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1	Delivery of melarsoprol using folate-targeted PEGylated cyclodextrin-based
2	nanoparticles for hepatocellular carcinoma
3	
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26 Abstract

27 Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, and has become one of the most lethal malignancies in the world. Although 28 29 chemotherapy remains a cornerstone of cancer therapy, the number of chemotherapeutic drugs approved for HCC is low, and emerging therapeutics are 30 31 needed. Melarsoprol (MEL) is an arsenic-containing drug, and has been applied in the 32 treatment of human African trypanosomiasis at the late stage. In this study, the potential 33 of MEL for HCC therapy was investigated for the first time using in vitro and in vivo 34 experimental approaches. A folate-targeted polyethylene glycol-modified amphiphilic 35 cyclodextrin nanoparticle was developed for safe, efficient and specific delivery of 36 MEL. Consequently, the targeted nanoformulation achieved cell-specific uptake, 37 cytotoxicity, apoptosis and migration inhibition in HCC cells. Furthermore, the targeted 38 nanoformulation significantly prolonged the survival of mice with orthotopic tumor, 39 without causing toxic signs. This study indicates the potential of the targeted 40 nanoformulation as an emerging chemotherapy option for treating HCC.

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42 Keywords: liver cancer, drug delivery, nanoparticle, arsenicals, chemotherapy.

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51 1. Introduction

52 Hepatocellular carcinoma (HCC) is the most common primary liver cancer, with estimated new cases and deaths over 680,000 and 620,000 respectively in 2020 53 54 worldwide (Sung et al., 2021). Since the majority of HCC patients are diagnosed at an 55 advanced stage, they can only be treated using systemic therapies, including 56 chemotherapy, targeted therapy (e.g., multi-kinase inhibitors), and immunotherapy (e.g., 57 immune checkpoint inhibitors) (Galle et al., 2021). However, the efficacy of these 58 therapies is low, and the overall survival of patients is only increased by 3 to 9 months 59 (Llovet et al., 2018). Chemotherapy is among the most effective strategies in many 60 types of cancer, but the number of chemotherapeutic drugs approved for HCC is low. 61 Therefore, therapeutic breakthroughs in chemotherapy are needed.

62 Melarsoprol (MEL) is an arsenic (As)-containing drug with a structure containing 63 melarsen oxide and dimercaptopropanol (Fig. 1), and has been exclusively applied for 64 late-stage human African trypanosomiasis (sleeping sickness) (Fairlamb and Horn, 65 2018). MEL is characterized as a highly lipophilic drug with a logP of 2.53 and a water solubility of 6 mg/L at 25 °C (Gibaud et al., 2005). The lipophilic nature allows MEL 66 67 to cross the blood brain barrier (BBB) for killing trypanosome parasites residing in the cerebrospinal fluid (CSF) (Fairlamb and Horn, 2018). However, the feasibility of MEL 68 69 in the treatment of sleeping sickness is dramatically limited by severe toxicities, such 70 as tissue necrosis around the injection location, neuropathy, and gastrointestinal upset 71 (Kennedy, 2006). The β -cyclodextrin (CD; a cyclic oligosaccharide with seven 72 macrocyclic rings of glucose units) has been widely applied to improve the solubility 73 and bioavailability of hydrophobic molecules. Recently, Kennedy and colleagues have 74 developed two CD derivatives that were termed as randomly methylated CD (RAME-75 CD) and hydroxypropyl CD (HP-CD) (Gibaud et al., 2005). MEL could be complexed with two CD derivatives at molar ratio of 1:1, forming RAME-CD.MEL and HPCD.MEL microcomplexes, respectively (Gibaud et al., 2005). Following systemic
administration, these microcomplexes were mainly delivered into the brain, in which
they exerted therapeutic function against the trypanosome infection in mice (Ben Zirar
et al., 2008) (Rodgers et al., 2011).

