

## Article

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## Accepted Manuscript

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1        **Liposome Mediated-CYP1A1 Gene Silencing Nanomedicine Prepared**  
2        **Using Lipid Film-Coated Proliposomes as a Potential Treatment Strategy**  
3        **of Lung Cancer**

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65 **ABSTRACT:**

66 The occurrence of lung cancer is linked with tobacco smoking, mainly through the  
67 generation of polycyclic aromatic hydrocarbons (PAHs). Elevated activity of cytochrome  
68 P4501A1 (CYP1A1) plays an important role in the metabolic processing of PAHs and its  
69 carcinogenicity. The present work aimed to investigate the role of CYP1A1 gene in PAH-  
70 mediated growth and tumor development *in vitro* and using an *in vivo* animal model. RNAi  
71 strategy was utilized to inhibit the overexpression of CYP1A1 gene using cationic  
72 liposomes generated using a lipid film-coated proliposome microparticles. Treatment of  
73 PAH-induced human alveolar adenocarcinoma cell line with cationic liposomes carrying  
74 CYP1A1 siRNA resulted in down regulation of CYP1A1 mRNA, protein as well as its  
75 enzymatic activity, triggering apoptosis and inhibiting multicellular tumor spheroids  
76 formation *in vitro*. Furthermore, silencing of CYP1A1 gene in BALB/c nude xenografts  
77 inhibited tumor growth via down regulation of CYP1A1 expression. Altogether, our  
78 findings showed that liposome-based gene delivery technology is a viable and stable  
79 approach for targeting cancer causing genes such as CY1PA1. This technology facilitated  
80 by the use of sugar particles coated with lipid films has demonstrated ability to generate  
81 anticancer effects that might be used in the future for therapeutic intervention and treatment  
82 of lung cancer.

83 **KEYWORDS:** Apoptosis, Cancer, CYP1A1, Lung, siRNA, Smoking, Tobacco

## 84 **1. INTRODUCTION**

85 Lung cancer has become a leading cause of death worldwide due to the increased  
86 environmental contamination with inhalable carcinogens occurring as byproducts of  
87 combustion processes and unhealthy habits such as tobacco smoking (Field and Withers,  
88 2012). Despite the efforts made to improve the life quality of cancer patients, a proper  
89 understanding of the pathogenesis of lung cancer is still missing, resulting in poor treatment  
90 outcomes and severe adverse effects of chemotherapy and radiotherapy (Brambilla and  
91 Gazdar, 2009). Susceptibility of lung to carcinogenesis is based on the metabolic imbalance  
92 between induction and detoxification pathways, with a significant role of external inducing  
93 factors (Hecht, 1999).

94 Polycyclic aromatic hydrocarbons (PAHs) produced by tobacco smoking are involved in  
95 the activation and development of lung cancer (Armstrong et al., 2004; Hecht, 1999).

96 Although the detailed mechanism of how this group of carcinogens disrupts the  
97 homeostasis of lung cells is still unclear, studies have concluded that PAHs can induce the

98 overexpression of cytochrome P4501A1 gene (CYP1A1), an important member of a large  
99 family of cytochrome P450 enzymes involved in the metabolism of PAHs (Shimada and

100 Fujii-Kuriyama, 2004). Consequently, many highly electrophilic metabolic intermediates

101 can be produced, causing irreversible damage to human tissues and inducing cancer  
102 occurrence (Shimada and Fujii-Kuriyama, 2004). Therefore, targeting of CYP1A1 gene  
103 may be a promising therapeutic strategy especially for smoking-related lung cancer  
104 (Androutsopoulos et al., 2009; Bruno and Njar, 2007). The induction of CYP1A1 primarily  
105 occurs when the inducer binds to the ligand-activated transcriptional factor aryl  
106 hydrocarbon receptor (AhR) (Guigal et al., 2000). Flavonoid galangin, an antagonist  
107 against AhR, has been considered as an inhibitor candidate to decrease the CYP1A1  
108 expression (Ciolino and Yeh, 1999). However, multi-targeted properties of this drug may  
109 lead to non-specific inhibition of the other members of the P450 gene family (Murakami et  
110 al., 2008; Sak and Everaus, 2015), suggesting a better and specific strategy is needed to  
111 target CYP1A1 gene for therapeutic intervention and treatment of lung cancer.

112 RNA interference (RNAi) is a gene silencing technology at the transcriptional level and  
113 works through specifically targeting mRNA via sequence-specific matches, resulting in  
114 degradation of the target mRNA (Agrawal et al., 2003). siRNA technology promises  
115 greater advantages over conventional drugs currently in the market for its high targeting  
116 selectivity and low toxicity; however, pharmacokinetic properties of siRNA are  
117 unpredictable and its cellular uptake is poor (Lorenzer et al., 2015). Accordingly, specific

118 siRNA-mediated silencing of CYP1A1 expression with improved kinetics and uptake by  
119 target cells is urgently warranted.

120 As widely used vehicles in nucleic acid delivery, non-viral vectors such as cationic  
121 liposomes are much safer than viral vectors (Khurana et al., 2013). Furthermore, compared  
122 to polymeric vectors, cationic liposomes may offer higher transfection and greater  
123 biocompatibility (Ruozi et al., 2003). Novel cationic lipids conjugated with functional  
124 targeting groups may offer a great potential for use in the preparation of cationic liposomes  
125 ( Ruozi et al., 2003; Kim et al., 2010b; Sun et al., 2018).

126 Liposomes manufactured using the traditional thin-film hydration technique with  
127 subsequent preparation as liquid dispersions are unstable during storage owing to the  
128 liability of phospholipids to hydrolysis and oxidation, with subsequent compromise of the  
129 validity of liposomes as drug carriers (Grit and Crommelin, 1993). Alternatively,  
130 proliposomes are stable powdered phospholipid formulations prepared by coating  
131 carbohydrate carrier particles with thin phospholipid films using modified rotary  
132 evaporators (Elhissi et al., 2006; Gala et al., 2015). Liposomes can be generated from  
133 proliposomes via the addition of aqueous phase and shaking (Elhissi et al., 2006; Gala et  
134 al., 2015). Several reports have established the suitability of manufacturing thin-film-based  
135 proliposome powders on a large scale, for instance by using fluidized-bed coating (Chen  
136 and Alli, 1987; Kumar et al., 2001; Gala et al., 2015). Liposomes generated from lipid film



137 coated sugars (i.e. proliposomes) have been widely investigated for drug delivery. For  
138 example, early reports have shown that oral delivery of non-steroidal anti-inflammatory  
139 drugs in liposomes generated from proliposomes can protect against gastric ulceration in  
140 experimental animals (Katare et al., 1990). Proliposomes have also been investigated for  
141 nasal delivery of propranolol hydrochloride and nicotine (Ahn et al., 1995; Jung et al.,  
142 2000a), and for parenteral administration of antifungal drugs (e.g. amphotericin B) (Payne  
143 et al., 1987), and anticancer agents such as methotrexate (Park et al., 1994) and doxorubicin  
144 (Wang et al., 2000), and for transdermal delivery of nicotine (Hwang et al., 1997; Jung et  
145 al., 2000b). We have previously shown that proliposomes made by coating sucrose with  
146 lipid films can generate inhalable liposomes when hydrated *in situ* within medical  
147 nebulizers (Elhissi et al., 2012). More recent investigators have shown that diltiazem HCL  
148 liposomes generated from proliposomes could be used for topical treatment of glioma  
149 (Mokhtar Ibrahim et al., 2013) and dermatitis (Jahn et al., 2014) using animal models.  
150 Proliposomes made by film-coating of sugar particles have recently been demonstrated to be  
151 compressible into tablets, with properties being dependent on formulation (Khan et al.,  
152 2018).

153

154 In this study, lipid film-based proliposome technology was employed for the preparation of  
155 cationic liposomes-siRNA (CL-siRNA) formulations for targeting the CYP1A1 gene. AhR-

156 mediated induced expression of CYP1A1 in A549 adenocarcinoma cell line was used to  
157 model smoking induction of CYP1A1 expression. The effects of CYP1A1 silencing with  
158 CYP1A1 CL-siRNA on CYP1A1 expression, CYP1A1 enzyme activity, cell apoptosis and  
159 tumor spheroids formation were verified in induced A549 cell lines. The effect of CYP1A1  
160 silencing on tumor regression was further investigated in the induced A549 tumors in  
161 xenograft BALB/c-nude mice.

