Bioorganic & Medicinal Chemistry Letters 20 (2010) 3680–3682

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Hybrid liposomes inhibit the growth of Cholangiocarcinoma by induction of cell cycle arrest in G_1 phase

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ARTICLE INFO

Article history:
Received 7 January 2010
Revised 14 April 2010
Accepted 21 April 2010
Available online 27 April 2010

Keywords:
Hybrid liposomes
Cholangiocarcinoma
G_1 phase cell cycle arrest

ABSTRACT

Specific accumulation and cell cycle arrest were observed in human cholangiocarcinoma cells by hybrid liposomes composed of 90 mol % L-α-dimyristoylphosphatidylcholine (DMPC) and 10 mol % polyoxyethylene(21)dodecyl ether ($C_{12}(EO)_{21}$) without affecting normal cholangiocytes.

Cholangiocarcinoma (CCA) is an aggressive and lethal cancer arising from biliary epithelia within either the intrahepatic or extrahepatic biliary tracts. This cancer is rare worldwide but it is found most frequently in the northeastern region of Thailand.[1] Infection with the liver fluke *Opisthorchis viverrini* is the most common risk factor for CCA in the Southeast Asia. The prognosis for CCA patients is quite poor due to the lack of an early diagnosis and the fact that the tumor is relatively resistant to chemotherapy.[2,3] Therefore, novel treatment strategies directed against CCA are needed.

Hybrid liposomes (HL), first developed by Ueoka et al.,[4,5] can be prepared by simply ultrasonicating a mixture of vesicular and micellar molecules in buffer solutions. HL composed of L-α-dimyristoyl phosphatidylcholine (DMPC) and polyoxyethylenealkyl ether have remarkable inhibitory effects on the growth of various tumor cells along with apoptosis in vitro and in vivo.[6,7] Furthermore, successful clinical treatments with drug-free HL to patients with lymphoma have been reported.[8]

In this study, we investigated the inhibitory effects of hybrid liposomes composed of DMPC and polyoxyethylene (*n*) dodecyl ether ($C_{12}(EO)_n$; $n = 21$, 23 and 25) on the growth of cholangiocarcinoma cells (CCA) in vitro.

The hybrid liposomes (HL-21, -23 and -25) were prepared by sonication of a mixture containing 90 mol % DMPC (Nippon Oil and fats Co. Japan) and 10 mol % $C_{12}(EO)_n$ ($n = 21$, 25: Nikko Chemicals Co., Osaka, Japan, $n = 23$: Sigma Chemical Co., St. Louis, MO) in 5% glucose solution as described previously.[6] The sample solutions were sterilized using membrane filter with 0.20 μm pore size. Dynamic light-scattering measurements with Otsuka Electronics ELS-8000 apparatus (Japan) showed that the size of HL was less than 100 nm in diameter and stable for more than 1 month. It is noteworthy that HL-*n* ($n = 21$, 23, 25) having less than 100 nm in diameter could avoid the reticular endothelial system in vivo[9] and should be appropriate for clinical applications.

At first, we examined the 50% inhibitory concentration (IC_{50}) of HL on the growth of two CCA cell lines (M213, M214) with MTT assay.[10] CCA cell lines were established from the primary tumors of CCA.[11] Cells (2.0×10^4 viable cells/ml) were seed into 96 well plate and left to adhere overnight. The cells were then incubated for 48 h in a humidified 5% CO_2 incubator at 37 °C in the absence or in the presence of different concentrations of HL. Subsequently, MTT (0.5 mg/ml final conc.) solution was added to each well. After 3 h of additional incubation, 100 μl of acidified isopropanol (HCl 34 μl/10 ml isopropanol) was added to dissolve the crystal. The absorption values at 595 nm were determined with an automatic ELISA plate reader (Multiskan JX, Thermo Electron, Vantaa, Finland). Values are normalized to the untreated (Control) samples. All assays were performed in triplicate. The results are summarized in Table 1. The IC_{50} value of HL-21, -23 and -25 were lower than

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doi:10.1016/j.bmcl.2010.04.091

Table 1
50% Inhibitory concentration of HL on the growth of CCA cells

IC_{50} (μM)							
M213				M214			
DMPC	HL-21	HL-23	HL-25	DMPC	HL-21	HL-23	HL-25
246	122	123	161	236	114	168	152

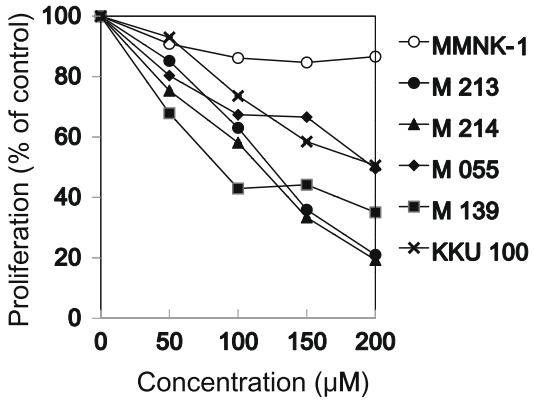


Figure 1. HL-21 inhibits the proliferation of CCA cells. CCA cells were incubated with various concentration of HL-21 for 48 h. Cell proliferation assays were done using MTT. Mean ± SD from three independent experiments.