81 Since As is perceived as a promising agent for cancer therapy, it is hypothesized 82 that MEL may achieve anti-HCC efficacy. Recently, a folate (FA)-targeted polyethylene 83 glycol (PEG)-modified amphiphilic CD nanoparticle (NP) has been developed in our 84 laboratories for in vitro and in vivo delivery of chemotherapeutic drugs (Zou et al., 2021) 85 (Sun et al., 2022). Since folate receptor (FR) is highly expressed on the membrane surface of liver cancer cells (Koirala et al., 2019), the FA-targeted PEGylated CD NP 86 87 was used in this study for delivery of MEL to HCC. Consequently, the targeted 88 nanoformulation (namely CD.MEL.PEG-FA) facilitated HCC-specific delivery of MEL via FR, achieving significantly higher cellular uptake, cytotoxicity, apoptotic cell 89 90 death and cell migration inhibition in comparison with the non-targeted counterpart 91 (CD.MEL.PEG). Following intravenous (i.v.) administration in orthotopic HCC mice, 92 CD.MEL.PEG-FA significantly retarded tumor development and promoted animal 93 survival, without causing obvious toxic effects.

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95 2. Materials and Methods

96 2.1. Materials

97 MEL ($C_{12}H_{15}AsN_6OS_2$, $M_W = 398.341$ g/mol; Fig. 1) was purchased from Toronto 98 Research Chemicals Inc. (Toronto, Canada). 1,2-distearoyl-sn-glycero-3-99 phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-mPEG₂₀₀₀) and 100 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)- 2000] (DSPE-PEG₂₀₀₀-Folate) were purchased from Xi'an Biological Technology Co.,
Ltd. (Xi'an, China). The other materials were obtained from Sigma-Aldrich unless
mentioned otherwise.

104 **2.2. Nanoformulation preparation**

105 The amphiphilic CD was produced as previously described (O'Mahony et al., 2012). As shown in Fig. 1, in order to prepare the targeted nanoformulation, 5 mg of 106 107 CD and 1 mg of MEL were dissolved in 50 mL of methanol (MeOH) and dried to form a thin film using a rotary evaporator. This content was rehydrated within 3 mL of 108 ultrapure water and sonicated at 37 °C for 1-2 h, in order to achieve the CD.MEL 109 110 nanocomplex. As previously described (Zou et al., 2021), 3.5 mg of DSPE-mPEG₂₀₀₀ 111 (DSPE-PEG) and 1 mg of DSPE-PEG₂₀₀₀-FA (DSPE-PEG-FA) (molar ratio \approx 4:1) were 112 dissolved in 0.5 mL of HEPES buffer (20 mmol/L, pH = 7.4) and incubated at 60 °C 113 for 30 min to obtain a solution. The solution was added into the CD.MEL nanocomplex and incubated at 60 °C with shaking for 1-1.5 h, in order to achieve the targeted 114 115 nanoformulation (CD.MEL.PEG-FA) (~ 5 mol% of FA in the outer surface per NP in 116 theory). The non-targeted nanoformulation (CD.MEL.PEG) was prepared as described above without the incorporation of DSPE-PEG-FA. The nanoformulations containing 117 rhodamine or DiR (0.05%, w/w) were prepared as described above by dissolving CD, 118 119 MEL and rhodamine (or DiR) in MeOH.

120 2.3. Physicochemical characterization of nanoformulation

121 The encapsulation efficiency (EE%) and loading capacity (LC%) of MEL were 122 measured using high performance liquid chromatography (HPLC; Shimadzu, Japan) 123 (C18 column; UV at 285 nm; mobile phase = acetonitrile and water, v:v = 70:30) and 124 calculated using the equations as follows: EE% = (weight of loaded MEL/weight of 125 feed MEL) x 100% and LC% = (weight of loaded MEL/weight of total nanoformulation)

126 x 100%.

The solution of nanoformulation containing 5 μg MEL was added onto a 400-mesh
carbon-filmed copper grid (Agar Scientific) (Guo et al., 2012). The grid was added with
2% (w/w) uranyl acetate, prior to the analysis using transmission electron microscopy
(TEM) (JEOL JEM-1230).

The particle size and zeta potential of nanoformulation were assessed using Malvern Nano-ZS as previously described (Luan et al., 2019). In addition, when the nanoformulation was stored at 4 °C in aqueous solution, the particle size was measured at different time points by Malvern Nano-ZS as previously described (Sun et al., 2022), in order to determine the stability.