162

## 163 2. MATERIALS AND METHODS

164

### 165 2.1. Materials

166 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and dioleoyl-  
167 phosphatidylethanolamine (DOPE) were purchased from Avanti Polar-Lipids Inc.  
168 (Alabaster, AL, USA). Cholesterol was obtained from Biotech Co. Ltd (Shanghai, China),  
169 and 3-methylcholanthrene (3-MC) was purchased from SUPELCO Co.  
170 (Pennsylvania, USA). Human CYP1A1 siRNA was chemically synthesized and purified  
171 via HPLC by RiboBio (Guangzhou, China). Goldview staining was purchased from  
172 Guangzhou Geneshun Biotech Ltd (Shanghai, China). RNA prep pure cell kit was  
173 purchased from TIANGEN (Beijing, China). The sequence for siRNA was as follows:  
174 siRNA against CYP1A1: sense, 5'-GGCCUGAAGAAUCCACCAG-3'; antisense, 3'-

175 CUGGUGGAUUCUUCAGGCC-5'. FAM-siRNA and the same sequence was obtained  
176 from Sangon Biotech (Shanghai, China). Lipofectamine2000 was obtained from Invitrogen  
177 (USA).

## 178 **2.2. Preparation of liposome-siRNA complexes using lipid-coated particulate-based** 179 **proliposomes**

180 Sorbitol particles (300-500  $\mu\text{m}$ ) were placed in 50 ml pear-shaped flask and attached to a  
181 modified rotary evaporator with a feed-line tube. The flask was partially immersed in a  
182 water bath (37°C). A chloroform solution containing DOTAP, DOPE and Cholesterol  
183 (3:4:3 mole ratio) was injected in portions (0.5 mL each) via the feed-line using a syringe  
184 and by releasing the vacuum for a few seconds using a valve fitted on top of the condenser  
185 to allow lipid solution to be drawn through the feed-line and be sprayed onto the sorbitol  
186 carrier particles. After each addition, complete evaporation of chloroform was allowed  
187 before injecting the next portion. After solvent was completely evaporated, the solid  
188 particles of proliposomes were collected and stored in glass vials in the freezer (-18°C).  
189 Proliposomes were hydrated with water to form liposomes (1 mg/mL) followed by probe-  
190 sonication. The sonicated cationic liposomes (CL) were mixed with siRNA in RNase-free  
191 water using vortex-mixing and incubated for 30 min at room temperature to form CL-  
192 siRNA complexes.

193

### 194 **2.3. Scanning electron microscopy (SEM) of lipid film coated proliposomes**

195 Microparticles made by coating sorbitol carrier with lipid film were positioned onto a  
196 carbon pad (Agar Scientific, UK), and coated with a thin film of gold using the sputter  
197 coater of the microscope (Bio-Rad, England). The morphology of the resultant  
198 microparticles was investigated under vacuum using the Quanta 200 scanning electronic  
199 microscope.

200

### 201 **2.4. Size analysis and zeta potential studies of CL-siRNA**

202 Size analysis and zeta-potential studies of CL and CL-siRNA complexes were conducted  
203 using Photon Correlation Spectroscopy (PCS) and laser Doppler velocimetry, respectively.  
204 The studies were performed using the Malvern ZetaSizer Nano ZS90 (Malvern Instruments  
205 Ltd, UK) upon selecting the right software for each type of analysis. Size and size  
206 distribution were expressed by the instrument as the mean hydrodynamic diameter and  
207 polydispersity index (PDI), respectively.

208

### 209 **2.5. Cell Culture Studies**

210 Human alveolar adenocarcinoma, A549 lung cancer cell line was obtained from American  
211 Type Culture Collection (Rockville, MD, USA). A549 cell line was cultured in RPMI 1640  
212 medium (HyClone, USA) supplemented with 10% fetal bovine serum (Minhai, China), 100

213 U/ml penicillin, 100 mg/ml streptomycin. Cell culture was performed in an incubator  
214 maintained at 37°C in a humidified environment containing 5% CO<sub>2</sub>.

215

216

## 217 **2.6. Agarose gel retardation assay**

218 To confirm formation of the complexes, the agarose gel retardation assay was employed to  
219 select the optimal charge ratio between cationic liposomes and negatively charged siRNA.  
220 CL-siRNA complexes were prepared at various molar ratios, and then run through a 2%  
221 agarose gel. The mobility of siRNA complexed with cationic liposomes was visualized by  
222 GoldView staining.

223

## 224 **2.7. RNase protection assay of siRNA in cationic liposome complexes**

225 An ideal siRNA delivery system is expected to protect siRNA against RNase enzymatic  
226 degradation. In order to monitor siRNA degradation by nuclease, firstly, siRNA-CL  
227 complexes were prepared at a final siRNA concentration of 5 µM and then incubated in the  
228 presence of 0.1mg/ml RNase for 30 min at 37°C. An aliquot (20 µl) was removed and snap-  
229 frozen at -80°C at every time point. All samples were thawed on ice when they were  
230 collected and immediately mixed with 5 µl of a 100 mM Triton X100 solution and 25 µl of  
231 RNA-extraction mixture (phenol/chloroform/isoamyl alcohol; 25:24:1). The siRNA was

232 precipitated with ethanol, electrophoresis was performed on agarose gel (2%) and  
233 visualization took place by GoldView staining (Buyens et al., 2008).

234

235

## 236 **2.8. Cell model with high expression of CYP1A1**

237 For the purpose of simulating the gene induction pathway to obtain a cell model with high  
238 CYP1A1 gene expression, an induction assay was carried out on A549 cells which are  
239 common for CYP1A1 gene research (Fazili et al., 2010). Cells were seeded in 12-well  
240 plates at a density of  $1 \times 10^4$  cells per well, followed by 24 h incubation at 37°C in a  
241 humidified environment containing 5% CO<sub>2</sub>, and cells were treated with 3-MC with a final  
242 concentration of 5 µM for further 24 h (3-MC was dissolved in DMSO). 3-MC is one of the  
243 most potent PAH carcinogens, which is usually used in the induction of CYP1A1 via the  
244 AhR mechanism (Abdelrahim et al., 2003). After the induction, the induced cells were  
245 collected and used in the subsequent experiments.

246

## 247 **2.9. Cellular uptake of siRNA in induced A549 lung cancer Cells**

248 Transfection of FAM-siRNA (what is FAM-siRNA) was performed in induced A549 cells.  
249 The induced A549 cells were seeded at a concentration of  $5 \times 10^5$  cells per well in six-well  
250 plates. The cells were grown to a confluency between 60% and 80% and washed with pre-

251 warmed (37°C ) PBS, and then they were incubated with 100 nM liposome-free FAM-  
252 siRNA or 100 nM FAM-siRNA-liposome complexes in serum-free medium. Following  
253 incubation for 4 h, the medium was replaced and the cells were washed with PBS twice,  
254 and then analyzed using flow cytometry (Beckman Coulter, USA) and examined under a  
255 fluorescence microscope. siRNA complexed with Lipofectamine2000 (Lipo2000) was used  
256 as a positive control in the experiments.

257

#### 258 **2.10. Silencing of 3-MC induced CYP1A1 gene in A549 lung cancer cell line**

259 For evaluation of the mRNA of CYP1A1 gene *in vitro* and *in vivo*, RNA was extracted  
260 from cells 24 h after transfection with CYP1A1-specific siRNA (n = 3) or from A549 lung  
261 tumor (n = 3), respectively, using RNA prep Pure cell kit. cDNA was then obtained by  
262 reverse transcription of the total RNA using the TIANscript RT kit and the CYP1A1 (sense,  
263 5'-GGCCUGAAGAAUCCACCAG-3'; antisense, 3'-CUGGUGGAUUCUUCAGGCC-5').  
264 mRNA levels were analyzed using the SosoFast™ EvaGreen Supermix on iCycler iQ™ 5  
265 system (Bio-Rad, USA) and  $\beta$ -actin was used as internal control. The PCR reaction was  
266 conducted at 95°C for 3 min followed by 40 cycles of 95°C for 5 s, and 56°C for 10 s in the  
267 iQ™5 Real-Time PCR Detection System. The expression of CYP1A1 was analyzed and  
268 normalized using the  $2^{\Delta Ct}$  method relative to the expression of  $\beta$ -actin.

269

**270 2.11. CYP1A1 enzyme assays**

271 To further study the silencing effect of siRNA on CYP1A1, the enzyme activity as well as  
272 the content of CYP1A1 was assessed. The CYP1A1 enzyme activity was determined by  
273 Human CYP1A1 fluorescence quantitative detection kits (Genmed Scientifics INC.USA).  
274 The CYP1A1 enzyme content was measured using Human CYP1A1 ELISA kits (R&D  
275 systems, USA). Both assays were performed following the relevant suppliers' instructions.

