A Novel 1,2-Dihydroquinoline Anticancer Agent and Its Delivery to Tumor Cells Using Cationic Liposomes

XIAOYUAN MA[1], YI WU[1], XUEWEI YANG[4], SHUANG YANG[4], YUHUAN LI[4], YUSHEN HUANG[6], ROBERT J. LEE[4,5], TIAN BAI[3] and YUNGANG LUO[2]

[1]*School of Pharmaceutical Sciences, Jilin University,*
[2]*The Second Hospital of Jilin University, Changchun, Jilin, P.R. China;*
[3]*College of Computer Science and Technology, Jilin University, Changchun, Jilin, P.R. China;*
[4]*School of Life Sciences, Jilin University, Changchun, Jilin, P.R. China;*
[5]*Division of Pharmaceutics, College of Pharmacy, The Ohio State University, Columbus, OH, U.S.A..;*
[6]*College of Agricultural and Life Sciences, University of Florida, Gainesville, FL, U.S.A.*

Abstract. *Aim: We screened nine 1,2-dihydro-quinolines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay in HepG2 and SMMC cells and identified ethyl-2-cyano-2-(2- (methoxycarbonyl)allyl)quinoline-1(2H)-carboxylate (compound 5) as a potential anticancer agent. In order to further develop its anticancer therapeutic potential, we incorporated this agent into cationic liposomes for delivery to tumor cells. The characteristics of liposomes, their cytotoxicity and cellular uptake by tumor cells were investigated. We demonstrated that cationic 1,2-dioleoyl-3- trimethyl-ammonium-propane containing liposomes (cLips) loaded with compound 5 has superior antitumor activity compared to neutral liposomes. These data suggest cLip–compound 5 to be a promising agent that warrants further evaluation.*

Many biologically active compounds contain quinoline and its derivatives (1, 2). Functionalized 2-substituted 1,2-dihydroquinolines are frequently found in active therapeutic agents (3, 4). Cationic liposomes (cLips) have been widely used to form stable electrostatic complexes with nucleic acid drugs and shown to have high delivery efficiency *in vitro* and *in vivo* (5-9) These cationic liposomes, however, have been reported to include red blood cell agglutination if they carry an excessive amount of cationic surface charge (10-13). 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) is a cationic lipid (14-17) that is frequently used in cLip formulations (18, 19).

In this study, we report the synthesis and screening of nine dihydroquinoline compounds. We then evaluated the delivery of the lead compound in DOTAP-based cLips in HepG2 and SMMC cells.

Correspondence to: Tian Bai, College of Computer Science and Technology, Jilin University, No.2699, Qianjin Street, Changchun, China, 130012. Tel: +0431 85166477, Fax: +0431 85166771, e-mail: baitian@jlu.edu.cn and Yungang Luo, The Second Hospital of Jilin University. No.218 Ziqiang Street, Changchun, China, 130041. Tel: +0431 89272357, Fax: +0431 89272357, e-mail:luoyungang@sohu.com

Key Words: Dihydroquinoline, liposomes, drug delivery, cationic liposomes.

Materials and Methods

Materials. Egg phosphatidylcholine (ePC) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Cholesterol and DOTAP were purchased from Lipoid (Ludwigshafen, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT,1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 7-nitrobenzofurazan-labeled (NBD-DOPE) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Gibco (Gibco BRL Co. Ltd, Gaithersburg, MD, USA). 4', 6-Diamidino-2-phenylindole (DAPI) was purchased from Invitrogen Molecular Probes (Carlsbad, CA, USA). HepG2 and SMMC cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA).

Synthesis of compounds. Morita-Baylis-Hillman (MBH) carbonate 2 was prepared according to previously published procedures (20, 21). Reissert compound 1 (R^1=COR) was prepared by the literature procedure (22).

1,4-Diazabicyclo(2.2.2)octane (DABCO) (20 mol%), Reissert compound **a** (0.2 mmol), MBH carbonate **b** (1.5 equiv), and CH_3CN or toluene (2.0 ml) were added in a 10 ml round-bottom flask and dried under a nitrogen atmosphere at room temperature. Upon completion, the reaction mixture was concentrated *in vacuo*. The crude mixture was purified by column chromatography [silica gel, EtOAc/petroleum ether (60-90˚C)] to provide product **c**, **d**, **e** or **f** (Figure 1).

Cell culture. HepG2 and SMMC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 100 IU penicillin and streptomycin at 37˚C, and in humidified atmosphere containing 5% CO_2.

