A combined miR-34a and arsenic trioxide nanodrug delivery system for synergistic inhibition of HCC progression after microwave ablation

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Abstract

Background

Microwave ablation (MWA) has become an alternative treatment for unresectable surgery for hepatocellular carcinoma (HCC), but it still faces the risk of recurrence and metastasis after treatment. Recent studies have found that miR-34a presents decreased gene expression in residual tumours after ablation therapy and can increase the therapeutic effect of arsenic trioxide on HCC, which brings new opportunities for its treatment.

Methods

A pH-sensitive charge inversion material was used to construct a nanotargeted delivery system that presents the synergistic effect of miR-34a and As₂O₃. We established in vitro and in vivo models of microwave ablation of HCC and performed in-depth research on dual drug systems to inhibit the rapid progression of HCC after microwave ablation in cell pyroptosis.

Results

The antitumour effect was enhanced with double-drug nanoparticles relative to a single formulation, and the therapeutic efficacy of the nanoparticles was more significant in a weakly acidic environment. Dual-drug nanoparticles could increase the N-terminal portion of GSDME, decrease the expression of Cyt-c and c-met.

Conclusions

Dual-drug nanoparticles may improve the therapeutic efficacy of HCC with insufficient ablation through Cyt-c and GSDME-N and decrease the expression levels of c-met. It is expected to provide new treatment methods for residual liver cancer after microwave ablation, prolong the survival of patients and improve quality of life.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours and presents rapid development and a poor prognosis^[1]. Surgical treatment is unfit for most patients, while microwave ablation (MWA) may be suitable for unresectable hepatocellular carcinoma^[2–6].

MWA is effective for smaller tumours, whereas insufficient ablation leaves residual tumours that develop into larger tumours^[2]. In addition, tumours adjacent to important structures are more frequently incompletely ablated to prevent damaging normal structures. Therefore, insufficient tumour ablation

limits the efficacy of MWA in the clinic. Studies have shown that there are many factors contributing to the rapid progression of residual tumours after incomplete thermal ablation, such as an abnormal miRNA regulatory networks, autophagy regulatory disorders and tumour-infiltrating T cell failures^[7–9].

Abnormal expression of key miRNAs in HCC is closely related to the malignant biological behaviours of tumours, among which a low expression level of miR-34a is associated with poor prognosis of HCC. Studies have found that the expression level of miR-34a in residual tumours after ablation is downregulated^[7]. In the pathological environment of HCC, miR-34a activation is blocked, thus leading to decreased miR-34a expression and subsequent cancer development. Restoration of the decreased miR-34a may be an alternative therapy for systemic treatment of HCC. miR-34a in combination with chemicals could achieve better efficacy, and this treatment has gained much attention^[10, 11].

In vivo, miRNAs are easily metabolized by nucleases in plasma and difficult to transport through membranes; therefore, their interference efficiency on genes is low. The above problems hamper the therapeutic efficacy of miRNAs. miRNA delivery carriers depend on high binding affinity for miRNA during circulation to prevent degradation, while the rapid release of miRNA into the cytoplasm triggers gene silencing. Nanoparticles with near neutral surface charge have favourable pharmacokinetic and biological distribution after systemic injection. At present, there are few studies on RNA delivery systems with nearly neutral zeta. miRNA-DSPE has disulfide bond binding between miRNA-DSPE in the shell, and at the same time, it is shielded by PEG to prevent nuclease degradation in the physiological environment^[12–14]. The combination of pH- and GSH-sensitive bonds between DSPE and drugs is beneficial to drug delivery to control drug release and improve efficacy. Redox potential has become an effective stimulant of intracellular drug release due to the significant difference in glutathione (GSH) concentration between cancer cells and normal extracellular matrix. In addition, combining PEGyl-based polymers was helpful for their long circulation and aggregation in tumour sites.

The main form of arsenic trioxide in water is arsenite ($\text{As}(\text{OH})_3$), and its trivalent arsenic exerts a key role in antitumour efficacy. Currently, arsenic trioxide is mainly loaded in liposomes^[15]. The tumour site is in a weak acid environment; thus, materials with charge reversal used in a weakly acidic environment could be prone to accumulate in the tumour through EPR and further improve the efficacy. PH-sensitive charge reversal polymers based on PAE poly(-amino ester) provide effective drug delivery and control of drug release depending on the pH gradient^[16–19].