276

**277 2.12. Apoptotic assays**

278 To examine the interactions between CYP1A1 gene regulation and the induced growth of  
279 tumor cells, different groups were designed in the cell apoptosis experiment. Induced A549  
280 cells were treated with CYP1A1-specific siRNA (100 nM) or complexed with cationic  
281 liposome in serum-free medium for 4 h and then further incubated in fresh completed  
282 medium. Cells were washed with PBS and digested in trypsin for suspension after  
283 incubation for 48 h, followed by double staining with FITC-Annexin V and propidium  
284 iodide using the cell apoptotic analysis kit (Beyotime, China) following the manufacturer's  
285 instructions. Flow cytometry was used for investigation of cell apoptosis (n = 3). Further  
286 studies were performed to investigate the apoptosis mechanism. Caspases are the critical  
287 proteins responsible for apoptosis. These proteins are classified as initiators or executioners  
288 depending on their point of entry into the apoptotic cascade. It has been confirmed that



289 there were two main apoptosis pathways mediated by caspases (Boatright and Salvesen,  
290 2003). Among all the family members in this pathway, caspase 3 was considered as the  
291 final executioner, and meanwhile, caspase 8 and caspase 9 are the key initiator proteins  
292 which exist in the extrinsic and intrinsic apoptotic pathways, respectively. The three  
293 caspases were firstly detected with Caspase Activity Assay Kits (Beyotime, China).

294

### 295 **2.13. Multicellular tumor spheroids (MCTSs) assays**

296 Multicellular tumor spheroids (MCTSs) may provide an appropriate model to identify the  
297 drug effect *in vitro* for its similarity to the tumor formation *in vivo* (Friedrich et al., 2009).

298 A549 cells were cultured in a modified tumor sphere medium. The medium is comprised of  
299 recombinant fibroblast growth factor (EGF) (10ng/ml), basic fibroblast growth factor  
300 (bFGF) (10ng/ml) and insulin (4U/L), and plated at a density of  $2 \times 10^3$  cells per well in 6-  
301 well plates. Spheres were formed after 8-10 days incubation. After 24 h 3-MC induction,  
302 spheres were treated with different groups of siRNA which were described in the gene  
303 silence study at a siRNA final dose of 100 nM for 4 h. After further 72 h incubation, the  
304 results were observed by microscope.

305

### 306 **2.14. In vivo efficacy of targeting CYP1A1 gene using liposomes generated from lipid** 307 **film-coated proliposomes**

308 The animal study protocol was approved by Institutional Animal Care and Use Committee  
309 of the Sichuan University in China. Male BALB/c nude mice (weighing 20-23 g) were used  
310 to investigate the antitumor efficacy of targeting CYP1A1 gene *in vivo*. Briefly,  $1 \times 10^7$   
311 A549 cells were re-suspended in 200  $\mu$ l serum-free RPMI 1640 medium and injected  
312 subcutaneously into the right flank of the nude mice. After 5 weeks tumor-bearing mice  
313 were randomly divided into four treatment groups (5 animals each). At days 1, 4, 7, 10, 13  
314 and 16, mice were intratumorally injected with 100  $\mu$ l 10% 3-MC solution. Then at days 2,  
315 5, 8, 11, 14 and 17, mice were intratumorally injected again but with PBS, free siRNA or  
316 CL-siRNA. Every treatment was based on the dose of 40  $\mu$ g siRNA per mouse. Calipers  
317 were used in this work to measure the tumor progression of every mouse. Tumor volumes  
318 were calculated as length $\times$ width $\times$ width $\times$ 0.5(mm<sup>3</sup>). At the day 18, three animals from each  
319 group including control were sacrificed, and the tumors were excised. The measurements of  
320 CYP1A1 gene silencing effect were conducted as described earlier.

321

### 322 **2.15. Statistical analysis**

323 Values were presented as mean ( $\pm$  SD) unless otherwise stated. The differences between  
324 groups were analyzed using the Student's *t*-tests and one-way analysis of variance  
325 (ANOVA) with Bonferroni tests for multiple-group analysis. A probability level of  $P <$   
326 0.05 was considered to indicate significant difference between the groups.

327

328

329

### 330 3. RESULTS

#### 331 3.1. Physical characterization of proliposomes, cationic liposomes (CL) and CL- 332 siRNA complexes

333 The surface morphology of proliposome powders prepared through coating sorbitol  
334 particles with lipid film was examined by scanning electron microscopy (SEM) (Figure 1).

335 The high porosity of sorbitol (Figure 1a) facilitated coating of the lipid on the carrier  
336 surfaces (Figure 1b). Our SEM observations using cationic lipids to coat sorbitol particles

337 is in concordance with the previous findings using neutral lipids such as  
338 dimyristoylphosphatidylcholine coated onto sorbitol particles (Payne et al., 1986). Our

339 study also further confirms that sorbitol is a highly suitable carrier for coating with lipid  
340 films and preparation of proliposomes because of its microporous structure. In another

341 study, we demonstrated that the film coating proliposome technology can be scaled up  
342 using fluid-bed coating equipment that can deposit a lipid film on carbohydrate particles

343 (e.g. sucrose), generating liposomes that can successfully entrap conventional small  
344 molecules, such as the antiasthma steroid beclometasone dipropionate (Gala et al., 2015). In

345 the present investigation, through a smaller scale of manufacturing using a modified rotary  
346 evaporator equipped with a feed tubeline, proliposomes made by coating sorbitol with  
347 cationic lipids were prepared. Upon hydration (including or excluding siRNA) and probe-  
348 sonication, cationic liposomes were generated. The measured size of the siRNA-free  
349 vesicles was as small as  $85\pm 3.2$  nm and the size distribution, expressed by PDI, was as low  
350 as 0.165. The uniform coating of sorbitol particles (Figure 1b) justifies the facilitated  
351 generation of liposomes in the nano-size range and the narrow size distribution (i.e. low  
352 PDI) (Figure 1d). Transmission electron microscopy (TEM) images confirmed the uniform  
353 round shape of the gene-free cationic liposomes, which were also similar to those  
354 incorporating siRNA, suggesting that the genetic material was complexed with the  
355 liposomes, with no apparent formation of siRNA aggregates (Figure 1c). 1,2-dioleoyl-3-  
356 trimethylammonium-propane (DOTAP) in the formulation conferred the liposomes with a  
357 positive surface charge of about +43 mV (sorbitol solution pH=7.5) (Figure 1d). For  
358 formulations incorporating siRNA, the integrity of siRNA was studied (Figure 1e). Varying  
359 charge ratios of CL to siRNA (N/P ratio) were prepared at fixed siRNA concentration (100  
360 nM). With the N/P ratio higher than 4, the migration of siRNA was completely retarded,  
361 indicating good binding efficiency of CL with siRNA and successful formation of the  
362 complexes (Figure 1e, f). On the other hand, size and zeta potential of CL-siRNA  
363 complexes were 90 nm and +30 mV, respectively at the N/P mole ratio of 4:1 (Figure 1d),

364 which contributed to the good dispersion properties and stability of CL-siRNA complexes.  
365 Considering all the results above, N/P = 4:1 was chosen as the optimal charge ratio for CL-  
366 siRNA complex formation.

367

368 Agarose gel assay is an established technique for checking the formation of complexes  
369 between liposomes and genetic materials (e.g. siRNA) (Kim et al., 2010a). To assess the  
370 ability of liposomes to protect siRNA from degradation, the stability of siRNA in RNase  
371 solution was tested. As shown in Figure 1f, free siRNA was completely degraded upon  
372 exposure to RNase. By contrast, when siRNA was incorporated into cationic liposomes, the  
373 genetic material was intact for at least 4 h, indicating that liposomes have provided short-  
374 term protection for siRNA against enzymatic degradation. In this study, we made powdered  
375 cationic formulations of proliposomes by film coating the sorbitol sugar with cationic lipid.  
376 This can readily generate liposomes complexing with siRNA via addition of aqueous phase  
377 and sonication just on the day of administration; hence, storage instability of liposome  
378 dispersions is avoided.

379

380 **3.2. Stimulation of CYP1A1 gene expression by 3-MC treatment in A549 lung cancer**  
381 **cell line**

382 After incubation with 3-MC (5  $\mu$ M) for 24 h, the induced A549 cells were collected to  
383 investigate the target gene CYP1A1 expression level. All samples were analyzed by RT-  
384 PCR, which suggested that the mRNA level of CYP1A1 in induced cells was about 7 times  
385 higher than that in the normal cells. Enhanced CYP1A1 expression was maintained for at  
386 least 48 h after single induction.