The solution of nanoformulation containing 500 μ g MEL in a dialysis bag (MWCO = 2 kDa; Solarbio[®], China) was incubated in 100 mL of release medium (0.01 M PBS containing 0.5% Tween 80; pH = 5.5 or 7.4) at 37 °C with stirring. At different time points, 5 mL of supernatant were collected, and 5 mL of fresh release medium were supplemented. The drug concentration within the supernatant was measured using HPLC as described above.

142 2.4. In vitro anti-HCC effects of nanoformulation

Hepa1-6 cells (a mouse HCC cell line with FR (Hu et al., 2017) (Maghsoudinia et al., 2021a) (Maghsoudinia et al., 2021b) (Gong et al., 2021)) were cultured in DMEM
with 10% FBS and 1% Penicillin-Streptomycin. Hepa1-6-Luc cells, which can stably
express the luciferase, were cultured in DMEM with 10% FBS, 1% PenicillinStreptomycin, and 1 µg/mL of puromycin (ThermoFisher). All the cells were
maintained at 37 °C with 5% CO₂ and 95% relative humidity.

Hepa1-6 cells (5 x 10³ cells per well) were seeded in 96-well plates for 24 h. After
this, cells were incubated with the nanoformulation at different concentrations of MEL

for 24 h. Subsequently, cells were washed twice with PBS and incubated with 20 μL of
MTT reagent (5 mg/mL in PBS) and 180 μL of fresh growth medium at 37 °C for 4 h.
The purple precipitate was dissolved by DMSO before the measurement at 570 nm
using Microplate Reader.

Hepa1-6 cells (2 x 10^4 cells per well) were seeded in 24-well plates with glass bottoms for 24 h. After this, cells were incubated with PBS or nanoformulations ([c] of MEL = 5 µmol/L MEL; 0.05 % rhodamine, w/w) for 8 h. Subsequently, cells were washed with PBS twice, treated with 4% paraformaldehyde for 20 min, and stained by DAPI (Beyotime Biotech, China). The cellular uptake was assessed using the confocal microscopy (Olympus FV3000) and analyzed using ImageJ to determine the relative fluorescence intensity (%).

162 Hepa1-6 cells (1 x 10^5 cells per well) were seeded in 6-well plates for 24 h. 163 Subsequently, cells were incubated with PBS or nanoformulations ([c] of MEL = 15 164 µmol/L) for 24 h, and apoptotic cells (%) were detected using Annexin V-FITC and 165 propidium iodide (PI) (YEASEN Biotech, China) using flow cytometry (BD 166 FACSCalibur).

167 Hepa1-6 cells (3 x 10^5 cells per well) were seeded in 6-well plates for 24 h. As 168 previously described (Yang et al., 2021), when cells reached the confluence, the cell 169 monolayer was washed with PBS and scraped with a pipette tip. Subsequently, cells 170 were incubated in serum-free growth medium and added with PBS or nanoformulations 171 ([c] of MEL = 15 µmol/L) for 24 h. The cell-free areas before and after treatment of 172 nanoformulations, as a measure of cell migration, were imaged under the light 173 microscope and analyzed using ImageJ.

174 2.5. *In vivo* toxicity and tumor distribution of nanoformulation

175 Six to eight week old male C57BL/6 mice were obtained from Beijing Vital River

176 Laboratory Animal Technology Company. The experiments were approved by the177 Animal Ethics Committee of Jilin University.

Healthy mice (n = 4) were i.v. injected with PBS or nanoformulations (containing ~ 1 mg/kg of MEL) on Day 1, 5, 9, 12 and 17, and the body weight was regularly recorded. On Day 25, the heart, liver, spleen, lung, kidneys were obtained for the analysis of histopathology, and the serum was collected to evaluate the hepatic/renal functions, as previously described (Guo et al., 2020).

183 As previously described (Yu et al., 2020) (Han et al., 2022), mice were anesthetized using 4% chloral hydrate, and 5×10^5 Hepa1-6-Luc cells were injected 184 into the liver lobe to develop orthotopic HCC (Day 0). Animals were intraperitoneally 185 186 administered 80 µL of luciferin (10 µg/µL; Pierce), and tumor development was 187 monitored using IVIS® System (Perkin Elmer). When the tumor intensity reached ~ 5 to 10×10^8 p/s/cm²/sr, the animals were used to detect tumor distribution. Mice (n = 4) 188 were i.v. injected with PBS or nanoformulations (containing ~ 1 mg/kg MEL; ~ 0.05 % 189 190 DiR, w/w). Twelve hours after i.v. injection, tumor distribution was analyzed using 191 IVIS® System (748 nm/780 nm).

