The Improved Anticancer Activities of Piperine Nanoliposome Conjugated CD133 Monoclonal Antibody Against NTERA-2 Cancer Stem Cells

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Abstract

Black pepper (Piper nigrum L.) is widely grown in the Chu-se district of Gia Lai province in Vietnam. The pepper, used as a spice, also serves as a traditional medicine in many countries. Black pepper contains many different substances; the most active of these is piperine, which exerts anti-oxidant, anti-inflammatory, and anti-cancer effects. However, piperine is a poorly absorbed alkaloid, and high concentrations may be toxic. Therefore, its medicinal uses remain limited. Here, we extracted piperine from black peppers collected at Chu Se, created piperine and anti-CD133 monoclonal antibodies (mAb^CD133) containing nanoliposomal complexes (PMCs), and evaluated their inhibitory effects on cancer stem cells (CSCs) in vitro. The physical properties of PMCs showed an approximated diameter of 170 nm, a PDI of 0.23, zeta potential of −9.38 mV, and an encapsulated efficiency of 73.33 ± 9.09%. The PMCs significantly inhibited NTERA-2 cell growth (IC₅₀ = 435.3 ± 4.3 µM), but were not toxic to healthy cells (IC₅₀ >500 µM). The PMCs remarkably affected the CSC surface marker expressed level of which the CD44⁺/CD133⁺ population was only 2.12% compared with 21.72% for blank nanoliposomes. The NTERA-2 antiproliferative activities of PMCs might be associated with their G2 cell cycle phase arresting and caspase-3 inducible activities (up to 1.51 times) (P < 0.05). The nanoliposomal complex also significantly inhibited the proliferation of NTERA-2 cells in three dimensional tumorspheroids with an IC₅₀ = 245.82 ± 11.44 µM and reduced the size by up to 41.50 ± 4.31% (P < 0.05). Thus, the PMCs proved their enhanced potential biomedical and pharmacological applications in targeted cancer therapies.

Keywords
cancer stem cells, CD133, monoclonal antibody, NTERA-2, piperine, PMCs
water-soluble, high concentrations are required for efficacy, and there are many side-effects. Pychaturawat reported that piperine at 300 mg/kg body weight was lethal to mice within 7 days, while piperine at 514 mg/kg caused respiratory failure within 1-3 minutes. Therefore, piperine use remains limited.

Nanoliposomes have served as carriers of drugs that retard tumor growth, and exhibit several advantages compared with conventional delivery systems. Clevers (2011) found that CSCs play important roles in drug resistance, metastasis, recurrence, and cancer treatment efficacy. As reported, CD133 is a common surface marker of CSCs in solid tumors. This biomarker was also recognized to play an important role in cancer metastasis, renewal, recurrence, and drug resistance. Therefore, in this study, we isolated and purified piperine from black pepper seeds harvested in the Chu-se area of Gia Lai Province in Vietnam. Then, pure piperine was entrapped in nanoliposomes to increase its bioavailability, and combined with monoclonal antibodies against CD133 (mAb^CD133). Our aim was to explore the possible anticancer medicinal utility of piperine-mAb^CD133 conjugated nanoliposomes (PMCs).

Material and Methods

Material

Black pepper seeds (Piper nigrum L.) were harvested at Chu-se district, Gia Lai province, Vietnam. The identity of the material was verified by botanist Dr Nguyen The Cuong of the Institute of Ecology and Biological Resources, VAST.

Pure piperine was isolated from the dried black pepper seeds. DMEM, fetal bovine serum (FBS), human CD133 monoclonal antibody conjugated with PE (CD133-PE), human CD133 monoclonal antibody, human CD44 monoclonal antibody with FITC (CD44-FITC), and Qubit Protein Assay kits were purchased from Thermo Fisher Scientific (Invitrogen; Carlsbad, CA, USA); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[(amino(polyethylene glycol)-2000-maleimide] (DSPE-PEG-2000-mal) from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA); and all other chemicals from Sigma Aldrich (St. Louis, MO, USA).

Bangham Thin Film Method for Production of Piperine-mAb^CD133 Conjugated Nanoliposomes

Lipids, including phosphatidylcholine (PS), cholesterol, and DSPE-PEG2000-maleimide, were dissolved in dichloromethane. Piperine was then mixed with the above lipid mixture in a 10:1:10.5 mol ratio of PS:cholesterol:DSPE-PEG2000-maleimide:piperine. The solution was vacuum evaporated to remove solvent for creating a thin-film lipid layer. The solvent was then thoroughly dispatched by nitrogen gas flushing. Subsequently, the thin film layer was treated with phosphate buffer saline (PBS, pH = 7.2) containing mAb^CD133 (1 µg/mL) at 37 °C for 30 minutes using a very low speed vortex mixer. The obtained solution was sonicated at 2 rpm for 20 s, rested for 10 s, and this was repeated 10 times. The ultrasonic solution was centrifuged at 12000 rpm for 30 minutes to collect the nanoliposomes, followed by filtering through a 0.22 µm PVDF membrane. The supernatant was collected to measure uncaptured piperine, as well as unconjugated mAb^CD133.