387

388 CL were compared with Lipo2000, a commonly used positive control for siRNA delivery,  
389 for evaluation of the siRNA delivering ability. FAM-labeled siRNA was prepared alone or  
390 mixed with CL or Lipo2000 at a final concentration of 100 nM. Both flow cytometry and  
391 confocal microscopy were used to investigate the uptake efficiency of the liposomes in  
392 A549 cells. The results indicated that both CL and Lipo2000 effectively delivered siRNA to  
393 cells (Figure 2a), and significantly improved the uptake efficiency compared with free  
394 siRNA solution (Figure 2b).

395

### 396 **3.3. Silencing of CYP1A1 gene expression in A569 lung cancer cell line**

397 Transfection of induced A549 cells with CL-CYP1A1-siRNA caused a 7-fold down-  
398 regulation of CYP1A1 gene expression. Similar results were obtained with transfection  
399 using CYP1A1-siRNA Lipo2000 control. On the other hand, free (i.e. naked) CYP1A1-  
400 siRNA and negative control siRNA (NC siRNA) did not show any marked silencing effect

401 on CYP1A1 gene expression (Figure 3a). All agents were tested in the induced cells, and  
402 the unstimulated A549 cells were used as a negative control. The silencing effects of CL-  
403 CYP1A1-siRNA on CYP1A1 protein levels and enzymatic activity was also seen (Figure  
404 3b and 3c), confirming successful retardation of gene expression target. CL-siRNA  
405 prepared using the film-coating proliposome technology caused a similar knockdown  
406 efficiency compared to the positive control Lipo2000. This clearly demonstrates that the  
407 facile approach of generating CL-siRNA using the proliposome technology was successful  
408 at providing a more stable powdered formulation than conventional liposomes. It was also  
409 capable of retarding the gene expression in levels similar to those of the established  
410 Lipo2000 transfection reagent.

411

#### 412 **3.4. Knockdown of CYP1A1 gene induces apoptotic cell death in 3MC- treated A549** 413 **cells**

414 The number of apoptotic cells was quantified by FITC-Annexin V and propidium iodide  
415 (PI) double-staining. CL-siRNA triggered apoptosis in induced A549 cells (Fig.4a). 3-MC  
416 induced cells without further treatment were used as the negative control in these  
417 experiments in order to eliminate the inducer influence on the results. Findings revealed  
418 that 3-MC induction had a little impact on the cellular growth, whereas the induced cells

419 tended to undergo apoptosis with CYP1A1 silencing through the intrinsic apoptotic  
420 pathway marked by elevated caspase 3 and caspase 9, but not caspase 8, activities (Figure  
421 4B), also confirmed by direct immunostaining (data not shown).

422

### 423 **3.5. The Effect of CYP1A1 gene silencing on sphere formation in A549 lung cancer** 424 **cell line**

425 Sphere formation assay was performed to investigate the effect of CYP1A1 silencing on  
426 formation of spheroid colonies *in vitro*. Untreated induced A549 cells successfully  
427 produced spheroid colonies when cultured in a modified tumor sphere medium. On the  
428 other hand, spheres treated with CYP1A1 siRNA delivered by cationic liposomes or  
429 Lipo2000 formulation showed a suppressive effect on the formation of sphere colonies. The  
430 other groups including those untreated and mock did not exhibit this effect (Figure 5).

431

### 432 **3.6. Antitumor efficacy of gene silencing of CYP1A1 in tumor-bearing nude mice** 433 **using particulate-based proliposome technology**

434 In order to investigate the impact of CYP1A1 silencing on tumor progression *in vivo*, we  
435 determined the antitumor efficacy of CL-siRNA in A549 xenograft nude mice model  
436 (Figure 6a). Results showed that growth rate of tumor with cationic liposome or Lipo2000



437 was significantly slower than that observed in the control groups including animals injected  
438 with PBS or naked (free) siRNA (Figure 6b/c). Moreover, the treatment caused down-  
439 regulation of the expression of CYP1A1 gene in the tumors as detected by RT-PCR on the  
440 third day after giving the intratumoral dose (Figure 6d). Thus, the reduction of CYP1A1  
441 gene in induced A549 cells mediated by siRNA gave a significant tumor growth inhibition.

442

#### 443 **4. DISCUSSION**

444 In this study we report that liposome-based gene delivery technology is a viable and stable  
445 approach for targeting the cancer causing gene CYP1A1. A major issue for liposomes is  
446 their instability as liquid dispersion, commonly when prepared using the thin-film  
447 hydration technique (Grit and Crommelin, 1993). This was overcome in the present study  
448 by using the film-coating proliposome technology to prepare powdered lipid formulations  
449 that, when needed, can be used to generate CL-siRNA complexes.

450 This technology, as demonstrated in our study, can potentially be considered for therapeutic  
451 intervention and treatment of lung cancer, one of the most common types of cancer and a  
452 leading cause of death (Torre et al., 2015). This approach comes as part of ongoing efforts  
453 to ameliorate the outcomes related to the undesirable pharmaceutical, pharmacokinetic and  
454 pharmacodynamic properties of lung cancer drugs, such as solubility, toxicity, stability, and

455 lack of selective effect on the cancerous cells (Tiwari et al., 2012). These properties can be  
456 enhanced by using drug vectors that are highly biocompatible and biodegradable  
457 (Zarogouldis et al., 2012).

458 Continuous exposure to tobacco smoking can induce the expression of CYP1A1, a gene  
459 present in extra hepatic tissues (Androustopoulos et al., 2009), that is involved in the  
460 metabolic activation of PAH produced from tobacco smoking. After the induction, high  
461 CYP1A1 gene expression can contribute to the carcinogenic derivatives production and  
462 may initiate neoplastic transformation (Whitlock, 1999). Stimulated bronchial epithelial  
463 cells express high levels of CYP1A1 gene when induced by tobacco or environmental  
464 pollutants, predisposing them to lung cancer (Mercer et al., 2006). Hence, A549 human  
465 alveolar basal epithelial cell line represents a valuable model for the mechanistic studies  
466 involving induction of the pulmonary CYP system (Giard et al., 1973). In this study, we  
467 constructed a cell model on the basis of AhR mechanism through which CYP1A1 can be  
468 activated to a high level using 3-MC as previously reported (Hukkanen et al., 2000). In the  
469 induced cells, a high CYP1A1 gene expression was observed, similar to that seen in  
470 cancerous cells exposed to air contaminants. In our study, the 3-MC concentration was  
471 optimized to exhibit low toxicity and relatively high induction efficiency.

472 Limited studies have reported the relationship between inhibition of CYP1A1 gene and  
473 lung cancer therapy (Androutsopoulos et al., 2009). Flavonoid (such as quercetin), for  
474 example, was previously reported to inhibit CYP1A1 induction (Ciolino and Yeh, 1999). In  
475 the present work, we used RNAi as the inhibition strategy in lung cancer cells. Successful  
476 therapy using siRNA depends on effective delivery and protection against RNase. Owing to  
477 its large molecular weight and anionic nature, the uptake of siRNA by cancer cells is very  
478 poor, making the use of appropriate delivery systems highly advantageous (Gala et al.,  
479 2015). To overcome these issues, we prepared cationic liposomes via the lipid-coating  
480 proliposome technology shown previously to be suitable for large scale production (Gala et  
481 al., 2015). Using fluidized bed coating, the solid proliposomes produced can be stored at -  
482 18°C until needed for subsequent generation of liposomes, providing stability for several  
483 months (data not shown). In addition mass production and storage stability of proliposomes  
484 (as liposome precursors), the cationic liposomes were able to protect siRNA from nucleases  
485 and facilitated efficient transportation of siRNA into the cytoplasm, resulting in gene  
486 silencing effects similar to those exhibited by the commercially established Lipo2000.  
487 Indeed, both our *in vitro* and *in vivo* results indicated that CYP1A1 gene silencing by  
488 siRNA can regulate the cancer in the induced cells. Our data showed that the down-  
489 regulation of *CYP1A1* gene induced cellular apoptosis and interfered with the formation of  
490 tumor spheres *in vitro* and inhibited tumor development in BALB/c nude xenograft model.