192 2.6. In vivo anti-HCC effects of nanoformulation

In addition, when tumor intensity reached ~ 5 to 10×10^8 p/s/cm²/sr, animals (n = 193 5) were i.v. treated with PBS or nanoformulations (containing $\sim 1 \text{ mg/kg MEL}$) on Day 194 195 9, 12 and 15. Tumor growth and animal survival were recorded. In addition, tumors were collected on Day 16 for western blotting experiment as previously described (Guo 196 et al., 2021). Briefly, tumors were homogenized and lysed using ProteinExt® 197 198 Mammalian Total Protein Extraction Kit (TransGen Biotech, China), and the concentration of proteins was quantified using the BCA Kit (Beyotime Biotech). 199 Subsequently, ~ 35 to 45 µg of proteins were loaded to the SDS-polyacrylamide gel and 200

electrophoresed at 100 V for 1-1.5 h. Proteins were then transferred onto a
polyvinylidene fluoride membrane (Invitrogen) at 200 mA for 1-1.5 h. The membrane
was incubated overnight with the primary antibodies (Table S1) at 4°C. The secondary
antibody (Table S1) was added to the membrane for 1.5 h, and the proteins were
detected using the enhanced chemiluminescence solution (YEASEN Biotech) and
quantified using ImageJ.

207 2.7. Statistical analysis

Results were presented as mean \pm standard deviation. The significance of differences was evaluated using one-way ANOVA (Bonferroni's Post-Hoctest) (GraphPad prism). The analysis of body weight, tumor growth and animal survival was evaluated using two-way ANOVA (Bonferroni's Post-Hoctest) (GraphPad prism). In all experiments, p < 0.05 was considered statistically significant.

213

214 **3.** Results and Discussion

215 3.1. Preparation and physicochemical characterization of CD.MEL.PEG-FA

216 As is a metalloid element with two oxidation states namely As (III) and As (V), and can form organic and inorganic arsenicals. Arsenicals have been historically used 217 as therapeutic agents for different diseases (e.g., dermatosis, hematological 218 219 malignancies and solid tumors) (Liu et al., 2021). For example, arsenic trioxide (As₂O₃, 220 an inorganic arsenical) has become the established component of first-line treatment 221 protocols for acute promyelocytic leukemia (one of the hematological malignancies) 222 (Yilmaz et al., 2021). In addition, MEL is an organic arsenical, and has been exclusively 223 applied for treating late-stage human African trypanosomiasis (a malignancy caused by protozoan parasites) (Fairlamb and Horn, 2018). MEL is capable of penetrating the 224 225 BBB to kill parasites inside the CSF (Fairlamb and Horn, 2018). Due to the capacity of As compounds for apoptotic induction (Liu et al., 2021), the potential of MEL for HCC therapy was assessed in this study. As shown in Fig. S1, MEL caused ~ 50% cell death (IC₅₀) in Hepa1-6 cells at ~ 24 μ mol/L, confirming the *in vitro* anti-HCC potential of MEL.

230 In the clinic, MEL is dissolved in propylene glycol and administered to patients *via* i.v. infusions. However, i.v. administration is painful as propylene glycol is highly 231 232 irritating to tissues. In addition, i.v. administration of MEL induces non-specific tissue 233 delivery, causing severe toxic effects and even death (Fairlamb and Horn, 2018). 234 Recently, Kennedy et al. have developed two CD derivatives (RAME-CD and HP-CD) 235 to improve solubility and bioavailability of MEL (Gibaud et al., 2005). Following oral 236 administration, the resultant MEL-containing microcomplexes were mainly delivered 237 into the brain, in which they provided therapeutic efficacy against the trypanosome 238 infection in mice, demonstrating the potential for treating human African 239 trypanosomiasis (Rodgers et al., 2011). However, systemic administration of these 240 microcomplexes may not be suitable for HCC therapy in that they are mainly found in 241 the brain, and lack the targeting ligand for facilitating MEL delivery to HCC cells (Ben 242 Zirar et al., 2008) (Rodgers et al., 2011).