Characterization of Piperine-mAb^CD133 Conjugated Nanoliposomes

The size (z-average), polydispersity indexes (PDI) and zeta potential of the nanoparticles were measured using a dynamic light scattering instrument (Zetasizer Nano-Z meter, Malvern Instruments, UK). The morphology of the obtained nanoparticles was observed by field emission scanning electronic microscopy (FESEM) (HITACHI S-4800 FESEM system).

Determination of Encapsulated Efficiency (EE)

Piperine integrated efficiency: Five mL supernatant was used to quantify the unconjugated piperine using an HPLC method with a suitable calibration curve. The encapsulated efficiency was calculated with the following formula: EE (%) = 100% - [(weight of unconjugated piperine)/(weight of initial piperine)] × 100.

Antibody conjugated efficiency: One mL of the supernatant was used to quantify the amount of unbound antibody using a Qubit Protein Assay kit by strictly following the guidance of the producer (Thermo Fisher Scientific, USA). The encapsulated efficiency was determined from the following equation: EE (%) = 100% - [(amount of unconjugated mAb^CD133)/(amount of initial mAb^CD133)] × 100.

Cell Lines and In Vitro Culture

NTERA-2 (pluripotent human embryonic carcinoma) cells and CCD-18Co cells (human colon normal) were kindly provided by Prof. CY Huang, Institute of Biopharmaceutical Sciences, National Yang Ming University, Taiwan and Prof. P. Wongtrakoongate, Mahidol University, Thailand. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, 100 IU/mL penicillin, and 2 mM L-glutamine. All cells were maintained in a humidified incubator with 5% CO2 at 37 °C.

MTT Anti-Proliferative Assay

The in vitro cytotoxic activities of the tested samples against NTERA-2 and CCD-18Co cells were evaluated by MTT anti-proliferative assays, as previously described.

Determination of Cellular Uptake Capacity of Piperine-mAb^CD133 Conjugated Nanoliposomes (PMCs)

NTERA-2 cells were seeded into wells of a 6-well plate (5 × 10⁴ cells/mL) and cultured for 24 hours in an incubator before
The samples were added. The study samples, including blank nanoliposomes, PMCs and PBS (pH = 7.2), were added to the cell seeded wells for 3 hours before being removed. The treated cells were then washed with PBS (pH = 7.2) and detached from the bottom of the wells using EDTA (1 mM). Treated cells were then collected into sterilized Eppendorf tubes and analyzed using a Novocyte flow cytometry system and NovoExpress software (ACEA Bioscience Inc.).

**Determination of PMCs’ Effects on CSC Surface Markers and Cell Cycle**

NTERA-2 cells were seeded into 6-well plates at $5 \times 10^4$ cells/mL for 24 hours, followed by treatment with the study samples, and a further 24 hours of incubation. Then, the cells were detached from the well bottom using EDTA 1 mM, and centrifuged at 1000 rpm for 5 minutes to obtain the cell pellets.

To examine the expression of CSC surface markers, CD44 and CD133 markers were employed. The prepared cell pellet was resuspended in DMEM containing FBS (1%), CD44-FITC and CD133-PE antibodies at 4 °C for 10 minutes, protected from light. The expression of cellular surface markers was analyzed using the flow cytometry Novocyt system and NovoExpress software (ACEA Bioscience Inc.).

To analyze the cell cycle, the treated cells were harvested and washed with cold PBS 1X, and fixed with 70% ethanol at 4 °C for 2 hours. The fixed cells were washed with cold PBS 1X twice, incubated with RNase A (1 mg/mL) in a 37 °C water bath for 15 minutes, and then stained with propidium iodide—PI (1 mg/mL) for 1 hour. The cell cycle arrest activities were determined by analyzing 10,000 cells/sample using the flow cytometry Novocyte system and NovoExpress software (ACEA Bioscience inc.).