491 Various murine models were established for the evaluation of novel therapeutics and  
492 examination of the molecular mechanisms underlying transformation, invasion and  
493 metastasis (Kellar et al., 2015). The A549 xenograft model was chosen in this study for the  
494 convenience of tumor measurement by making the cancer cells readily accessible (Kellar et  
495 al., 2015). Therefore *in vivo* results remain preliminary in nature and inconclusive.  
496 However, the emerging data confirm the validity of CL-siRNA-CYP1A1 as a proof of  
497 concept for targeting lung cancer, future experiments will explore different experimental  
498 designs including optimizing dosage and scheduling regimen to improve efficacy.

499

## 500 **5. CONCLUSION**

501 This study has shown that CYP1A1 gene can be a potential target for treatment of lung  
502 cancer. Cationic liposomes generated from film-coated proliposomes provided excellent  
503 siRNA carriers, with subsequent ability to silence the CYP1A1 gene both *in vitro* and *in*  
504 *vivo*. Further investigations to evaluate the aerosolization properties of CL-siRNA in animal  
505 models using the proliposome approach are warranted. This study will open the doors to  
506 further investigations in multiple therapeutic directions in the field of drug delivery and  
507 cancer treatment.

508

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#### 516 **DECLARATION OF CONFLICTS OF INTERESTS**

517 The authors declare no conflicts of interests.

518

#### 519 **REFERENCES**

520

521 Abdelrahim, M., Smith, R., 3rd, Safe, S., 2003. Aryl hydrocarbon receptor gene silencing  
522 with small inhibitory RNA differentially modulates Ah-responsiveness in MCF-7 and  
523 HepG2 cancer cells. *Molecular pharmacology* 63, 1373-1381.

524

525 Agrawal, N., Dasaradhi, P.V., Mohmmed, A., Malhotra, P., Bhatnagar, R.K., Mukherjee,  
526 S.K., 2003. RNA interference: biology, mechanism, and applications. *Microbiology and*  
527 *molecular biology reviews* : MMBR 67, 657-685.

528

529 Ahn, B.N., Kim, S.K., Shim, C.K., 1995. Preparation and evaluation of proliposomes  
530 containing propranolol hydrochloride. *Journal of microencapsulation* 12, 363-375.

531

532 Androutsopoulos, V.P., Tsatsakis, A.M., Spandidos, D.A., 2009. Cytochrome P450  
533 CYP1A1: wider roles in cancer progression and prevention. *BMC cancer* 9, 187.

534

- 535 Armstrong, B., Hutchinson, E., Unwin, J., Fletcher, T., 2004. Lung cancer risk after  
536 exposure to polycyclic aromatic hydrocarbons: a review and meta-analysis. *Environmental*  
537 *health perspectives* 112, 970-978.  
538
- 539 Boatright, K.M., Salvesen, G.S., 2003. Mechanisms of caspase activation. *Current Opinion*  
540 *in Cell Biology* 15, 725-731.  
541
- 542 Brambilla, E., Gazdar, A., 2009. Pathogenesis of lung cancer signalling pathways: roadmap  
543 for therapies. *The European respiratory journal* 33, 1485-1497.  
544
- 545 Bruno, R.D., Njar, V.C., 2007. Targeting cytochrome P450 enzymes: a new approach in  
546 anti-cancer drug development. *Bioorganic & medicinal chemistry* 15, 5047-5060.  
547
- 548 Buyens, K., Lucas, B., Raemdonck, K., Braeckmans, K., Vercammen, J., Hendrix, J.,  
549 Engelborghs, Y., De Smedt, S.C., Sanders, N.N., 2008. A fast and sensitive method for  
550 measuring the integrity of siRNA-carrier complexes in full human serum. *Journal of*  
551 *controlled release : official journal of the Controlled Release Society* 126, 67-76.  
552
- 553 Chen, C.M., Alli, D., 1987. Use of fluidized bed in proliposome manufacturing. *Journal of*  
554 *pharmaceutical sciences* 76, 419.  
555
- 556 Ciolino, H.P., Yeh, G.C., 1999. The flavonoid galangin is an inhibitor of CYP1A1 activity  
557 and an agonist/antagonist of the aryl hydrocarbon receptor. *British journal of cancer* 79,  
558 1340-1346.  
559
- 560 Elhissi, A.M., Ahmed, W., Taylor, K.M., 2012. Laser diffraction and electron microscopy  
561 studies on inhalable liposomes generated from particulate-based proliposomes within a  
562 medical nebulizer. *J. Nanosci. Nanotechnol.* 12, 6693-6699.  
563
- 564 Elhissi, A.M., Karnam, K.K., Danesh-Azari, M.R., Gill, H.S., Taylor, K.M., 2006.  
565 Formulations generated from ethanol-based proliposomes for delivery via medical  
566 nebulizers. *J. Pharm. Pharmacol.* 58, 887-894.  
567
- 568 Fazili, I.S., Jiang, W., Wang, L., Felix, E.A., Khatlani, T., Coumoul, X., Barouki, R.,  
569 Moorthy, B., 2010. Persistent induction of cytochrome P4501A1 in human hepatoma cells  
570 by 3-methylcholanthrene: evidence for sustained transcriptional activation of the CYP1A1  
571 promoter. *The Journal of pharmacology and experimental therapeutics* 333, 99-109.  
572

- 573 Field, R.W., Withers, B.L., 2012. Occupational and environmental causes of lung cancer.  
574 Clinics in chest medicine 33, 681-703.  
575
- 576 Friedrich, J., Seidel, C., Ebner, R., Kunz-Schughart, L.A., 2009. Spheroid-based drug  
577 screen: considerations and practical approach. Nature protocols 4, 309-324.  
578
- 579 Gala, R.P., Khan, I., Elhissi, A.M., Alhnan, M.A., 2015. A comprehensive production  
580 method of self-cryoprotected nano-liposome powders. Int. J. Pharm. 486, 153-158.  
581
- 582 Giard, D.J., Aaronson, S.A., Todaro, G.J., Arnstein, P., Kersey, J.H., Dosik, H., Parks,  
583 W.P., 1973. In vitro cultivation of human tumors: establishment of cell lines derived from a  
584 series of solid tumors. Journal of the National Cancer Institute 51, 1417-1423.  
585
- 586 Grit, M., Crommelin, D.J., 1993. Chemical stability of liposomes: implications for their  
587 physical stability. Chemistry and physics of lipids 64, 3-18.  
588
- 589 Guigal, N., Seree, E., Bourgarel-Rey, V., Barra, Y., 2000. Induction of CYP1A1 by serum  
590 independent of AhR pathway. Biochemical and biophysical research communications 267,  
591 572-576.  
592
- 593 Hecht, S.S., 1999. Tobacco smoke carcinogens and lung cancer. Journal of the National  
594 Cancer Institute 91, 1194-1210.  
595
- 596 Hukkanen, J., Lassila, A., Paivarinta, K., Valanne, S., Sarpo, S., Hakkola, J., Pelkonen, O.,  
597 Raunio, H., 2000. Induction and regulation of xenobiotic-metabolizing cytochrome P450s  
598 in the human A549 lung adenocarcinoma cell line. American journal of respiratory cell and  
599 molecular biology 22, 360-366.  
600
- 601 Hwang, B.-Y., Jung, B.-H., Chung, S.-J., Lee, M.-H., Shim, C.-K., 1997. In vitro skin  
602 permeation of nicotine from proliposomes. Journal of controlled release 49, 177-184.  
603
- 604 Jahn, A., Song, C.K., Balakrishnan, P., Hong, S.-S., Lee, J.-H., Chung, S.-J., Kim, D.-D.,  
605 2014. AAPE proliposomes for topical atopic dermatitis treatment. Journal of  
606 microencapsulation 31, 768-773.  
607
- 608 Jung, B.H., Chung, B.C., Chung, S.-J., Lee, M.-H., Shim, C.-K., 2000a. Prolonged delivery  
609 of nicotine in rats via nasal administration of proliposomes. Journal of controlled release  
610 66, 73-79.