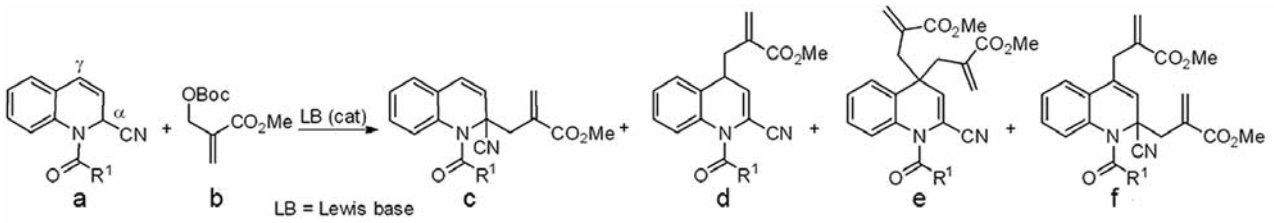

Figure 1. *The scheme of compound synthesis.*

MTT screening assay. A total of nine compounds (Figure 2) were evaluated in HepG2 and SMMC cells using the MTT assay (23). Cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and grown for 24 h. HepG2 and SMMC cells were then incubated with compounds at 250 μg/ml and 1.5 μg/ml per well, respectively. Plates were incubated for 4 h and 100 μl of fresh medium was added to each well. Cells were incubated for another 20 h. Then, 10 μl of MTT solution (5 mg/ml) was added to each well, which was incubated at 37˚C for 4 h. Formazan crystals were dissolved by the addition of 100 μl of dimethyl sulfoxide. Cell viability data were obtained by measuring OD_{490} nm on a microplate reader. The results were summarized as the mean±SD of six replicates for each sample. Compound 5 had the highest cytotoxicity among compounds tested, and therefore, was selected for further evaluation.

Preparation of liposomes. Due to its poor water solubility, compound 5 liposomes were prepared by an ethanol injection method. Briefly, neutral liposomes (nLips) were prepared from ePC and cholesterol at the following ratios by mass: ePC:cholesterol (63:35). Cationic liposomes (cLips) were prepared from DOTAP, ePC and cholesterol at the following ratios by mass: DOTAP:ePC:cholesterol (45:18:35). Briefly, 0.3 μmol compound 5 were added to 1.6 μmol lipid mixture. Then the mixture (7 mg/ml) was slowly added to HEPES buffer (20 mM HEPES, pH 7.4) under vortexing to achieve a final ethanol concentration of 14%. The solution was then sonicated with a bath-type sonicator for 20s. The mixture was then passed through a sterile membrane to remove unentrapped compound 5.

For fluorescence labelling of the lipid membrane, NBD-DOPE (1 mole% of total lipids) was added to the lipid mixture to make fluorescence-labelled liposomes, which were stored in the dark at 4˚C.

Characterization of liposomes. The particle size and ζ-potential of liposomes were measured with Zeta-sizer Nano ZS (Malvern Instruments Ltd, Malvern, UK) (24). In addition, the size and surface morphology of the cLip–compound 5 were investigated by field-emission scanning electron microscopy (FE-SEM) (JSM-6700F; JEOL, Tokyo, Japan) (25). The sample was fixed on a brass stub using double-sided adhesive tape and was coated with a thin layer of gold and then images were taken at 3.0 kV accelerating voltage.

Cytotoxicity assay (detailed method has already been described above). MTT assay was used to evaluate the viability of HepG2 cells and SMMC cells after treatment with nLip–compound 5, cLip–compound 5 and free compound 5. Cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and grown for another 24 h. After the medium was replaced with DMEM without FBS, the formulations were then added and the plate was incubated for a further 4 h. Then fresh medium was added and the cells were incubated for another 20 h. Cell viability was determined *via* the MTT assay. Firstly, 10 μl of MTT (5 mg/ml) reagent was added to each well. After incubation for 4 h, formazan crystals were dissolved by adding 100 μl of DMSO. Cell viability data were obtained by measuring OD490 nm on a microplate reader. The results are summarized as the mean±SD of six replicates for each sample.

Cellular uptake by flow cytometric analysis. The cellular uptake of liposomes by HepG2 and SMMC cells was determined by an EPICS XL flow cytometer (Beckman Coulter Inc., Brea, CA, USA) (26). Cells (1×10^5) were seeded in a 24-well plate and cultured for 24 h. Then, culture medium was replaced with nLip–compound 5, cLip–compound 5 or free compound 5 diluted in fresh culture medium without FBS and cultured for another 4 h. The cells were then washed three times with phosphate-buffered saline (PBS) and fixed with 350 μl 4% formaldehyde solution. The mean fluorescence intensity of positive cells with NBD-DOPE was analyzed by EPICS XL flow cytometer (Beckman Coulter Corp. Brea, CA, USA) and the data were analyzed with Cell Quest software.

Confocal microscopy and analysis of cellular internalization. Cells (1.5×10^5) were seeded in a 35 mm glass-bottom culture dishes and cultured for 24 h. Then culture medium was replaced with nLip–compound 5, cLip–compound 5 or free compound 5 diluted in fresh culture medium without FBS and cultured for 4 h. After the incubation, the cells were then washed three times with PBS and fixed with 400 μl 4% formaldehyde for 8 min. The cells were then washed with PBS. Cellular nuclei were stained with 2 μg/ml DAPI for 3 min, and then cells were washed again. Internalization of liposomes was observed on a Zeiss 710 LSMNLO confocal microscope (Carl Zeiss, Jena, Germany) (27).