DMPC liposomes in the growth of both CCA cells. As HL-21 was the most effective among the three HL, we used HL-21 for the following experiments. We next confirmed the effect of HL on CCA cells (M213, M214, M055, M139 and KKU100) and immortalized cholangiocyte cell line, MMNK-1, which was established by Maruyama et al.[12] using simian virus 40 large T (SV40T) and human telomerase reverse transcriptase (hTERT) transduction. As shown in Figure 1, HL-21 also inhibited cell growth of other CCA cell lines in a dose-dependent manner. However, HL-21 does not exert the inhibition of MMNK-1 cell.

Next, we examined fusion and accumulation of HL-21 including 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBDPC, Avanti Polar Lipid, USA, [DMPC] = 86 mol % [$C_{12}(EO)_n$] = 10 mol %, [NBDPC] = 4 mol %) as a fluorescence probe[13] into CCA and MMNK-1 cell membranes using a flow cytometer (LSR II, BD Bioscience, San Jose, CA). Cells (1×10^5 cells/ml) were treated with 100 μM HL21/NBDPC for 30, 60, 120 and 180 min. The results are shown in Figure 2. Increases in accumulation of HL-21/NBDPC into CCA cells were observed with time dependent manner. In contrast, accumulation of HL-21/NBDPC into MMNK-1 cells was low and constant. The fluorescence intensity for CCA cells were about 10-fold higher than MMNK-1 cells 180 min after adding HL-21/NBDPC. These results suggest that HL-21 could selectively fuse and accumulate into CCA cells but not normal cholangiocytes.

We analyzed DNA contents of HL-treated CCA cells by flow cytometry, which can detect the apoptotic subdiploid cells as well as the state of cell cycle. Cells (1×10^5 cells/ml) were seed in 12 well plate and left to adhere overnight. The cells were then incubated in the absence or in the presence of different concentrations of HL-21. After 24 h, adherent cells were trypsinized and washed with phosphate-buffered saline (PBS). Cells were fixed in 70%

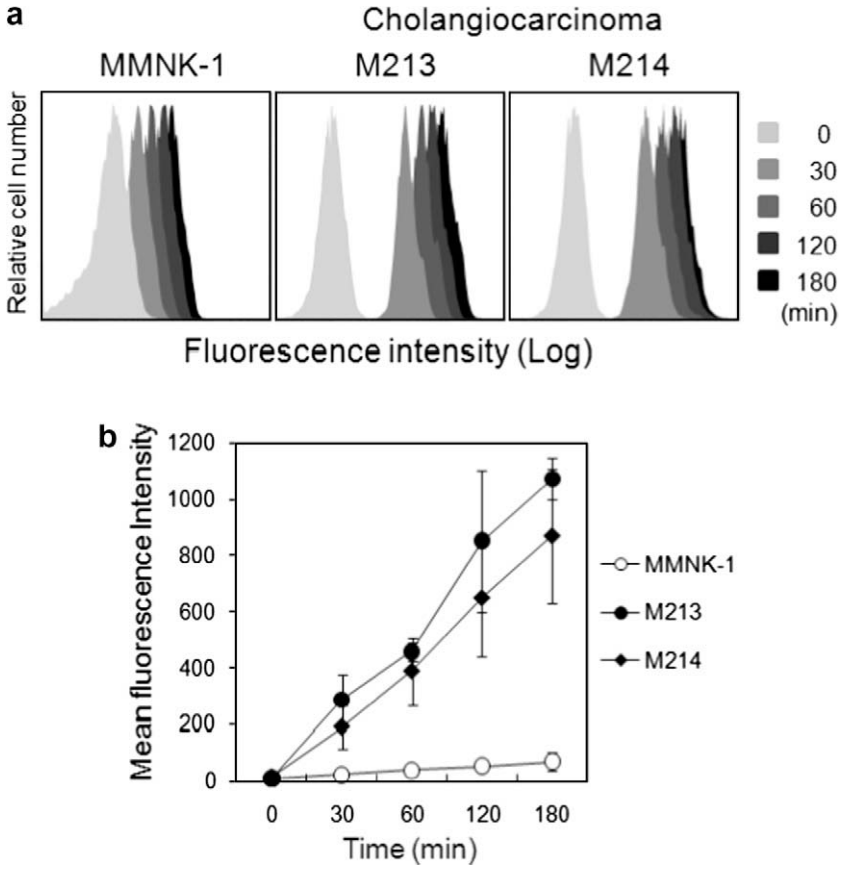
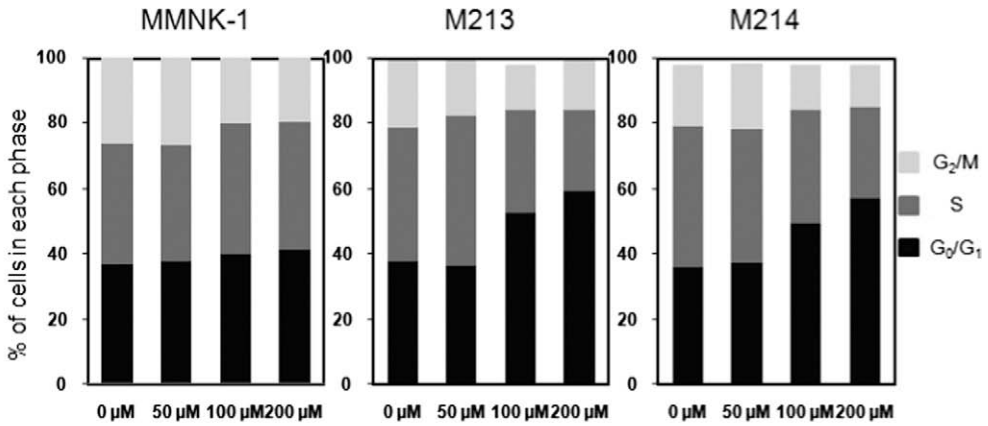