- 611  
612 Jung, B.H., Chung, B.C., Chung, S.J., Lee, M.H., Shim, C.K., 2000b. Prolonged delivery of  
613 nicotine in rats via nasal administration of proliposomes. *Journal of controlled release* :  
614 official journal of the Controlled Release Society 66, 73-79.
- 615  
616 Katare, O.P., Vyas, S.P., Dixit, V.K., 1990. Effervescent granule based proliposomes of  
617 ibuprofen. *Journal of microencapsulation* 7, 455-462.
- 618  
619 Kellar, A., Egan, C., Morris, D., 2015. Preclinical murine models for lung cancer: Clinical  
620 trial applications. *BioMed Research International*, Article ID 621324, 17 pages.
- 621  
622 Khan, I., Yousaf, S., Subramanian, S., Albed Alhnan, M., Ahmed, W., Elhissi, A., 2018.  
623 Proliposome tablets manufactured using a slurry-driven lipid-enriched powders:  
624 Development, characterization and stability evaluation. *Int. J. Pharm.* 538, 250-262.
- 625  
626 Khurana, B., Goyal, A.K., Budhiraja, A., Aora, D., Vyas, S.P., 2013. Lipoplexes versus  
627 nanoparticles: pDNA/siRNA delivery. *Drug delivery* 20, 57-64.
- 628  
629 Kim, H.-K., Davaa, E., Myung, C.-S., Park, J.-S., 2010a. Enhanced siRNA delivery using  
630 cationic liposomes with new polyarginine-conjugated PEG-lipid. *International journal of*  
631 *pharmaceutics* 392, 141-147.
- 632  
633 Kim, H.K., Davaa, E., Myung, C.S., Park, J.S., 2010b. Enhanced siRNA delivery using  
634 cationic liposomes with new polyarginine-conjugated PEG-lipid. *International journal of*  
635 *pharmaceutics* 392, 141-147.
- 636  
637 Kumar, R., Gupta, R.B., Betageri, G.V., 2001. Formulation, characterization, and in vitro  
638 release of glyburide from proliposomal beads. *Drug delivery* 8, 25-27.
- 639  
640 Lorenzer, C., Dirin, M., Winkler, A.M., Baumann, V., Winkler, J., 2015. Going beyond the  
641 liver: progress and challenges of targeted delivery of siRNA therapeutics. *J. Cont. Rel.* 203,  
642 1-15.
- 643  
644 Mercer, B.A., Lemaitre, V., Powell, C.A., D'Armiento, J., 2006. The Epithelial Cell in  
645 Lung Health and Emphysema Pathogenesis. *Current Respiratory Medicine Reviews* 2, 101-  
646 142.
- 647



- 648 Mokhtar Ibrahim, M., Tawfique, S.A., Mahdy, M.M., 2013. Liposomal diltiazem HCl as  
649 ocular drug delivery system for glaucoma. *Drug development and industrial pharmacy* 40,  
650 765-773.  
651
- 652 Murakami, A., Ashida, H., Terao, J., 2008. Multitargeted cancer prevention by quercetin.  
653 *Cancer letters* 269, 315-325.  
654
- 655 Park, J.M., Ahn, B.N., Yoon, E.J., Lee, M.G., Shim, C.K., Kim, C.K., 1994. The  
656 pharmacokinetics of methotrexate after intravenous administration of methotrexate-loaded  
657 proliposomes to rats. *Biopharmaceutics & drug disposition* 15, 391-407.  
658
- 659 Payne, N.I., Browning, I., Hynes, C.A., 1986. Characterization of proliposomes. *Journal of*  
660 *pharmaceutical sciences* 75, 330-333.  
661
- 662 Payne, N.I., Cosgrove, R.F., Green, A.P., Liu, L., 1987. In-vivo studies of amphotericin B  
663 liposomes derived from proliposomes: effect of formulation on toxicity and tissue  
664 disposition of the drug in mice. *The Journal of pharmacy and pharmacology* 39, 24-28.  
665
- 666 Ruozi, B., Forni, F., Battini, R., Vandelli, M.A., 2003. Cationic liposomes for gene  
667 transfection. *Journal of drug targeting* 11, 407-414.  
668
- 669 Sak, K., Everaus, H., 2015. Multi-Target Cytotoxic Actions of Flavonoids in Blood Cancer  
670 Cells. *Asian Pacific journal of cancer prevention : APJCP* 16, 4843-4847.  
671
- 672 Shimada, T., Fujii-Kuriyama, Y., 2004. Metabolic activation of polycyclic aromatic  
673 hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. *Cancer science* 95, 1-6.  
674
- 675 Sun, X., Chen, Y., Zhao, H., Qiao, G., Liu, M., Zhang, C., Cui, D., Ma, L., 2018. Dual-  
676 modified cationic liposomes loaded with paclitaxel and survivin siRNA for targeted  
677 imaging and therapy of cancer stem cells in brain glioma. *Drug Deliv.* 25, 1718-1727.  
678
- 679 Tiwari, G., Tiwari, R., Sriwastawa, B., Bhati, L., Pandey, S., Pandey, P., Bannerjee, S.K.,  
680 2012. Drug delivery systems: An updated review. *International journal of pharmaceutical*  
681 *investigation* 2, 2-11.  
682
- 683 Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., Jemal, A., 2015. Global  
684 cancer statistics, 2012. *CA: a cancer journal for clinicians* 65, 87-108.  
685

686 Wang, J.P., Maitani, Y., Takayama, K., Nagai, T., 2000. Pharmacokinetics and antitumor  
687 effect of doxorubicin carried by stealth and remote loading proliposome. *Pharmaceutical*  
688 *research* 17, 782-787.

689

690 Whitlock, J.P., Jr., 1999. Induction of cytochrome P4501A1. *Annual review of*  
691 *pharmacology and toxicology* 39, 103-125.

692

693 Zarogouldis, P., Karamanos, N.K., Porpodis, K., Domvri, K., Huang, H., Hohenforst-  
694 Schimdt, W., Goldberg, E.P., Zarogoulidis, K., 2012. Vectors for inhaled gene therapy in  
695 lung cancer. Application for nano oncology and safety of bio nanotechnology. *International*  
696 *journal of molecular sciences* 13, 10828-10862.

## 697 **FIGURE LEGENDS**

698

699 **FIGURE 1. Characterization of liposome-siRNA complex.** (a) Scanning electron  
700 microscopy images of blank sorbitol and (b) Image of proliposome particles after coating  
701 with the lipid. (c) Transmission electron microscopy image of cationic liposomes generated  
702 from proliposomes. (d) Size and zeta potential of CL-siRNA complex at different cationic  
703 liposome to siRNA ratios. (e) The mobility of siRNA complexed with cationic liposomes at  
704 various molar ratios, ranging from 1-10 liposome to siRNA, by agarose gel retardation  
705 assay visualized by Goldview staining. (f) Stability of CL-siRNA complex against RNase.  
706 Cationic liposomes were complexed with siRNA at different molar ratios to study the  
707 degradation of siRNA by RNase by incubation with RNase at 37<sup>0</sup>C for up to 6 hours. The  
708 CL and siRNA N/P ratio was kept 4:1 in all samples and siRNA alone was used as negative  
709 control.

710

711 **FIGURE 2. Cellular uptake of CL-siRNA by A549 lung cancer cell line. (a)**

712 Representative images of A549 cells transfected with FAM-siRNA, CL-FAM-siRNA or

713 Lipo2000-FAM-siRNA. Cells were treated with 5  $\mu$ M 3-MC to induce CYP1A1 expression

714 then incubated either with 100 nM liposome-free FAM-siRNA or 100 nM FAM-siRNA-

715 liposome complexes in serum-free medium. After transfection, cells were stained with 4',6-

716 diamidino-2-phenylindole (DAPI) and fluorescence images were taken by confocal

717 microscope. (b) The cellular uptake efficiency of CL-siRNA in the induced cells was also

718 measured by flow cytometry (n=3).

719

720 **FIGURE 3. Targeting of CYP1A1 gene using gene silencing approach. A549 lung**

721 cancer cells were treated with 3-MC then transfected with CYP1A1-siRNA using liposome

722 (CL/siRNA) or Lipofectomine 2000 (Lipo2000/siRNA). Non-stimulated A549 cells were

723 used as a negative control whereas 3-MC stimulated A549 transfected with naked siRNA

724 were used as a positive control. (a) Expression of CYP1A1 gene of was analyzed by

725 quantitative RT-PCR using  $\beta$ -actin as internal control. Data are shown as normalized fold

726 expression relative to the untreated control (n = 3), \* p<0.05. (b) CYP1A1 enzyme activity

727 was measured by Human CYP1A1 enzyme activity fluorescence quantitative detection kits

728 (n=3), \* p<0.05. (c) CYP1A1 enzyme content was detected with Human CYP1A1 ELISA  
729 kits (n=3), \*p<0.05.

730

731 **FIGURE 4. Knockdown of CYP1A1 gene causes apoptosis in lung cancer cells.** (a)

732 A549 lung cancer cells were treated with 3-MC for 24 hours then transfected with CL-

733 CYP1A1-siRNA or CYP1A1 lipofectamine-2000. Cells were then stained with fluorescein-

734 conjugated annexin-V and propidium iodide (PI) and analyzed by flow cytometry.