This research intends to construct a nanodelivery system with a synergistic effect from carrying As_2O_3 and miR-34a to inhibit tumour progression after microwave ablation. The nanodelivery system was prepared using amphiphilic triblock copolymer (mPEG-PLA-PAE), miR-34a-DSPE, mPEG-DSPE and arsenic trioxide using double emulsion methods. Hydrophilic mPEG fragments form protective shells on the outside of nanoparticles, thus providing a stable drug delivery system and extending the circulation time in the body. Hydrophobic PAE segments ($\text{pH} > \text{pK}_a$) contribute to increased micelle stability, and hydrophilic PAE segments with high positive potential ($\text{pH} < \text{pK}_a$) can increase cell internalization and uptake. We used sublethal cancer cells to mimic insufficient ablation to conveniently detect the efficacy.

We believe that these particles hold great promise for clinical translation to inhibit the progression of HCC ablation.

2. Materials And Methods

mPEG-PLA-PAE was obtained from Xi'an Ruixi Biotechnology Co., Ltd. DSPE-PDP was purchased from Shanghai Langxu Biotechnology Co., Ltd. mPEG-DSPE was provided by Shanghai Fansuo Biotechnology Co. Ltd. [Arsenic trioxide](#) was kindly provided by the Chinese Academy of Sciences. RIPA lysis buffer was purchased from Beyotime Institute of Biotechnology. Monoclonal antibodies against GSDME, GPX4, and Cyt-c were obtained from Abcam. Monoclonal antibodies against GAPDH and Caspase3 were purchased from Cell Signaling Technology. β -actin-HRP and Cyt-c were obtained from Proteintech.

Preparation of As/miR-34a-mPEG-PLA-PAE nanoparticles

mPEG-PLA-PAE NPs were prepared as previously described. As/miR-34a NPs (As/miR-34a-mPEG-PLA-PAE nanoparticles) were prepared by a double emulsion method as follows. miR34a-SH is conjugated to DSPE-PDP as previously reported. Briefly, a 0.1 ml aqueous solution of As_2O_3 (1 mg/ml) and a 0.2 ml DSPE-miR34a (20 mg/ml) were emulsified in 0.5 ml of dichloromethane containing 20 mg mPEG-PLA-PAE and 4 mg mPEG-DSPE copolymer using an ultrasonic processor at 400 W for 2 min. Next, the water/oil (w/o) emulsion was added to 1.7 mL of pluronic F68 water solution (1 mg/mL) and the mixture was sonicated at 400 W for 2 min. Subsequently, the resulting w/o/w emulsion was stirred at room temperature to evaporate the organic phase.

Characterization of nanoparticles

Nanoparticle morphology at pH 7.4 and pH 6.5 was observed by transmission electron microscopy (TEM). The potential of nanoparticles under different acidities (pH=7.4, 6.5, 5.5) was determined by a Nano-ZS analyser. As/miR-34a NPs were incubated with GSH at pH 7.4 or pH 6.5, and then the DNA gel block method was used to determine miRNA stability after serum incubation. The film dialysis method was used to measure the amount of the drug released at different time points to evaluate the drug release properties as described in a previous report. Inductively coupled plasma mass spectrometry (ICP-MS) was used to determine the encapsulation efficiency and drug loading of As_2O_3 (As) according to a previous procedure.

Sublethal heat treatment HCC cell lines as an insufficient microwave ablation model

Huh7, PLC/PRF/5, and Hep1-6 cells at 5×10^5 cells/cm³ were inoculated into DMEM containing 10% foetal bovine serum (FBS) and cultured (37°C) overnight. Then, they were treated at 47°C for 10 minutes. After heating, DMEM was replaced three times a day until the 3rd day to remove debris and dead cells. Then, the cell number was adjusted to 1×10^6 on the 5th day after heating, the surviving HCC cells were subcultured into new culture dishes, and the cell lines were named H-Huh7, H-PLC, and H-Hep1-6. This

procedure was performed three times. c-Met expression was detected by western blot. H-Huh7, H-PLC, and H-Hep1-6 cells at 5×10^3 cells/cm³ in 96-well plates were cultured overnight according to a procedure similar to that for studying viability using the MTT method. As₂O₃ and miR34a (according to the agent procedure) were applied using the same methods for determining cell viability and c-met expression. Different concentrations of blank nanoparticles were added to LO2 human liver cells to determine viability using the MTT method.