**Determination of Caspase 3 Inducible Activity**

The caspase 3 induced activity of nanoliposomes was clarified using a Caspase-3 Colorimetric Assay Kit (Biovision Inc.), as previously described.14

**Determination of 3D Tumorspherical Inhibitory Effects of PMCs**

3D tumorspheres of NTERA-2 cells were produced using the hanging drop method, as detailed previously.13 In brief, NTERA-2 cells (1500 cells) in 20 µL medium were dropped onto the bottom of the lid of a 60 mm tissue culture dish. The lids were then inverted onto 5 ml medium filled bottom dishes and further incubated at 37 °C in 5% CO$_2$ at 95% humidity. After 3 days of incubation, the cells assemble into a single spherical body. Cell growth in the spheroids under the influence of the study samples was measured using a MTT dye protocol and compared with the negative control. The growth areas of spheroids were also analyzed using ImageJ software (NIH, Bethesda, MD, USA).

**Table 1. Characteristics of the Obtained PMCs and Other Nanoliposomes.**

| Types                  | Piperine-mAb^CD133 nanoliposome (PMCs) | Piperine nanoliposome | Blank nanoliposome |
|------------------------|----------------------------------------|-----------------------|--------------------|
| Size (nm)              | 171.2 ± 15.2                           | 175.2 ± 10.9          | 130.0 ± 12.7       |
| PDI                    | 0.23 ± 0.03                             | 0.32 ± 0.04           | 0.26 ± 0.03        |
| Zeta potential (mV)    | −9.38 ± 0.72                            | −13.81 ± 1.22         | −12.27 ± 1.04      |
| EE (%)                 | 73.33 ± 9.09                            | 85.41 ± 9.22          | -                  |

*Note.* Blank liposome including PC, cholesterol and DSPE-PEG2000.
significant inhibitory activity toward CSCs with an IC₅₀ (inhibitory concentration at 50%) value of 435.3 ± 4.3 µM, compared to that of piperine free in water (IC₅₀ >500 µM). The complex also presented a less toxic effect to healthy cells (17.77%) at the highest treated concentration in comparison with that of free piperine (in DMSO). As such, PMCs reflected their successful CSC targeting and improved bioavailability. Wang found that chimeric antigen receptor-directed CD133 therapy for patients with hepatocellular carcinomas and colorectal cancers was effective in clinical trials.15 From this study, we also realized that the PMCs inhibited CSCs more effectively than did unconjugated piperine. Placement of piperine within liposomes bearing anti-CD133 mAbs increased piperine bio-availability and improved targeting. Further molecular and preclinical experiments are required for validation of these results (Figure 1).

Cellular Uptake Capacity of Piperine-mAb^CD133 Conjugated Nanoliposomes (PMCs)

After PMCs were produced and evaluated for cytotoxic activities, the complex was examined for its cellular uptake capacity on NTERA-2 cells. The uptake rate was 13.8%, whereas those of the negative control and blank were only 1.47% and 1.30%, respectively.

Table 2. Cell Growth Inhibitory Activity of Studied Samples.

| Conc. of piperine (µM) | Nanoliposome—blank | Piperine—DMSO | PMCs | Piperine—water |
|-------------|---------------------|---------------|------|----------------|
| 500         | CCD-18Co NTERA-2    | CCD-18Co NTERA-2 | CCD-18Co NTERA-2 | CCD-18Co NTERA-2 |
| 100         | 6.77 ± 0.35 7.47 ± 0.77 | 40.41 ± 0.37 67.99 ± 5.95 | 17.77 ± 3.03 55.02 ± 1.29 | 11.79 ± 2.67 −0.25 ± 0.04 |
| 20          | 2.13 ± 0.13 4.53 ± 0.57 | 5.82 ± 0.49 22.97 ± 1.53 | −0.47 ± 0.25 16.74 ± 1.80 | 4.72 ± 2.00 3.37 ± 0.21 |
| 4           | 0.65 ± 0.07 1.44 ± 0.18 | 2.04 ± 0.18 3.68 ± 0.26 | −2.04 ± 0.37 −0.72 ± 1.30 | 1.94 ± 0.67 −4.29 ± 0.14 |
| IC₅₀        | >500                | >500           | >500             | >500            |

Figure 1. FE-SEM morphological images (HITACHI S-4800, Japan) of (A) piperine and mAb^CD133 conjugated nanoliposomes (EE = 73.33 ± 9.09%); (B) piperine entrapped nanoliposomes (EE = 85.41 ± 9.22%); (C) Blank nanoliposomal particles.

Figure 2. Cellular uptake capacity of (A) normal saline buffer, which served as the negative control; (B) blank nanoparticles; (C) piperine-mAb^CD133 conjugated nanoliposomes, tested on NTERA-2 cells (5 × 10⁴ cells/mL) after 24 hours of treatment and analyzed using Novocyte flow cytometry system and NovoExpress software.
respectively (Figure 2). The results showed that PMCs have an efficient capacity to enter NTERA-2 CSC cells.