735 Percentages of apoptotic cells are presented as mean  $\pm$  SD (n = 3). (b) Quantification of the

736 active caspase 3, caspase 8 and caspase 9 in 3-MC-induced A549 lung cells transfected

737 with CL- CYP1A1-siRNA as performed by Flow Cytometry using caspase activity assay

738 kits as described in the methods section. Data are presented as mean  $\pm$  SD (n = 3).

739

740 **FIGURE 5. Effect of CYP1A1 gene silencing on A549-mediated spheroid colonies.**

741 Spheroid colonies were generated as described in the methods section. Sphere cells were

742 treated with 3-MC for 24 h and subsequently then transfected with CL-CYP1A1-siRNA or

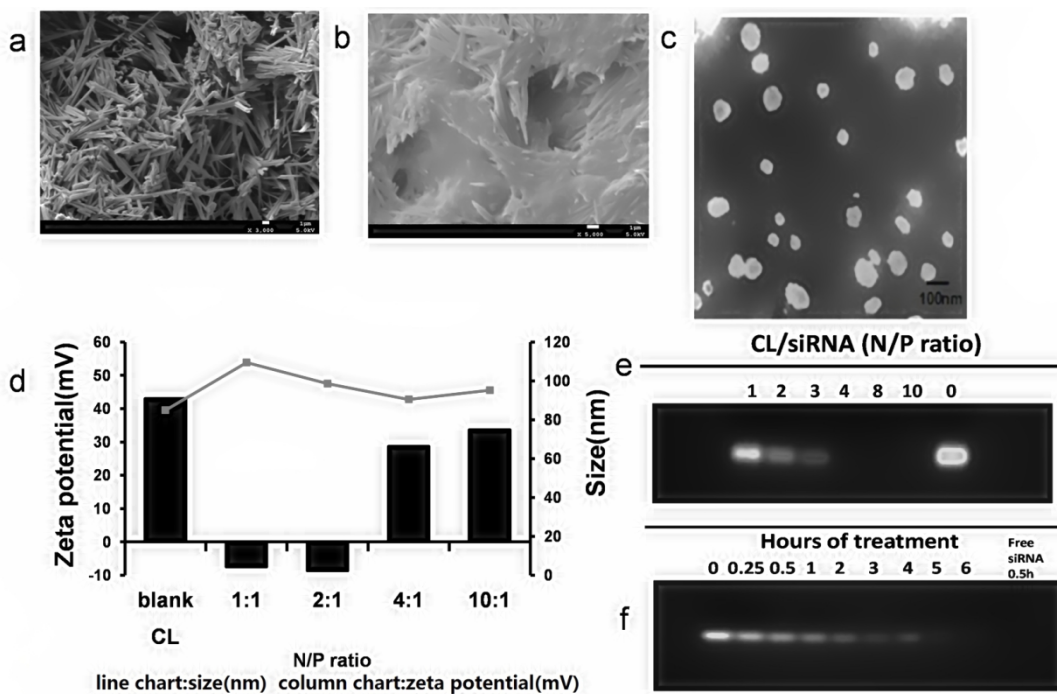
743 CYP1A1 lipofectamine-2000 for 72 h. Representative images shown are from three

744 different experiments.

745

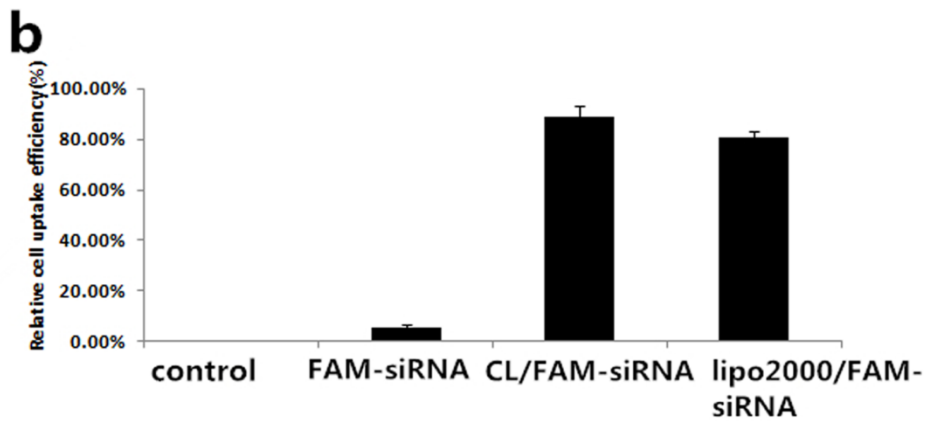
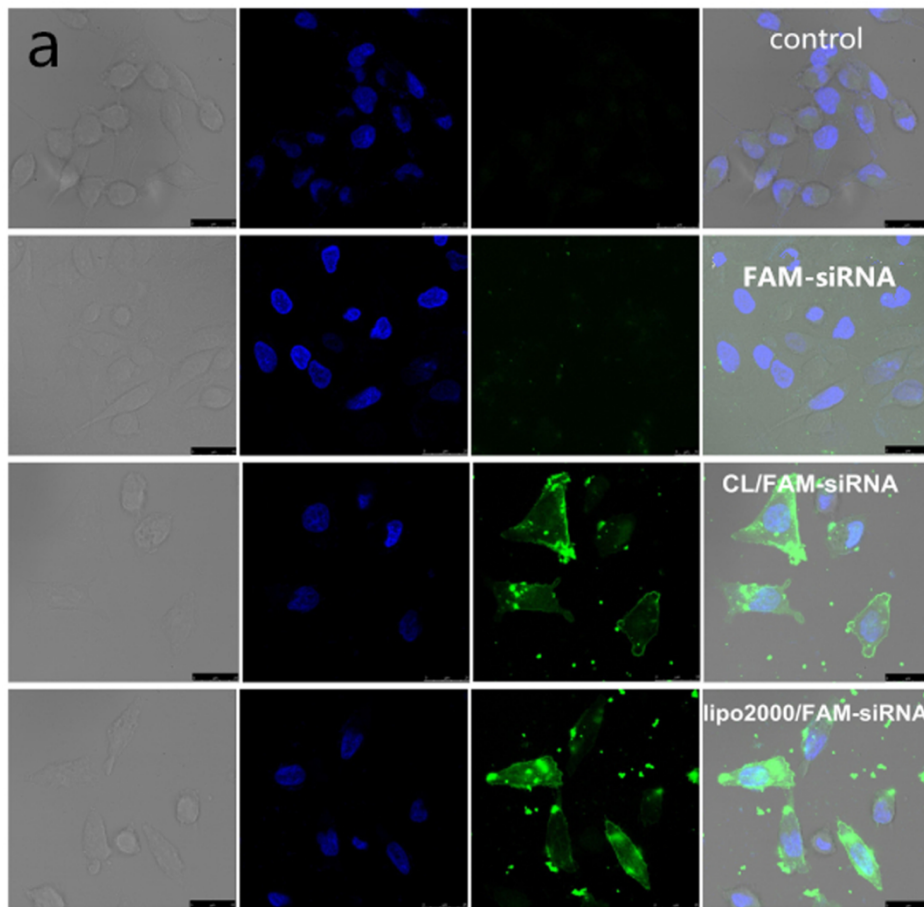
746 **FIGURE 6. Inhibition of tumor growth using CYP1A1-siRNA in mouse model system.**