Figure 2. Specific accumulation of HL-21 including NBDPC on CCA cells. Fluorescent intensity of NBDPC incorporated into HL-21 on CCA cells. CCA cells (1×10^5/ml) were cultured in the presence or absence of 100 μM of HL-21 including NBDPC for 30, 60, 120 and 180 min. After incubation, cells were analyzed by flow cytometry. Mean ± SD from three independent experiments.



	MMNK-1				M213				M214			
G_0/G_1	37.2	38.1	40.2	41.5	36.9	34.2	54.2	59.5	35.4	37.4	56.4	57.1
S	36.7	35.5	39.8	38.9	40.4	47.9	30.1	24.6	43.8	42.5	31.1	27.8
G_2/M	27.0	28.3	22.1	20.8	21.9	17.5	13.9	17.2	18.6	18.9	11.9	14.4

Figure 3. HL-21 induced cell cycle arrest at G1 phase. Cells were incubated with indicated concentration of HL-21 for 24 h. Adherent cells were harvested by trypsinization. Cells were stained with propidium iodide and analyzed by flow cytometry. Percentage of cell in each phase is indicated. Data are representative of three independent experiments.

ethanol, treated with RNase A and stained with propidium iodide (PI).[10,14] The DNA content of each cell was analyzed on LSR II flow cytometer (BD Bioscience, San Jose, CA). Data were analyzed with FlowJo software (Tree Star, San Carlos, CA) and were displayed as the percentage of each cell cycle phase. Treatment with HL-21 induced significant increase in the G_1 population and decreases in S and G_2/M populations in M213 and M214 CCA cells and no changes were observed in MMNK-1 cells (Fig. 3), indicating that this agent induced the blockage of G_1/S transition or G_1 arrest. However, HL-21 treatment did not induce hypodiploid sub-G_1 peak, a feature indicative of apoptotic cell death in CCA cell lines (<3% until 48 h). HL has been shown to induce apoptosis rapidly for various tumor cells with the activation of caspases.[6,7,13,15] CCA is highly resistant to apoptosis by overexpression of Bcl-2 family proteins (Bcl-2, Bcl-xL, Mcl-1),[16–18] hence we can speculate that HL treatment induced only G1 arrest for CCA. It is noteworthy that HL selectively accumulated and induced cell cycle arrest of CCA cells, while HL were not much accumulated into the immortalized cholangiocyte cell line (MMNK-1) (Figs. 2 and 3). HL have been shown to fuse and accumulate into the tumor cells having high membrane fluidity selectively[15] and they have little adverse effects both in vivo and in vitro.[6–8]

We next examined the in vivo effects of HL in a xenografted mouse model. Severe immunodeficient, NOD/Scid/Jak3 deficient mice (NOJ mice)[19] were inoculated intraperitoneally with 4×10^6 M213 cells. A dose of 200 mg/kg HL21 in PBS or PBS alone was administered via intraperitoneal injection on day 3 after cell inoculation and everyday thereafter for 14 days by the schedule as described.[13] The HL21-treated mice had tendency to lower tumor weight (0.35 ± 0.22 g, $n = 8$) compared with the non-treated mice (0.47 ± 0.31 g, $n = 8$), although this did not reach statistical significance ($p = 0.069$). As CCA is highly resistant to anti-cancer reagents due to high expression of anti-apoptotic Bcl-2 family proteins, HL alone may not be effective to suppress CCA in vivo, and Bcl-2 family antagonist[18] can facilitate apoptosis in combination with HL and other anti-cancer reagents.

In conclusion, our study provides the first evidence that HL-21 selectively accumulates and elicits G_1 phase cell cycle arrest on CCA cells. These results suggest that HL can use as key reagent for the targeting chemotherapy of CCA in combination with Bcl-2 family antagonists and other anti-cancer agents.

Acknowledgments

This work was supported in part by Grants-in-Aid for Science Research on Innovative Areas (Nos. 21107522, 20107007, 20360377) and Grants-in-Aid for Science Research (C) (No. 21591209) from the Ministry of Education, Science, Sports, and Culture (MEXT) of Japan.

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