Recent advances in nanotechnology and drug formulation expedite the design and 243 244 application of nano drug delivery systems (NDDS) for chemotherapeutic drugs in 245 cancer therapy (Guo and Huang, 2020) (Sun et al., 2021) (Shan et al., 2022) (Guo and Huang, 2022) (Guo et al., 2023). Thus, developments of emerging NDDS may 246 potentially improve the safety and efficacy of MEL in the treatment of HCC. In order 247 248 to facilitate in vivo anti-HCC potential of MEL, a NDDS was developed in this study by loading MEL into the amphiphilic CD modified with PEGylated FA that could 249 250 specifically bind to FR on HCC cells (Fig. 1). It has been reported by our laboratories

that the CD could self-assemble into NPs for encapsulating chemotherapeutic drugs,
whereby chemotherapeutic drugs were loaded within the hydrophobic cavity and
domain of the CD (Zou et al., 2021) (Sun et al., 2022). In this study, HPLC results
demonstrated that MEL could be encapsulated within the CD at various weight ratios
(WR) of CD and MEL (results not shown), and the optimal EE% (~ 85 wt%) and LC%
(~ 14 wt%) of MEL were achieved by the CD at WR5 (Fig. 2A).

257 Subsequently, the "postinsertion" technique that allows the incorporation of 258 DSPE-PEG-targeting ligands into the preformed liposomes and liposome-like 259 nanostructures (Evans et al., 2017) (Swart et al., 2022), was used to achieve the 260 incorporation of DSPE-PEG and DSPE-PEG-FA onto the CD.MEL nanocomplex (Fig. 261 1). HPLC results demonstrated that CD.MEL.PEG and CD.MEL.PEG-FA achieved 262 similar EE% (~ 88 and 88 wt%) and LC% (~ 11 and 11 wt%) as compared to CD.MEL 263 (Fig. 2A), indicating that the encapsulation of MEL within CD remained unaffected by the modification of DSPE-PEG and DSPE-PEG-FA. In addition, CD.MEL.PEG and 264 265 CD.MEL.PEG-FA displayed a significantly higher particle sizes (~ 108 and 110 nm) and reduced surface charge (~ 8 and 9 mV) as compared to CD.MEL (~ 95 nm and 40 266 mV), suggesting the successful incorporation of DSPE-PEG and DSPE-PEG-FA which 267 masks the surface charge (Fig. 2A). The polydispersity index (PDI) of CD.MEL, 268 269 CD.MEL.PEG and CD.MEL.PEG-FA was ~ 0.22, 0.31 and 0.32 respectively (Fig. 2A), 270 indicating the generation of monodisperse nanoformulations. Indeed, CD.MEL.PEG-271 FA displayed a uniform spherical morphology (Fig. 2B), which was similar to the morphologies observed by CD.MEL and CD.MEL.PEG (data not shown). 272

The release efficiency of MEL from the targeted nanoformulation was assessed under neutral and acidic environments. As shown in Fig. 2C, $\sim 10\%$ of MEL were released after 1 h incubation in neutral environment (pH = 7.4), whereas the drug

276 release was significantly (p < 0.01) increased (~ 30%) after 1 h incubation in acidic 277 environment (pH = 5.5). After 24 h incubation, \sim 50% and 90% of MEL were released in neutral (pH = 7.4) and acidic (pH = 5.5) environments, respectively. These results 278 279 show that the targeted nanoformulation achieved significantly higher drug release at 280 acidic pH, which was similar to different amine-modified nanoformulations that were developed by other research groups (Gao et al., 2010) (He et al., 2017) (Zhuo et al., 281 282 2020). The mechanism underlying the pH-sensitive drug release of our targeted 283 nanoformulation and others is due to the protonation of the amine groups in response 284 to the acidic environments (Gao et al., 2010) (He et al., 2017) (Zhuo et al., 2020). In 285 addition, following storage at 4 °C, the particle size of the targeted nanoformulation in 286 water remained unchanged (~110 to 110 nm) for two weeks (Fig. 2D). The non-targeted 287 nanoformulation also achieved similar drug release and stability to those recorded by 288 FA-targeted counterpart. However, the aggregation was detected with the CD.MEL nanocomplex during the drug release and stability experiments. These results indicate 289 290 that the PEG incorporation efficiently masked the surface of the CD.MEL nanocomplex 291 to improve the particle stability. Due to the instability, CD.MEL was not used in the 292 following in vitro and in vivo experiments.

