precise treatment strategies for pancreatic cancer.

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