Effects on NTERA-2 Surface Markers and Cell Cycle of Piperine-mAb\(^{\text{CD133}}\) Conjugated Nanoliposomes (PMCs)

Also, the CSCs typical surface markers, such as CD44 and CD133, under PMCs treatment were assessed. After 24 hours of exposure, the proportion of cells expressing CD44\(^{-} /\) CD133\(^{+}\) (2.12%) was much lower than that of the negative control (21.72%), and somewhat less than after treatment with piperine alone (5.18%) (Figure 3).

CSCs express unique surface membrane proteins, including CD44 and/or CD133.\(^{16-18}\) These antigen factors could assist CSCs to prevent apoptosis. Activated T cells (ATCs) with 2 specific antibodies (MS133 cells) exerted possible anti-tumor effects in vitro and in vivo by targeting these proteins.\(^{19}\) In our research, the PMCs simultaneously reduced the expression levels of CD44 and CD133, which may indicate that they target these unique antigens of NTERA-2 cells. The molecular mechanisms by which CD44/CD133 markers assist hepatic CSCs to evade therapy (including the auto-resistance induced by interferon-gamma) have been explored.\(^{20}\) Ding also reported that CD133 on liver cancer cells counteracted apoptosis by affecting the levels of transforming growth factor \(\beta\) (TGF-\(\beta\)).\(^{21}\) As shown in Figure 4, PMCs induced a 1.51 fold change of caspase 3. Together with this nanocomplex effect on strongly decreasing the expression of CD44\(^{-} /\) CD133\(^{+}\) level compared to the control on NTERA-2 cells, the PMCs might help to inhibit the ability of cancer stem cells to resist apoptosis. Therefore, the nanopiperine-antibody complex significantly reduced the potency of CSCs.

In one study, Siddiqui found that piperine suppressed growth by modulating the mitochondrion-mediated apoptosis that induces ROS synthesis and cell cycle arrest.\(^{4}\) Jafri also reported that growth inhibition of Hela cells under piperine treatment was related with G2/M phase arrest and sub-G1 accumulation.\(^{22}\) From this study, PMCs at 20 \(\mu\)M again presented their effects on the NTERA-2 cell cycle with G2/M arrest (31.96%), while nanoliposome piperine showed its effect on G1 arrest (Figure 5).

**PMCs Inhibited the In Vitro Growth of NTERA-2 Tumorspheroids**

We formed 3D tumorspheres using NTERA-2 cells. The proliferation of cells inside the tumorspheres was assessed by MTT assay, while the 3D tumor areas were analyzed using ImageJ software. Under the treatment of PMCs, the cell proliferation inside 3D tumors was strongly inhibited (Figure 6); the IC\(_{50}\) of PMCs was 245.82 ± 11.44 \(\mu\)M, while that of nanoliposome piperine was 281.72 ± 5.67 and of piperine (in DMSO) 301.99 ± 15.85 \(\mu\)M. These results show that PMCs possessed significantly stronger activities on inhibition of NTERA-2 cells in 3D tumorspheroids. Accordingly, after 3 days of incubation, the sphere area in wells with PMCs was only 58.50 ± 4.31% compared with that of the control, meaning approximately 41.50% inhibition. In wells with nanoliposome piperine, cell
growth in tumorspheres was only slightly inhibited (82.65 ± 2.63%). Thus, PMCs with piperine and mAb^CD133 significantly inhibited 3D tumorspherical growth. The inhibitory effect of PMCs (41.50%) was better than that of nanopiperine without the CD133- mAb (17.35%).

Conclusions

We fabricated a nanoliposomal complex of piperine and an anti-CD133 monoclonal antibody (PMCs). The obtained nanoparticles were about 100-200 nm in diameter, with a PDI of 0.23, a zeta potential of −9.38 mV, and an encapsulated efficiency of 73.33 ± 9.09%, while mAb^CD133 was supposed to be fully conjugated. The PMCs inhibited the CSC growth rate by 55.02%, and that of healthy cells by 17.77%. The PMCs remarkably affected the CSC surface marker expressed level with CD44^+/CD133^+ populations (2.12%) compared with that of blank nanoliposome (21.72%). The nanoliposomal complex also inhibited the proliferation of NTERA-2 cells in tumorspheroids with an IC_{50} = 245.82 ± 11.44 µM and reduced the size by up to 41.50 ± 4.31%. The PMCs showed effects on the NTERA-2 cell cycle by arresting at the G2 phase (31.96%) and induced caspase-3 activity by up to 1.51 times. Piperine is poorly absorbed, and may not be effective unless delivered in a targeted manner. Our CSC-targeting fabricated nanoliposomal method significantly increased the bioavailability of piperine.

Declaration of Conflicting Interests

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