Statistical analysis. The data were analyzed for statistical significance using Student's *t*-test and *p*-values less than 0.05 were regarded as significant. Where indicated, the results are presented as the mean±SD.

Results

MTT screening assay. Many 1,2-dihydroquinolines are bioactive compounds. For cytotoxicity assay, tumor cells (HepG2 and SMMC cells) were treated with different concentrations of nine compounds. The cytotoxicity data (Figure 3) show that all nine compounds inhibited tumor cells at a high concentration. Cells treated with a low concentration showed that compound 5 had the highest

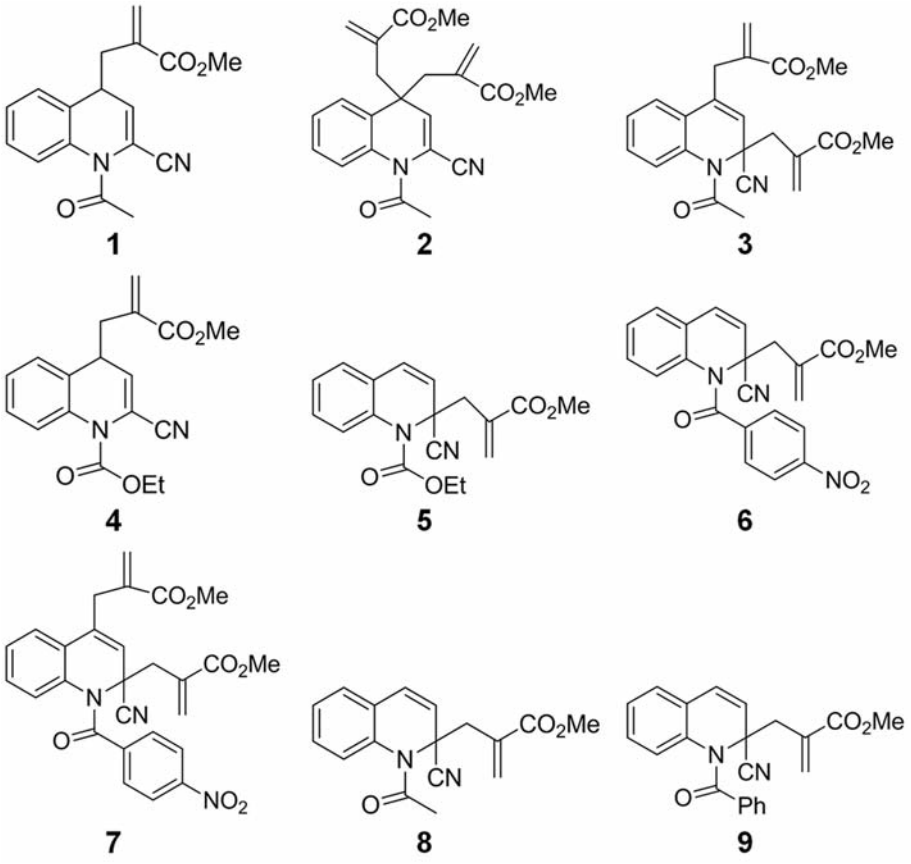

Figure 2. *Chemical structure of test compounds*.

cytotoxicity for tumor cells among tested compounds. Therefore compound 5 was selected for further evaluation.

Characterization of liposomes. Liposomes were prepared as described above. Particle size and zeta potential of nLip–compound 5 and DOTAP cLip–compound 5 are shown in Table I. ζ-Potential of nLips was –6.45±0.50 mV. In contrast, the value was 41.23±0.87 nm for cLips, which was highly positive. There was no significant difference in particle size between the nLip–compound 5 and cLip–compound 5. The morphology of the cLip–compound 5 was observed using FE-SEM and the results are shown in Figure 4. The FE-SEM image shows that most cLip–compound 5 was spherical particles with similar size and uniform dispersion.

Cytotoxicity assay. The cytotoxicity of nLip–compound 5, cLip–compound 5 or free compound 5 was tested in HepG2 and SMMC cells (Figure 5). Cell viabilities of the various formulations indicated the order of cytotoxicity to be cLip–compound 5> compound 5> nLip–compound 5 in HepG2 cells and cLip–compound 5> nLip–compound 5> compound 5 in SMMC cells (Figure 6A and B). The cytotoxicity of cLip–compound 5 was significantly greater than that of nLip–compound 5 in tumor cells.