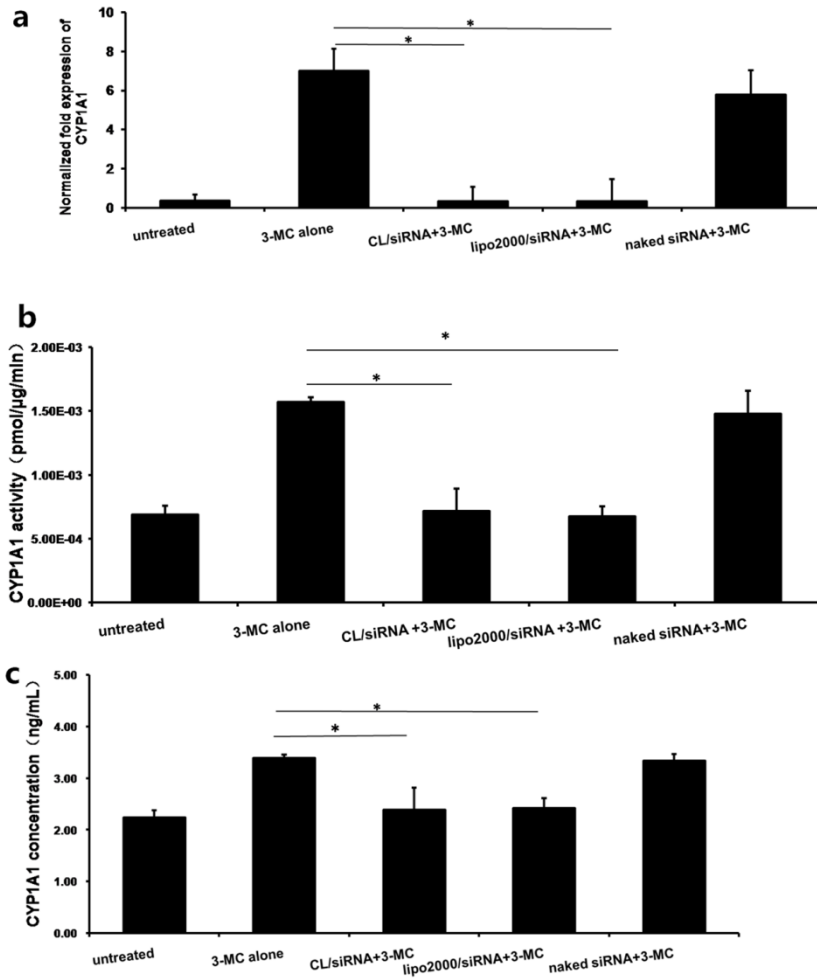
747 BALB/c-nude mice were injected with 10 million A549 cells in serum free medium  
748 subcutaneously into right flank. The tumor bearing mice were divided into four treatment  
749 group (n=5). (a) All mice were injected with 3-MC and after 10 days were treated with (i)  
750 PBS (ii) CL-CYP1A1-siRNA (iii) Naked-siRNA and (iv) Lipofectamin-complexed  
751 CYP1A1-siRNA. (b) The volume of each tumor was measured at the indicated time points  
752 as described in methods. Results are expressed as mean, (n = 5),  $\pm$  SD. (c) Mice were  
753 sacrificed after 18 days with six intratumoural injection of CYP1A1 siRNA and images of  
754 each tumors were taken as shown (n=5). (d) Total RNA were isolated from tumor of each  
755 mice. Expression of CYP1A1 gene were quantified by RT-PCR (Data expressed as mean  $\pm$   
756 SD; n=3).



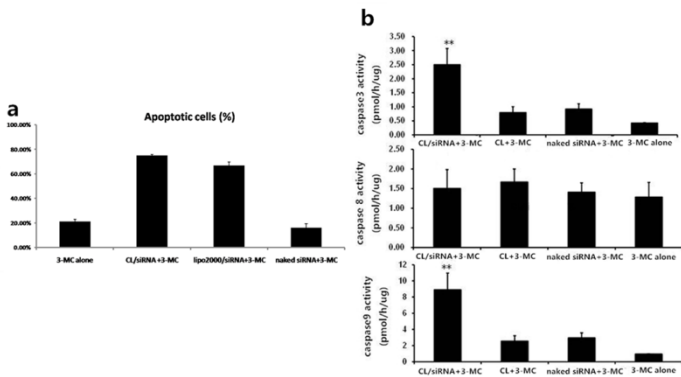
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ACCEPTED MANUSCRIPT





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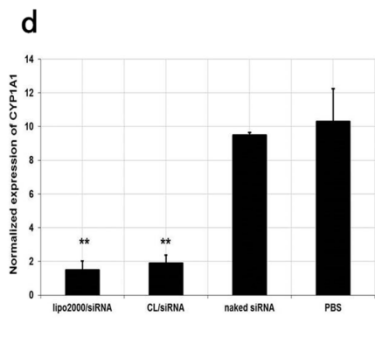
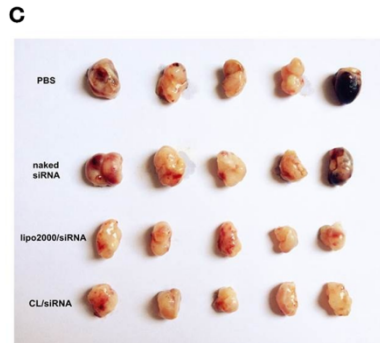
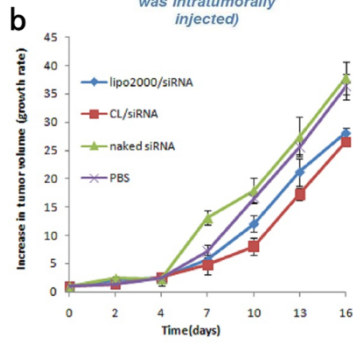
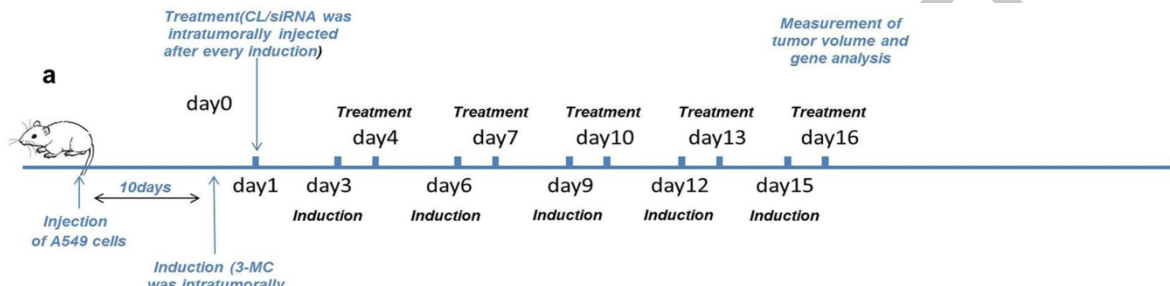
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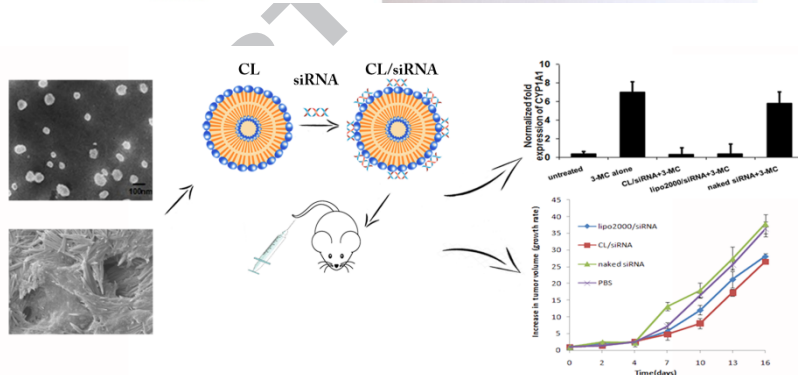


untreated                      mock                      CL/siRNA                      Lipo2000/siRNA

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764 Declaration of interests

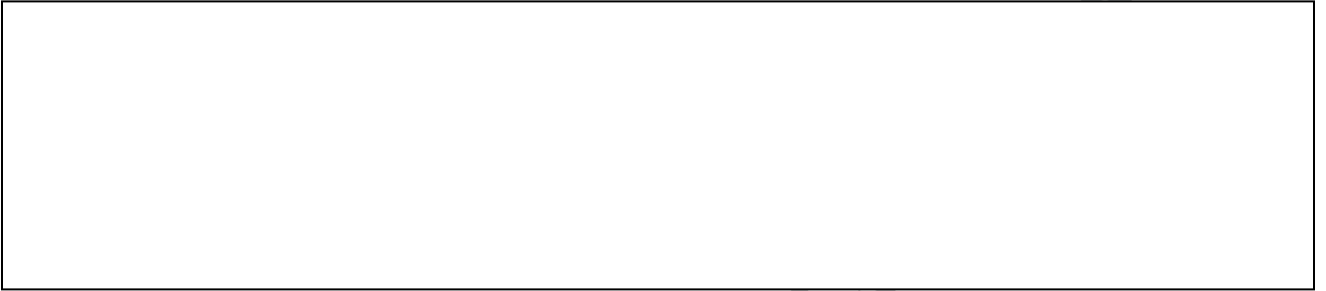
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766 ☒ The authors declare that they have no known competing financial interests or personal  
767 relationships that could have appeared to influence the work reported in this paper.

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769 The authors declare the following financial interests/personal relationships which may be  
770 considered as potential competing interests:

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ACCEPTED MANUSCRIPT