293

3.2. In vitro anti-HCC effects of CD.MEL.PEG-FA

The *in vitro* anti-HCC property of the targeted nanoformulation (CD.MEL.PEG-FA) was first studied using the MTT assay in Hepa1-6 cells, which is a mouse HCC cell line that has been widely used to determine the targeted delivery efficacy of FAmodified nanoformulations (Hu et al., 2017) (Maghsoudinia et al., 2021a) (Maghsoudinia et al., 2021b) (Gong et al., 2021). As shown in Fig. S1, MEL on its own achieved an IC₅₀ at ~ 24 µmol/L (24 h incubation), while the cytotoxicity was significantly increased (p < 0.05) by CD.MEL.PEG-FA (IC₅₀ \approx 15 µg/mL of MEL) (Fig. 301 S1). The anti-HCC activity mainly resulted from cell-specific delivery of MEL *via* the 302 FA-FR-associated pathway, as NPs without MEL caused no cell death (Fig. S1). Indeed, 303 CD.MEL.PEG-FA at the same concentrations of MEL also induced significantly (p <304 0.05) higher cytotoxicity as compared to CD.MEL.PEG (Fig. 3A), confirming the 305 function of FA as a targeting ligand for HCC-specific drug delivery.

To further confirm the active targeting delivery, the cellular uptake of CD.MEL.PEG and CD.MEL.PEG-FA containing rhodamine was investigated using confocal microscopy. As shown in Fig. 3B, the cellular uptake achieved by the targeted nanoformulation containing rhodamine was significantly (the relative fluorescence intensity \approx 38% after 8 h incubation; p < 0.05) higher than PBS (0%) and the nontargeted counterpart (\sim 20%) (Fig. 3B), further confirming the role of the FA targeting ligand in facilitating HCC-specific drug delivery.

313 In addition, the targeted nanoformulation caused significantly higher HCC cell apoptosis (~ 50% after 24 h incubation; p < 0.01) as compared to PBS (< 1%) and the 314 non-targeted counterpart (~ 20%) (Fig. 3C). Moreover, the targeted nanoformulation 315 316 significantly inhibited HCC cell migration (the cell-free area after treatment vs. the cellfree area before treatment > 90% after 24 h incubation; p < 0.01) as compared to PBS 317 (~ 40%) and non-targeted counterpart (~ 50%) (Fig. 3D). Therefore, results in Fig. 3 318 319 indicate that MEL-mediated apoptosis is the main mechanism for CD.MEL.PEG-FA to 320 suppress the HCC cell growth and migration.

321 3.3. *In vivo* toxicity and tumor distribution of CD.MEL.PEG-FA

The *in vivo* toxicity of the targeted nanoformulation was evaluated in the healthy mice (n = 4). It is known that the i.v. administration of MEL is painful and causes severe tissue damage (Fairlamb and Horn, 2018). Indeed, toxic signs (e.g., body weight loss, hunched posture and ruffled hair coat) were observed in mice following i.v. injections 326 of MEL at a dose of 1 mg/kg (Fig. S2). In contrast, no body weight loss was observed 327 in mice i.v. administrated with CD.MEL.PEG-FA relative to PBS (Fig. 4A). In addition, 328 results of H&E staining experiments demonstrated that no histopathology was found in 329 the heart, liver, spleen, lung and kidneys of mice after i.v. injections of CD.MEL.PEG-330 FA as compared to PBS (Fig. 4B). Moreover, no alteration in alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea nitrogen (BUN) and creatinine (CRE) 331 332 was detected, indicating that no hepatic and renal injury was caused by CD.MEL.PEG-333 FA when compared with PBS (Fig. 4C). These results show that no systemic toxicity 334 was caused by the targeted nanoformulation under the conditions tested.