Table I. *Size and ζ-potential of neutral liposomes (nLip)–compound 5 and cationic liposomes (cLip)–compound 5 at pH 7.4. Data are shown as means and standard deviation (n = 3).*

Physical properties	nLip–Compound 5	cLip–Compound 5
Size (nm)	120.43±1.76	112.43±2.51
ζ-Potential (mV)	−6.45±0.50	41.23±0.87

Cellular uptake studies of DOTAP cLip–compound 5. To evaluate the delivery efficiencies of liposomes, flow cytometry was used to quantify the liposomal uptake by HepG2 and SMMC cells for nLip–compound 5 and cLip–compound 5 (Figure 6). As shown in Figure 6A, the uptake of cLip–compound 5 was much higher than that of

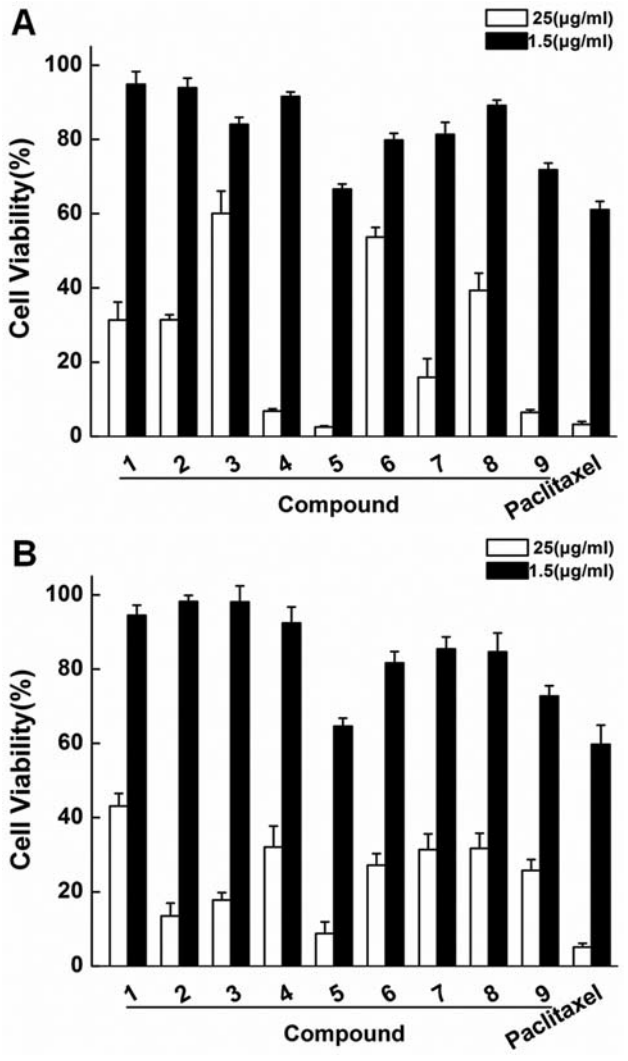

Figure 3. *Cytotoxicity tests of compounds. Cells were treated with different compounds for 4 h, and grown for another 20 h. A: HepG2 cells, B: SMMC cells. Data represent the mean±standard deviation (n=6).*

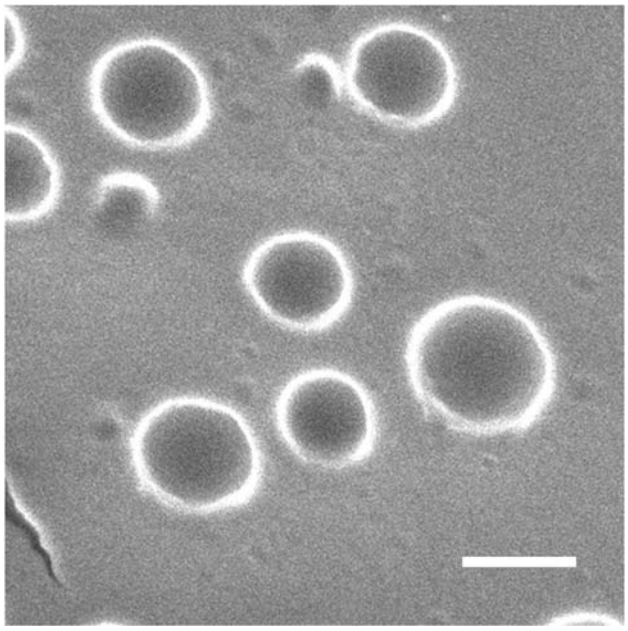

Figure 4. *Field-emission scanning electron microscopy images of cationic liposomes containing compound 5. Scale bar=100 nm.*