As/miR-34aNPs inhibit the growth of HCC cell lines subjected to sublethal heat treatment

H-Huh7, H-PLC, and H-Hep1-6 cells were inoculated into 96-well plates at a fusion rate of approximately 50%. PBS, miR-34aNPs and AsNPs (As₂O₃NPs) were added to the culture at pH 7.4 for 48 h. As/miR-34aNPs were added at pH 6.5 and pH 7.4. Finally, MTT was added, and the cells were cultured for 4 h.

H-huh7 insensitivity to ferroptosis after treatment with As₂O₃

H-Huh7 cells were inoculated into 6-well plates at a fusion rate of approximately 50%. RSL3 and RSL3+Vit. E were added to the culture for 48 h and then observed by microscopy. H-Huh7 cells were treated with RSL3, RSL3+Vit. E, RSL3+DFOM, As, As+Vit. E, As+DFOM, RSL3, RSL3+As, and RSL3+As for 48 h. Then, the MTT method was used to detect cell viability as described above. GPX4 was detected after treatment with As₂O₃ and RSL3.

Cell uptake of nanoparticles in H-Huh7 and H-PLC

Cy3-labelled nanoparticles were prepared according to the procedure. Cy3NPs and free cy3 were inoculated into H-Huh7 and H-PLC cells in a normal physiological environment. Cy3NPs were inoculated into H-Huh7 and H-PLC at pH 6.5, further verifying that the acid-sensitive charge reversal mechanism was beneficial to cell uptake. Cellular uptake was observed by confocal microscopy.

Antitumour mechanisms of As/miR-34aNPs in vitro

H-Huh7, H-PLC, and H-Hep1-6 cells were inoculated into 6-well plates at a fusion rate of approximately 50%. PBS, miR-34aNPs and AsNPs (As₂O₃NPs) were added to the culture at pH 7.2 for 48 h. As/miR-34a@NPs were added at pH 6.5 and pH 7.2. The expression of C-met, caspase3, GSDME, Cyt-c, and cell medium Cyt-c was detected by western blot.

As/miR34aNPs trigger Cyt-c release

HEK293T cells were transiently transfected with GSN-GFP or GSC-GFP and then observed by fluorescence microscopy after treatment with As₂O₃ for 24 h. GSN-mCherry and GSC-mCherry were transiently transfected into HEK293T cells. Then, mitochondria and mCherry were observed by confocal microscopy, MitoTracker (Green) was used to stain mitochondria, and Hoechst 33342 was used to mark nuclei. H-Huh7 cells were treated for 48 h with PBS, AsNPs and miR34aNPs at pH 7.4 and As/miR34aNPs

at pH 7.4 and pH 6.5. The expression levels of Cyt c and Cyt c in the culture media (s-Cyt c) were detected by western blot.

Cell uptake of nanoparticles in vivo

Six-week-old BALB/c female nude mice were obtained from Slack Laboratory Animal Co., Ltd. (Shanghai, China). All animal experimental procedures were carried out according to protocols approved by the Animal Care and Use Committee of the Shanghai Institute of Biochemistry and Cell, Chinese Academy of Sciences. An insufficient microwave ablation HCC model was established by subcutaneous inoculation of the sublethal heat-treated HCC cell line H-Huh7 into nude mice. When the tumour grew to a size of 200 mm³, Cy3NPs were administered to mice via the tail vein and then the mice were sacrificed after 48 h. Tumour sections were prepared to observe the fluorescence distribution by confocal microscopy.

Antitumour therapy in an insufficient microwave ablation model

H-Huh7 suspension was inoculated on the right back of mice to establish a subcutaneous transplanted tumour model. When the tumours grew to 200 mm³, the mice were randomly divided into four groups with six mice per group. All miR-34a formulations were equivalent to 1 mg/kg body weight, and As formulations were equivalent to 0.5 mg/kg body. All formulations were administered intravenously once a week. Tumour growth was monitored and recorded by callipers eight times over three weeks. The tumour volume (V) was calculated based on the following formula: $V = [\text{major axis}(\text{minor axis})^2]/2$. Then, the mice were sacrificed and the tumours were excised and photographed. To evaluate the potential cytotoxicity of formulations in vivo, at the end of the treatment, serum biochemical analysis was carried out. Liver function was estimated with the serum levels of ALT, AST, and ALP. Kidney function was verified based on the serum levels of BUN and CRE.