To confirm tumor targeting delivery, the tumor distribution of CD.MEL.PEG-FA was determined in Hepa1-6-Luc-derived orthotopic HCC mice. Results show that CD.MEL.PEG-FA was significantly (p < 0.05) located in the liver tumor area as compared to CD.MEL.PEG, and this was confirmed by the overlapping of NP (DiR dye) and the tumor (luciferase in tumor cells) (Fig. 4D). These results indicate that the targeted nanoformulation, due to targeting the FR expressed on HCC cells, can promote tumor targeting delivery.

342 3.4. In vivo anti-HCC effects of CD.MEL.PEG-FA

Subsequently, the therapeutic efficacy of the targeted nanoformulation was 343 344 assessed using Hepa1-6-Luc-derived orthotopic HCC mice (n = 5). Due to the fact that 345 free MEL caused toxic signs (Fig. S2), animals were i.v. treated with PBS, 346 CD.MEL.PEG, and CD.MEL.PEG-FA (Fig. 5A). As shown in Fig. 5B, CD.MEL.PEG significantly (p < 0.05) suppressed tumor growth as compared to PBS, while tumor 347 348 growth was further (p < 0.01) retarded by CD.MEL.PEG-FA relative to PBS and CD.MEL.PEG. As a result, CD.MEL.PEG-FA significantly (p < 0.01) prolonged the 349 350 survival of diseased animals relative to PBS and CD.MEL.PEG (Fig. 5C). In addition, 351 CD.MEL.PEG-FA caused significantly higher apoptosis (*p* < 0.05) in tumor cells than 352 PBS and CD.MEL.PEG, which was confirmed by the increased expression of Bax and 353 cleaved-Caspase 3 proteins (two key apoptosis-triggering proteins) and the reduced 354 expression of Bcl-2 protein (a key apoptosis-inhibitory protein) (Fig. 5D). Thus, results 355 in Fig. 5 indicate that the anti-HCC efficacy of the targeted nanoformulation resulted 356 mainly from tumor-specific delivery of MEL and from MEL-mediated apoptosis.

357 Recent developments of emerging NDDS effectively promote anticancer efficacy 358 and alleviate toxic effects of arsenic and its compounds (Fei et al., 2020) (Fu et al., 359 2020). Since As₂O₃ has become the pioneer arsenic-containing drug, the design and 360 formulation of As₂O₃ NDDS have attracted more attention in the fields of drug delivery 361 and cancer therapy (Sonksen et al., 2022). However, NDDS for MEL delivery have 362 been much less studied. One example includes that MEL-containing nanosuspensions 363 were prepared using Pluronic F-68® (poloxamer 188) or Pluronic F-127® (poloxamer 364 407) and mannitol (Ben Zirar et al., 2008). However, these nanosuspensions have not 365 been applied for HCC therapy as they do not have the HCC-targeting ligand and may 366 not specifically deliver MEL to the tumors (Ben Zirar et al., 2008). Consequently, the FA-targeted PEGylated amphiphilic CD NP developed in this study is to our knowledge 367 the first nano delivery system to achieve safe, efficient and specific delivery of MEL 368 369 against HCC (Fig. 4 and 5).

370

371 4. Conclusions

In this study, an amphiphilic CD was used to develop a FA-targeted PEGylated nano delivery vector for formulating MEL (Fig. 1). The resultant NDDS (CD.MEL.PEG-FA) demonstrated favorable physicochemical properties, in terms of drug loading (EE% \approx 88 wt% and LC% \approx 11 wt%), particle size (~ 110 nm), zeta

potential (~ 9 mV), pH-sensitive drug release (higher MEL release in acidic condition),
and storage stability (no aggregation for two weeks) (Fig. 2). These physicochemical
properties were compliant with the requirements for favorable *in vitro and in vivo*delivery fates (Fig. 3 and 4). Indeed, the tumor growth was significantly retarded
following i.v. administration of CD.MEL.PEG-FA (Fig. 5), indicating the HCC-specific
delivery of MEL, without inducing obvious toxic signs. Our study demonstrates great
potential of CD.MEL.PEG-FA as an emerging chemotherapy option for treating HCC.