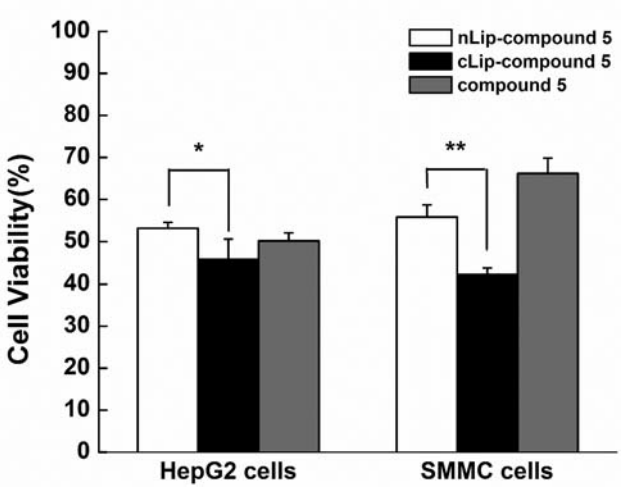

Figure 5. *Cytotoxicity tests of different formulations on HepG2 cells and SMMC cells. Data represent the mean±standard deviation (n=6) (*p<0.05, **p<0.01).*

the nLip–compound 5 in HepG2 cells. The mean fluorescence intensities of treated cells are shown in Figure 6C. In SMMC cells, cLip–compound 5 also exhibited higher cellar uptake than nLip–compound 5 (Figure 6C). The values of the group of cLip–compound 5 were about 10-times those of nLip–compound 5 (Figure 6C). These data indicated that cLip–compound 5 mediated more efficient uptake *in vitro*.

Confocal microscopic evaluation of cLip–compound 5 cellular uptake. The cellular uptake of the nLip–compound 5 and cLip–compound 5 was further evaluated by confocal microscopy. In Figure 7, it can be seen that compound 5 delivered by the cLips accumulated to significant extents in both HepG2 (Figure 7A and B) and SMMC cells (Figure 7C and D). In contrast, a relatively small amount of nLip-delivered Cmpd 5 was observed in the cells. DAPI was used for labelling the nuclei. Confocal microscopy analysis indicated that association of cLips to HepG2 and SMMC cells was much greater than that of nLips.