Statistical analysis

All values were presented as the mean $\pm$ S.D. Statistical significance was determined by one-way analysis of variance using SPSS software (version 17.0, IBM Inc, Chicago, IL, USA). The differences were considered significant at $p < 0.05$ and highly significant at $p < 0.01$.

3. Results

3.1 Sublethal heat treatment model to mimic insufficient microwave ablation of HCC

First, heat treatment methods were performed to mimic insufficient microwave ablation of HCC. Cell growth after incubation for 48 h was examined by MTT. H-PLC and H-huh7 grew more rapidly than the wild type (Fig. 1A). Western blotting was used to detect the changes in c-met after sublethal treatment. c-met was upregulated in both cell types. Then, miRNA was added to regulate related gene expression. c-met was downregulated after treatment with miR34a for 48 h (Fig. 1B). The results showed that

compared with As or miR34a alone, the survival rate of H-huh7 and H-PLC in the As/miR34a group was significantly lower. The As/miR34a group had a better antiproliferative effect than the single formulation group, indicating that combination treatment may have a stronger antitumour effect (Fig. 1C). A previous report indicated that As₂O₃ triggers pyroptosis and induces GSDME cleavage (Figure S1). However, its insensitivity to ferroptosis in HCC is not very clear.

3.2 Characterization of As/miR34a@NPs

The morphology of the nanoparticles was observed by TEM. As shown in Fig. 2A, the nanoparticles were small and nearly spherical and had good dispersion. As shown in Fig. 2B, nanoparticles under normal physiological conditions showed a negative charge but shifted to a positive charge at pH 6.5, and arsenic was released rapidly in a slightly acidic environment (Fig. 2B-C). These results suggested that the drug delivery system has the potential to increase drug accumulation at tumour sites with long circulation and increase drug uptake in weak acid environments with charge reversal.

The release characteristics of the miRNA were similar to those of arsenic trioxide. These formulations slowed the degradation of miRNA in serum, and the miRNA was released rapidly under the action of 10 mM GSH (Fig. 2E). The acidic sensitivity of these NPs contributes to their accumulation in tumour tissues through the EPR effect in the circulation. Drug safety is an important aspect that must be evaluated to determine the potential clinical use of new therapies. The normal cell line LO2 has been widely used to detect toxicity. The viability of LO2 cells after treatment with blank nanoparticles for 48 h indicated that the blank nanoparticles had low toxicity to normal cells (Fig. 2D).

3.3. Cell uptake of NPs in insufficient ablation cells

Effective uptake of chemicals can be beneficial to achieving effective antitumour efficacy. In general, ensuring that most of the drug enters the cancer cell can lead to better antitumour activity. In this section, cellular uptake of nanocarriers was observed by confocal microscopy. Compared to free miR-cy3, the fluorescence intensity of miR-cy3-NPs was stronger at a pH of 7.4. In a more acidic environment (pH 6.5), the intensity of NPs was enhanced due to their charge inversion (Fig. 3). This finding indicates that the charge inversion of nanoparticles induced by pH alteration could effectively promote chemical uptake. The uptake of drugs could be enhanced under slightly acidic conditions.

3.4 As/miR34aNPs trigger insufficient ablation cell pyroptosis

Cells with characteristic large bubbles and LDH release are recognized as the key standards of pyroptosis. As/miR34aNPs treatment induced more dying cells blowing characteristic large bubbles from the plasma membrane and whole cells characteristic of typical swelling. However, cells with pyroptosis features induced by As/miR34aNPs at pH 6.5 were more obvious than those at pH 7.4 (Fig. 4A, S2). At the same time, LDH release induced by the combination treatment was more profound than that by the single formulation, and its release was greater under an acidic environment (Fig. 4B, C). To evaluate the inhibitory effect of drug-loaded nanoparticles on the proliferation of sublethal cells, an MTT assay was

used in this study to evaluate the inhibitory effect of drug-loaded nanoparticles on the proliferation of H-Huh7 and H-PLC cells. The results showed that compared with the single formulation, the survival rate of H-huh7 and H-PLC in As/miR34aNPs was significantly lower, indicating that the combined treatment may have a stronger antitumour effect. In a slightly acidic environment, the combined treatment could exert a significant therapeutic effect (Fig. 4D, E). The charge reversal capacity of As/miR34aNPs makes them an ideal nanodelivery carrier to maximize therapeutic efficacy by inducing ferroptosis.