383

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395

396 Conflicts of interest

397 Authors declares no conflict of interest.

398

399 Figure legends

400 **Figure 1.** The formulation schematic of CD.MEL.PEG-FA.

Figure 2. Physicochemical characterization of CD.MEL.PEG-FA. (A) The EE%, LC%, particle size, surface charge and PDI of nanoformulations (n = 4). (B) TEM image of FA-targeted nanoformulation (scale bar = 100 nm). (C) The release of MEL from FA-targeted nanoformulation in 0.01 M PBS (pH = 5.5 and 7.4) at 37 °C (n = 5, * p < 0.05). (D) The particle size of FA-targeted nanoformulation in aqueous solution stored at 4 °C (n = 5, * p < 0.05, ** p < 0.01 and NS = no significance).

Figure 3. In vitro characterization of CD.MEL.PEG-FA in Hepa1-6 cells. (A) Cell 407 viability after 24 h treatment of CD.MEL.PEG and CD.MEL.PEG-FA at different 408 concentrations (n = 4, * p < 0.05 and NS = no significance). (B) The cellular uptake 409 after 8 h incubation of CD.MEL.PEG and CD.MEL.PEG-FA (5 µmol/L MEL; 0.05 % 410 411 rhodamine, w/w) (n = 3, * p < 0.05 and ** p < 0.01) (scale bar = 10 μ m). (C) Apoptotic 412 cells (%) after 24 h treatment of CD.MEL.PEG and CD.MEL.PEG-FA (15 µmol/L MEL) (n = 4, * p < 0.05 and ** p < 0.01). (D) Relative scratch area (%) before and after 413 24 h treatment of CD.MEL.PEG and CD.MEL.PEG-FA (15 μ mol/L MEL) (n = 4, * p 414 415 < 0.05 and NS = no significance).

416 Figure 4. In vivo toxicity and tumor distribution of CD.MEL.PEG-FA. (A) The body weight of healthy C57BL/6 mice over a 25-day period after i.v. injections of PBS 417 and CD.MEL.PEG-FA (1 mg/kg MEL) on Day 1, 5, 9, 12 and 17 (n = 4). (B) The heart, 418 liver, spleen, lung and kidneys were collected on Day 25 and assessed using H&E 419 420 staining assay (scale bar = $100 \mu m$). (C) The liver/kidney functions (alanine 421 aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (CRE)) on Day 25 (n = 4). (D) Tumor distribution of DiR-labeled 422 423 nanoformulations at 12 h following i.v. injection in Hepa1-6-Luc derived orthotopic HCC mice. 424

425 Figure 5. *In vivo* anti-HCC activity of CD.MEL.PEG-FA in Hepa1-6-Luc derived

426 orthotopic HCC mice. (A) HCC development and treatment schedule (red arrow = i.v. 427 injection). (B) The tumor growth following the treatment of PBS, CD.MEL.PEG and CD.MEL.PEG-FA (1 mg/kg MEL) over a 35-day period following the treatment 428 schedule as shown in Fig. 5 A (n = 5, * p < 0.05). (C) Animal survival (median survival: 429 PBS for ~ 26 days, CD.MEL.PEG for ~ 42 days, and CD.MEL.PEG-FA for ~ 64 days) 430 (n = 5, * p < 0.05). (D) The protein expression in the Bax/Bcl-2/caspase 3 signaling 431 cascade within the tumors one day (Day 16) following the treatment schedule as shown 432 in Fig. 5 A (n = 4, * p < 0.05 and ** p < 0.01). 433

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Figure 1.

Α									
Nanoformulation	WR	EE%	LC%	Size (nm)	Charge (mV)	PDI			
CD.MEL	5:1	85 ± 2	14 ± 3	95 ± 5 nm	40 ± 5 mV	0.22			
CD.MEL.PEG	5:1:2.5	88 ± 4	11 ± 3	108 ± 3 nm	8 ± 3 mV	0.31			
CD.MEL.PEG-FA	5:1:2.5	88 ± 3	11 ± 2	110 ± 2 nm	9 ± 3 mV	0.32			



Figure 2.



Figure 3.



Figure 4.



Figure 5.