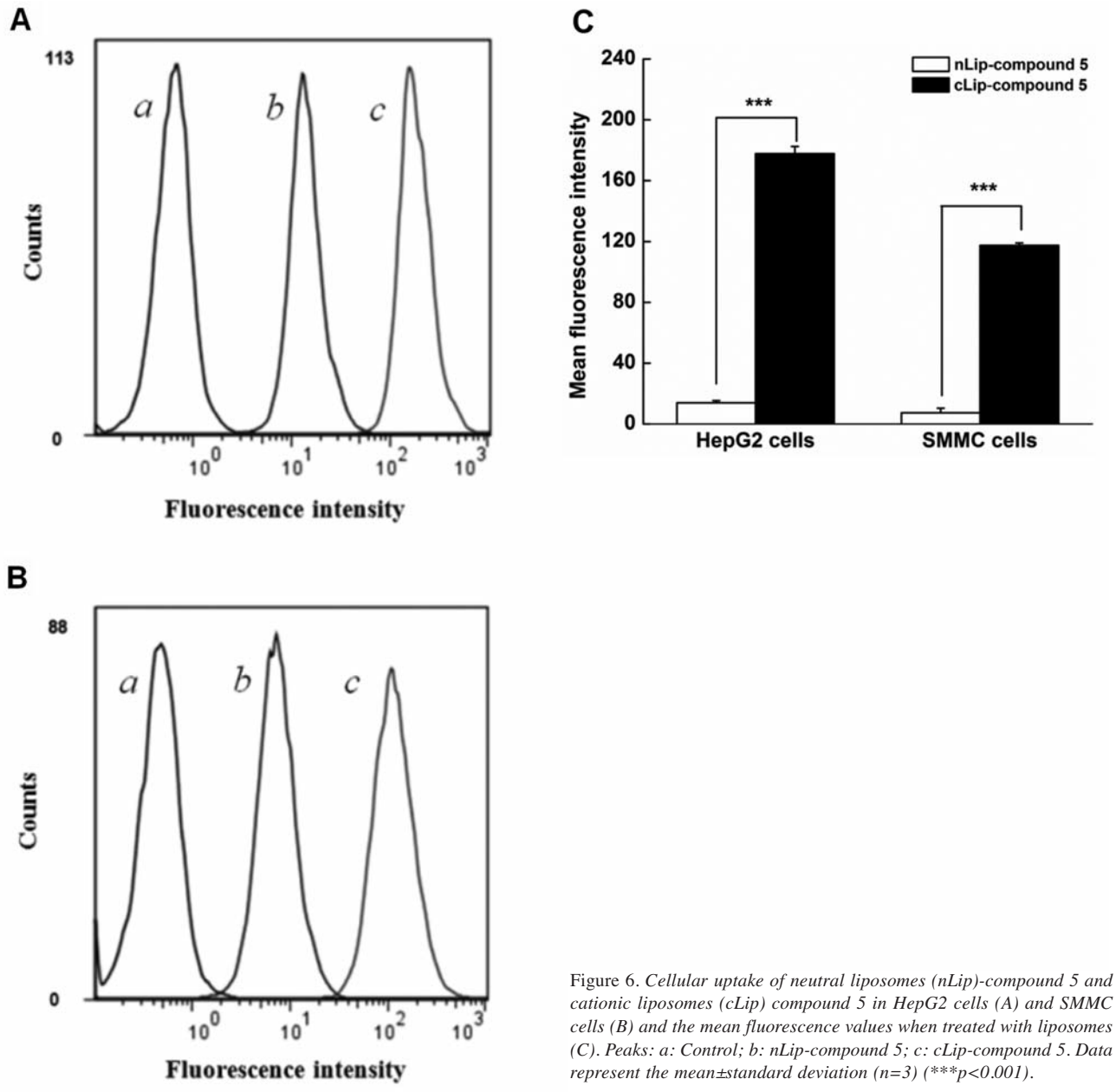

Figure 6. *Cellular uptake of neutral liposomes (nLip)-compound 5 and cationic liposomes (cLip) compound 5 in HepG2 cells (A) and SMMC cells (B) and the mean fluorescence values when treated with liposomes (C). Peaks: a: Control; b: nLip-compound 5; c: cLip-compound 5. Data represent the mean±standard deviation (n=3) (***p<0.001).*

Discussion

We screened nine compounds to select a potential anticancer agent using two hepatocellular carcinoma cell lines (HepG2 and SMMC cells). All nine compounds showed anticancer properties at high concentration. Compound 5 had the highest cytotoxicity in two cell lines among the nine compounds (Figure 3).

In order to improve the solubility of compound 5, we prepared liposomes by an ethanol injection method. The characteristic of the liposomes, cytotoxicity, cellular uptake of liposomes in tumor cells were all investigated.

FE-SEM, particle size and zeta potentials of the liposomes containing compound 5 showed that cLip–compound 5 exhibited excellent properties as a delivery vehicle with positive surface charges (Table I, Figure 4). In addition, cLip–compound 5 showed higher cytotoxicity than nLip–compound 5 in tumor cells (Figure 5). We also demonstrated that cLips were able to increase the uptake of compound 5 compared with nLips in HepG2 and SMMC cells (Figures 6 and 7).