GSDME is another member of the gasdermin family. This protein has a gasdermin-N domain similar to that of GSDMD and can induce pyroptosis in cancer cells with GSDME expression. GSDME is a key determining protein of H-Huh7 and H-PLC in pyroptosis induced by arsenic trioxide. The levels of GSDME-F and GSDME-N proteins were detected by western blot for 48 h in HCC after drug treatment. The results showed that drug-loaded nanoparticles could increase the proportion of the N-terminal part of GSDME, while the antitumour efficacy of dual drug nanoparticles in the weakly acidic environment was more significant (Fig. 4F, G). The expression of GSDME-N and cleaved caspase3 was increased after treatment with As/miR34aNPs (S3). c-met is closely related to the prognosis of tumour patients. The results showed that the double drug-loaded nanoparticles could reduce the expression levels of c-met and the effect of the nanoparticles was more significant in the weakly acidic environment.

3.5. As/miR34aNPs trigger Cyt c release

The GSDME-N portion was expressed at low levels under normal conditions; however, its expression was higher after treatment with chemicals. 293T cells were treated with As₂O₃ after transient transfection with GSDME-N-GFP. As shown in Fig. 5A, the intensity of As treatment was brighter than that of the control group. However, the intensity of GSC-GFP-transfected cells after treatment with As was almost unchanged (Fig. 5A). We observed overlap between GSDME-N mCherry puncta and MitoTracker Green-stained mitochondria in GSDME-N-EGFP-expressing HEK293T cells (Fig. 5B). Similar results were observed in the GSDME-C portion. In previous results, GSDME-N targeted the mitochondria to release the death protein Cyt c. In this part, we found that As₂O₃ treatment may enhance GSDME-N stability and then trigger more Cyt c release. Cyt c release induced by the combination treatment was more profound than that by the single formulation, and its triggered release occurred at a higher level under an acidic environment (Fig. 5C).

3.6. Combination therapy inhibits the progression of insufficient ablation HCC in vivo

To estimate the use of a dual drug formulation as a potential therapy for inhibiting the progression of insufficient microwave ablation, sublethal HCC cells were used as an insufficient microwave ablation cell model according to a previous procedure. An in vivo antitumour efficacy study was performed using a subcutaneous H-Huh7 cell-derived xenograft mouse model. The effective concentration of drugs in tumours and the taking up of drugs by tumour cells are keys to the effectiveness of anticancer treatments. We used Cy3 to trace nanoparticles via i.v. injections. Cy3-labelled nanoparticles showed higher intratumoural accumulation than cy3 alone (Fig. 6B), which was confirmed by fluorescence

measurements in tumour samples. We further investigated the antitumour activity of As/miR34aNPs in vivo. When the tumour size reached approximately 200 mm³, the tumour-bearing nude mice (H-huh7) were divided into four groups and treated with different i.v. injections: Control, AsNPs, miR34aNPs and As/miR34aNPs. Then, the tumour volumes were measured and plotted every three days (Fig. 6A). The results showed that in the PBS-treated group, the tumours grew rapidly, whereas in the single formulation group, effective tumour suppression was observed, and it was better than that of the control group. However, the As/miR34aNP treatment exhibited greater antitumour efficacy than the single formulations. As/miR34aNPs increased c-met, Cyt-c, and cleaved caspase3 levels and the N-terminal proportion of GSDME (Fig. 6C, 6D). To evaluate the potential cytotoxicity of the formulations in vivo, serum biochemical analysis was carried out. Liver function was estimated with the serum levels of aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). Kidney function was verified with serum blood urea nitrogen (BUN) and creatinine (CRE). Inconspicuous changes in the biochemistry index in the four formulation groups indicate that this nanomedicine exhibits few side effects, suggesting its good biosafety in vivo (Fig. 6E, S4).

4. Discussion

Insufficient heat therapy, such as microwave ablation, can lead to the aggressive progression and rapid growth of residual tumours. Traditional treatment, such as chemotherapy, can prevent the progression of residual tumour cells. The miRNA regulatory network was abnormal after thermal ablation of tumours, in which miR34a expression was decreased. Low expression of miR34a is closely related to poor prognosis, and miR34a addition can lower the dosage of other chemicals to inhibit tumour growth. miR34a combined with As₂O₃ can reduce c-met protein expression, which is of positive significance for microwave ablation therapy and may be used as a potential candidate drug for clinical treatment.