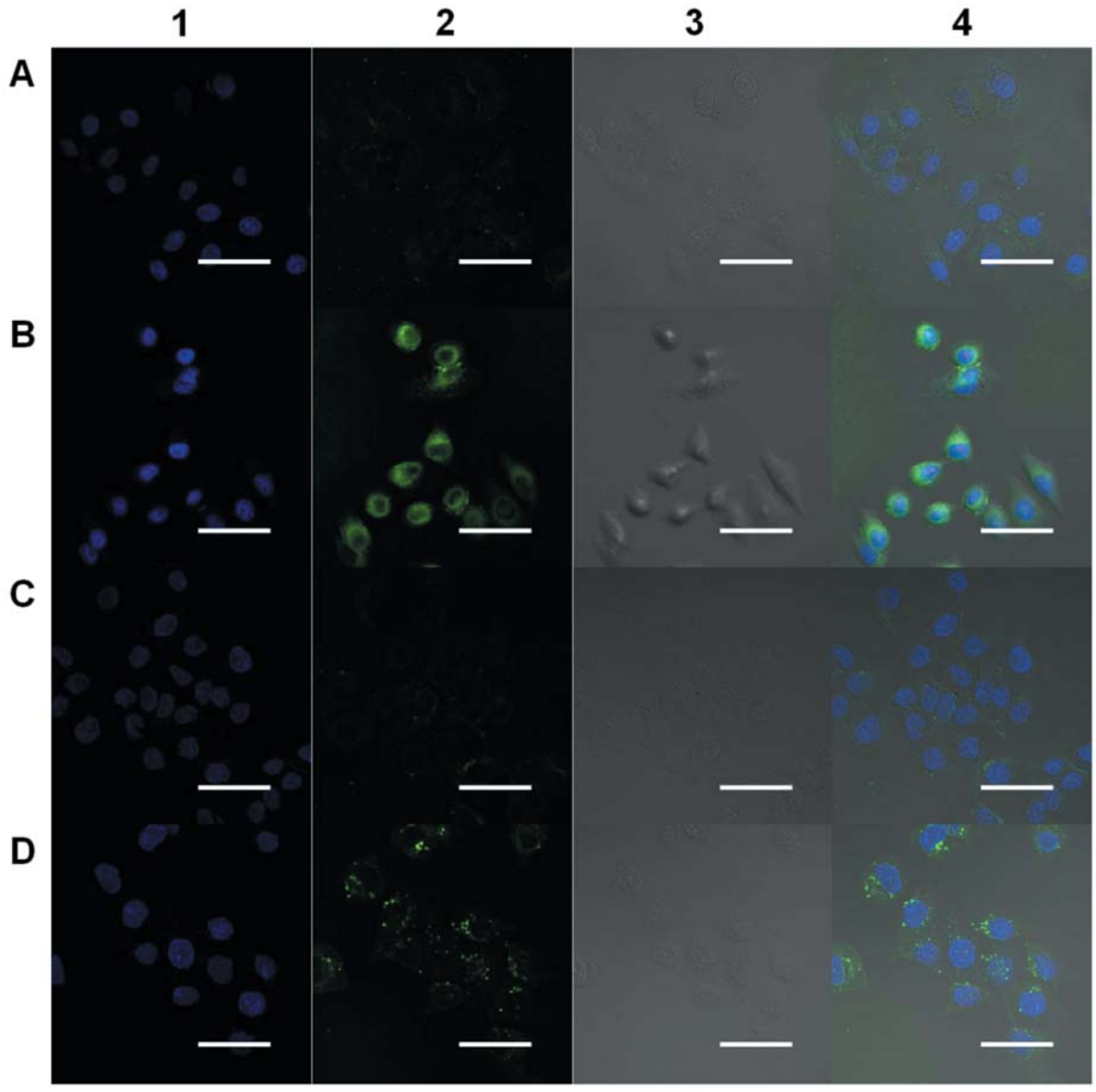

Figure 7. *Intracellular localization of neutral liposomes–compound 5 in HepG2 cells (A) and SMMC cells (B), and cationic liposomes–compound 5 in HepG2 cells (C) and SMMC cells (D). 1: 4',6-Diamidino-2-phenylindole; 2: 7-nitrobenzofurazan-labeled 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; 3: differential interference contrast image; 4: merged image (scale bars: 50 μm).*

In this study, we focused on the screening of potential anticancer agent and the development of a cationic liposome delivery systems. cLip–compound 5 showed high anti-tumor effect, furthermore, compared to nLip–compound 5, cLip–compound 5 showed remarkable increase in delivery efficiency of compound 5 in the tumor cells. In summary, cLip–compound 5 represents a novel preparation for cancer therapy. Further on this matter studies are clearly warranted.

References

1 Zarghi A, Ghodsi R, Azizi E, Daraie B, Hedayati M and Dadrass OG: Synthesis and biological evaluation of new 4-carboxyl quinoline derivatives as cyclooxygenase-2 inhibitors. Bioorg Med Chem *17*: 5312-5317, 2009.

2 Radi ZA and Khan NK: Comparative expression and distribution of c-fos, estrogen receptor alpha (ER alpha), and p38 alpha in the uterus of rats, monkeys, and humans. Toxicol Pathol *34*: 327-335, 2006.