Arsenic trioxide use in the treatment of acute promyelocytic leukaemia has progressed, and treatments for other tumours have also progressed. Arsenic trioxide has several antitumour mechanisms, and G2/M phase arrest and apoptosis and inhibition of invasion and migration have been well investigated^[20, 21]. Investigating novel antitumour mechanisms of these chemicals may be beneficial for improving therapy. In this study, we found that miR34a could sensitize arsenic trioxide to trigger pyroptosis. Removal of arsenic trioxide and its products from the blood by the reticuloendothelial system prevents the therapeutic dose from accumulating at the site of the tumour. To overcome this obstacle, arsenic trioxide and other arsenic compounds have been successfully loaded in polymer vesicles, liposomes, and other nanoparticles. To inhibit or eradicate residual cancer cells, nanoparticles have been widely used as carriers to deliver drugs with persistent release characteristics. However, miRNAs are prone to rapid degradation by nucleases in plasma in vivo, and they show weak transport ability across cell members, short half-lives and low interference rates on genes. These obstacles hinder miRNA antitumour efficacy and limit further research. In this study, we used DSPE as a skeleton to load with chemicals. The combination of pH and GSH-sensitized bonds between DSPE and drugs is beneficial for controlling drug release during drug delivery and improving efficacy.

If nanocarriers or drugs resist entering tumour cells, their ability to kill tumour cells will be greatly limited. pH-sensitive charge inversion polymers based on PAE poly(-amino ester) have effective drug delivery capacity and trigger drug release depending on the pH gradient. PEG-based nanodelivery systems facilitate long circulation in the body, thereby increasing PEG-based nanocarrier accumulation at tumour sites. The nanocarrier can more easily enter cells than the free drug, and the uptake of the nanocarrier can be enhanced under a weakly acidic tumour environment, which can be beneficial for effectively inhibiting tumour growth and reducing side effects.

Cell death in all multicellular organisms plays an important role in growth, survival and homeostasis. Pyroptosis is induced by chemicals, toxic agents and even viral infection and represents a novel form of death discovered in recent years. It has the characteristics of membrane pore formation, with typical swelling and cytoplasmic content release. In this study, As/miR34a@NPs generated more cells that showed typical swelling and bubble formation. GSDMD has been demonstrated to play a key role in membrane pore formation and triggers pyroptosis during inflammatory caspase-1/-11/-4/-5 activation^[22]. GSDME is another member of the gasdermin superfamily similar to GSDMD. It has the same function as GSDMD in the formation of membrane pores. GSDME activation triggers pyroptosis in cells with high GSDME expression under chemical treatment^[23–25]. The GSDME gene is inactivated by epigenetic mechanisms in many types of cancer, including colorectal, stomach and breast cancer. At present, there are few studies on liver cancer cell pyroptosis. HepG2 cells express GSDME protein, form GSDME-N fragments after cytochrome C treatment and cause pyroptosis^[23]. In a previous report, GSDME-N was released into the cell plasma and mitochondria. We discovered that both GSDME-N and GSDME-C could reside in mitochondria. GSDME cleavage is triggered by caspase-3 activation after treatment with chemicals. GSDME-N releases to the membrane and mitochondria. Drug treatment could stabilize GSDME-N and trigger Cyt-c release and then cleave GSDME indirectly to form a loop (Fig. 7). Dual-drug nanoparticles induced more cells with pyroptosis characteristics, while the ratio of GSDME-N/GSDME increased, indicating that dual-drug nanoparticles may improve the therapeutic efficacy of HCC with insufficient ablation through GSDMD-N.

5. Conclusion

A novel nanoplatform with a synergistic combined strategy was designed that showed effective therapeutic efficacy in preventing the progression of HCC after insufficient ablation. The drug carriers showed good biocompatibility and could be taken up by tumour cells in a slightly acidic tumour microenvironment. Drug treatment may stabilize GSDME-N, which triggers Cyt-c release. Dual drug-loaded nanoparticles may improve antitumour therapy by downregulating c-met expression, upregulating Cyt-c expression and increasing the GSDME-N ratio. We believe that the novel combined therapy approach may be a promising avenue in the field of microwave ablation.

Abbreviations

GSDME-N: the N-terminal proportion of GSDME; GSDME-C: the C-terminal proportion of GSDME; Vit. E: vitamin e; As: arsenic trioxide

Declarations

Acknowledgments

Not applicable.