3 Kaila N, Janz K, DeBernardo S, Bedard PW, Camphausen RT, Tam S, Tsao DHH, Keith JC, Nickerson-Nutter C, Shilling A, Young-Sciame R and Wang Q: Synthesis and biological evaluation of quinoline salicylic acids as P-selectin antagonists. J Med Chem *5*: 21-39, 2007.
4 Cody R, Stewart D, DeForni M, Moore M, Dallaire B, Azarnia N and Gyves J: Multicenter phase II study of brequinar sodium in patients with advanced breast cancer. Am J Clin Oncol *16*: 526-528, 1993.
5 Uchida H, Tanaka T, Sasaki K, Kato K, Dehari H, Ito Y, Kobune M, Miyagishi M, Taira K, Tahara H and Hamada H: Adenovirus-mediated transfer of siRNA against survivin induced apoptosis and attenuated tumor cell growth *in vitro* and *in vivo*. Mol Ther *10*: 162-171, 2004.
6 Fuessel S, Herrmann J, Ning SL, Kotzsch M, Kraemer K, Schmidt U, Hakenberg OW, Wirth MP and Meye A: Chemosensitization of bladder cancer cells by survivin-directed antisense oligodeoxynucleotides and siRNA. Cancer Lett *232*: 243-254, 2006.
7 Yang Z, Yu B, Zhu J, Huang X, Xie J, Xu S, Yang X, Wang X, Yung BC, Lee LJ, Lee RJ and Teng L: A microfluidic method to synthesize transferrin-lipid nanoparticles loaded with siRNA LOR1284 for therapy of acute myeloid leukemia. Nanoscale *6*: 9742-9751, 2014.
8 Xie J, Teng L, Yang Z, Zhou C, Liu Y, Yung BC and Lee RJ: A polyethylenimine-linoleic acid conjugate for antisense oligonucleotide delivery. BioMed Res Int *2013*: 710502, 2013.
9 Zhou C, Yang Z and Teng L: Nanomedicine based on nucleic acids: pharmacokinetic and pharmacodynamic perspectives. Curr Pharm Biotechnol *15*: 829-838, 2014.
10 Yang S, Guo ZH, Yang XW, Xie J, Lee RJ, Jiang D and Teng LS: Enhanced survivin siRNA delivery using cationic liposome incorporating fatty acid-modified polyethylenimine. Chem Res Chin Univ *31*: 401-405, 2015.
11 Kurosaki T, Kitahara T, Kawakami S, Higuchi Y, Yamaguchi A, Nakagawa H, Kodama Y, Hamamoto T, Hashida M and Sasaki H: γ-Polyglutamic acid-coated vectors for effective and safe gene therapy. J Control Release *142*: 404-410, 2010.
12 Martino S, di Girolamo I, Tiribuzi R, D'Angelo F, Datti A and Orlacchio A: Efficient siRNA delivery by the cationic liposome DOTAP in human hematopoietic stem cells differentiating into dendritic cells. J Biomed Biotechnol, 2009.
13 Doh KO, Kim BK, Lee TJ, Park JW and Seu YB: Synthesis of NBD-labeled DOTAP analog to track intracellular delivery of liposome. J Microbiol Biotechnol *23*: 131-135, 2013.
14 Simberg D, Weisman S, Talmon Y and Barenholz Y: DOTAP (and other cationic lipids): Chemistry, biophysics, and transfection. Crit Rev Ther Drug Carrier Syst *21*: 257-317, 2004.
15 Kitagawa S, Kasamaki M and Hyodo M: Cationic vesicles consisting of 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and phosphatidyl-cholines and their interaction with erythrocyte membrane. Chem Pharm Bull *52*: 451-453, 2004.
16 Ott G,Singh M, Kazzaz J, Briones M, Soenawan E, Ugozzoli M and O'Hagan DT: A cationic sub-micron emulsion (MF59/DOTAP) is an effective delivery system for DNA vaccines. J Control Release *79*: 1-5, 2002.
17 Regelin AE, Fankhaenel S, Gurtesch L, Prinz C, von Kiedrowski G and Massing U: Biophysical and lipofection studies of DOTAP analogs. BBA-Biomembranes *1464*: 151-164, 2000.
18 Chen X A, Wang XH, Wang YS, Yang L, Hu J, Xiao WJ, Fu A, Cai LL, Li X, Ye X, Liu YL, Wu WS, Shao XM, Mao YQ, Wei YQ and Chen LJ: Improved tumor-targeting drug delivery and therapeutic efficacy by cationic liposome modified with truncated bFGF peptide. J Control Release *145*: 17-25, 2010.
19 Gross N, Ranjbar M, Evers C, Hua J, Martin G, Schulze B, Michaelis U, Hansen LL and Agostini HT: Choroidal neovascularization reduced by targeted drug delivery with cationic liposome-encapsulated paclitaxel or targeted photodynamic therapy with verteporfin encapsulated in cationic liposomes. Mol Vis *19*: 54-61, 2013.
20 Sun WS, Ma XZ, Hong L and Wang R: Asymmetric organocatalytic allylic substitution of Morita-Baylis-Hillman carbonates with allylamines for the synthesis of 2,5-dihydropyrroles. J Org Chem *76*: 7826-7833, 2011.
21 Du YH, Lu XY and Zhang CM: A catalytic carbon-phosphorus ylide reaction: Phosphane-catalyzed annulation of allylic compounds with electron-deficient alkenes. Angew Chem Int Ed *42*: 1035-1037, 2003.
22 Feng JQ, Lu XY, Kong AD and Han XL: A highly regio- and stereo-selective [3+2] annulation of allylic compounds and 2-substituted 1,1-dicyanoalkenes through a catalytic carbon-phosphorus ylide reaction. Tetrahedron *63*: 6035-6041, 2007.
23 Yang XW, Yang S, Chai HY, Yang ZG, Lee RJ, Liao WW and Teng LS: A novel isoquinoline derivative anticancer agent and its targeted delivery to tumor cells using transferrin-conjugated liposomes. Plos One *10*: e0136649, 2015.
24 Wang X, Huang X, Yang Z, Gallego- Perez D, Ma J, Zhao X, Xie J, Nakano I and Lee LJ: Targeted delivery of tumor suppressor microRNA-1 by transferrin-conjugated lipopolyplex nanoparticles to patient-derived glioblastoma stem cells. Curr pharm biotechnol *15*: 839-846, 2014.
25 Yang Z, Awuti G, Cao YZ, Wu JB, Wang J, Lu W, Zhang X. and Zhang Q: Preparation and physicochemical characterization of quercetin-HP-beta-CD inclusion complexes. J Chin Pharm Sci *15*: 69-75, 2006.
26 Yu B, Wang X, Zhou C, Teng L, Ren W, Yang Z, Shih CH, Wang T, Lee RJ, Tang S and Lee LJ: Insight into mechanisms of cellular uptake of lipid nanoparticles and intracellular release of small RNAs. Pharm Res *31*: 2685-2695, 2014.
27 Yang Z, Sun W and Hu K: Adenosine A(1) receptors selectively target protein kinase C isoforms to the caveolin-rich plasma membrane in cardiac myocytes. Biochim Biophys Acta *1793*: 1868-1875, 2009.

Received February 8, 2016
Revised March 28, 2016
Accepted April 1, 2016