Authors' contributions

JH performed the experiments. WCP helped with data analysis, and data interpretation. JH, ZYJ, ZHL designed research, revised and finalized the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

All remaining data are available within the article and supplementary files, or available from the authors upon request.

Ethics approval and consent to participate

The study was authorized by the Ethic Committee of the Shanghai Institute of Biochemistry and Cell, Chinese Academy of Sciences. Written informed consent was provided from each subject. All animal experimental procedures were carried out according to protocols approved by the Animal Care and Use Committee.

Consent for publication

All authors have agreed to publish this manuscript.

Competing Interests

The authors declare no competing financial interest.

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Figures

Figure 1

Insufficient microwave ablation of HCC. (A) Cell viability of Huh7 and PLC cells after incubation at 47°C for 10 min three times, and the corresponding residual cells were named H-Huh7 and H-PLC. (B) Upper panel: c-met expression of H-huh7 and H-PLC was detected by western blot; lower panel: H-huh7 and H-PLC were treated with miR34a. (C) Cell viability of H-huh7 and H-PLC after treatment with As₂O₃(As), miR34a and their combination. *p < 0.05, **, p < 0.01 compared with the control.

Figure 2

Characteristics of As/miR34a@NPs. A. TEM images under different pH conditions; B. zeta potential of As/miR34a@NPs measured by DSL at different pH values; C. Cell viability of blank nanoparticles was examined by MTT. D. drug release curve (pH 7.4 or pH 6.5, with or without GSH treatment); E. miRNA agarose gel electrophoresis experiment. As/miR34a@NPs were incubated with serum at different pH values for 24 h (pH 7.4 or pH 6.5, with or without GSH treatment).

Figure 3

(A) Cell uptake of nanocarriers by H-huh7 cells observed by confocal microscopy. (B) Cell uptake of nanocarriers by H-PLC cells observed by confocal microscopy.

Figure 4

As/miR34aNP induce sublethal HCC pyroptosis. (A) Representative microscopic images of H-Huh7 and H-PLC cells treated with PBS, AsNPs and miR34aNP in a normal physiological environment and As/miR34aNP in a normal and acidic environment. White arrowheads indicate the characteristic bubbles in the cell membrane (400×). (B, C) LDH release was assessed to measure cell membrane destruction. (D, E) Cell viability was detected by the MTT method. (F, G) Expression levels of caspase3, GSDME, c-met and β-actin were detected by western blot. * $p < 0.05$, ** $p < 0.01$ compared with the control.

Figure 5

miR34aNP triggers Cyt c release. (A) Representative microscopic images of HEK293T cells treated with As₂O₃ after transient transfection with GSN-GFP or GSC-GFP. (B) GSN-mCherry and GSC-mCherry were transiently transfected into HEK293T cells. Then, mitochondria and mCherry were observed by confocal microscopy, MitoTracker was used to stain mitochondria, and Hoechst 33342 was used to mark nuclei. (C) H-Huh7 cells were treated for 48 h with PBS, AsNPs and miR34aNP in a normal physiological environment and As/miR34aNP in a normal and acidic environment. The expression levels of Cyt c and Cyt c in the culture media (s-Cyt c) were examined by western blot.

Figure 6

In vivo antitumour performance of different formulations in H-huh7 tumour-bearing nude mice. (A) Tumour growth curves of different groups treated with the four formulations (Control, AsNPs, miR34aNP and As/miR34aNP). Data are shown as the mean $\pm$ SD (n = 6). (*p < 0.05, **, p < 0.01 compared with the control). (B) Intratumoural fluorescence of Cy3-labelled miRNA (red) visualized by CLSM 24 h postinjection. The nucleus is stained with DAPI (blue). (C) Expression levels of GSDME, c-met, and Cyt-c in vivo were detected by western blot. (D) Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN) were detected to evaluate the side effects of different formulations in vivo after the treatment.

Figure 7

As/miR34aNP uptake by tumour cells was enhanced in a slightly acidic tumour microenvironment. This formulation could inhibit c-met expression to exert antitumour efficacy. GSDME cleavage is triggered by caspase-3 activation after treatment with As/miR34aNP. GSDME-N releases to the membrane and mitochondria. Drug treatment could stabilize GSDME-N and trigger Cyt-c release and then cleave GSDME indirectly to form a loop.

Supplementary Files

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