Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 16 (2006) 782–785
Bioorganic & Medicinal Chemistry Letters

Two methylene groups in phospholipids distinguish between apoptosis and necrosis for tumor cells

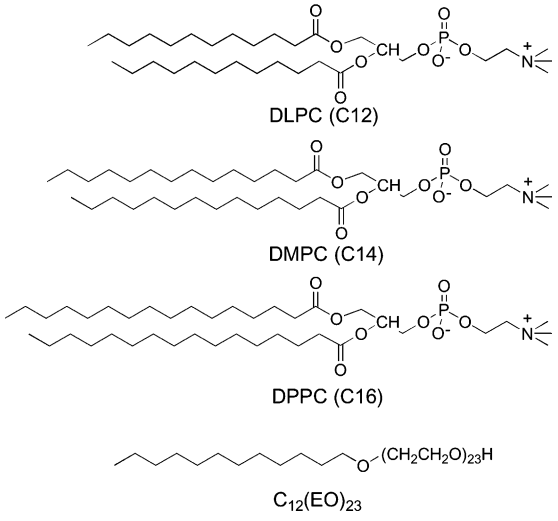
Hideaki Nagami,[a] Koji Nakano,[a] Hideaki Ichihara,[b] Yoko Matsumoto[a,b] and Ryuichi Ueoka[a,b,*]

[a]*Graduate Course of Applied Chemistry, Sojo University, Ikeda, Kumamoto 860-0082, Japan*
[b]*Graduate Course of Life Science, Sojo University, Ikeda, Kumamoto 860-0082, Japan*

Received 21 September 2005; revised 1 November 2005; accepted 8 November 2005
Available online 6 December 2005

Abstract—High inhibitory effects of hybrid liposomes (HL) themselves composed of DMPC/10 mol % $C_{12}(EO)_{23}$ on the growth of HL-60 cells were obtained without any drug. Induction of apoptosis was obtained by the HL of DMPC/10 mol % $C_{12}(EO)_{23}$. On the other hand, necrosis was observed for the HL of DLPC/10 mol % $C_{12}(EO)_{23}$. In the case of DPPC/10 mol % $C_{12}(EO)_{23}$, neither apoptosis nor necrosis was observed.

Liposomes are closed vesicles that are formed when phospholipids (constituents of biological membranes) are dispersed in water at relatively low concentrations. Since Bangham et al. discovered liposomes in 1965,[1] these liposomes have been studied for chemical and medical applications. Especially, liposomes have contributed significantly to drug delivery, as well as analysis of cellular function, due to their mimicry of biological membranes and closed properties.[2,3] Hybrid liposomes, which have been developed by Ueoka et al., can be prepared by the treatment of phospholipids and micellar molecules with sonication, and contain no organic solvent as compared with conventional liposomes.[4] The physical properties of these liposomes such as shape, size, membrane fluidity, and the temperature of their phase transition can be controlled by changing the constituents and compositional ratios. Significantly prolonged survival in rats was obtained using hybrid liposomes as drug carriers in the treatment of brain tumors.[5] It was also demonstrated that hybrid liposomes without drugs should inhibit the proliferation of various tumor cells in vitro and in vivo.[6–13]



In this study, the inhibitory effects of hybrid liposomes composed of phospholipids having the same hydrophilic head group (phosphatidylcholine group) and different hydrophobic alkyl chains (L-α-dilauroylphosphatidylcholine (C12: DLPC), L-α-dimyristoylphosphatidylcholine (C14: DMPC), L-α-dipalmitoylphosphatidylcholine (C16: DPPC)), and polyoxyethylenedodecyl ether ($C_{12}(EO)_{23}$) on the growth of human leukemia (HL60) cells in vitro were examined.

Keywords: Liposome; Antitumor effect; Apoptosis; Phospholipid.
* Corresponding author. Tel.: +81 96 326 3952; fax: +81 96 326 0522; e-mail: ueoka@life.sojo-u.ac.jp

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.11.034

Hybrid liposomes were prepared by sonication of a mixture of vesicular (DLPC, DMPC or DPPC) and micellar ($C_{12}(EO)_{23}$) molecules in 5% glucose solution at 45 °C in a nitrogen atmosphere and, subsequently, filter-sterilized through a 0.20 μm pore filter.

The inhibitory effects of hybrid liposomes on the growth of HL60 cells were examined on the basis of WST-1 method.[14] The inhibitory effects of liposomes composed of lipid molecules only (DLPC, DMPC, and DPPC), micelles ($C_{12}(EO)_{23}$), and hybrid liposomes composed of lipid molecules and micelles on the growth of HL60 cells were examined.[15] The results are shown in Figure 1. It is attractive that the remarkably high inhibitory effects of DLPC liposomes and hybrid liposomes of DLPC/10 mol % $C_{12}(EO)_{23}$ and DMPC/10 mol % $C_{12}(EO)_{23}$ on the growth of HL60 cells were obtained. Moderate inhibitory effects were observed in the case of hybrid liposomes of DPPC/10 mol % $C_{12}(EO)_{23}$. In previous experiments, the markedly prolonged survival of mice was obtained in the treatment with DMPC liposomes.[10] On the other hand, the DPPC liposomes had no life-prolonging effects. In the case of DLPC liposomes, survival was negative.

So, we examined morphological change in HL60 cells after the treatment with hybrid liposomes composed of DLPC/$C_{12}(EO)_{23}$, DMPC/$C_{12}(EO)_{23}$, and DPPC/$C_{12}(EO)_{23}$ using time laps video. As shown in Figure 2, the formation of bleb and corpuscle indicating a characteristic feature of apoptosis, was observed for hybrid liposomes of DMPC/10 mol % $C_{12}(EO)_{23}$. On the other hand, swelling of cells and dissolving of cell membrane, that is necrosis, were observed for hybrid liposomes of DLPC/10 mol % $C_{12}(EO)_{23}$. It is worthy to note that two methylene groups of acyl chain in phosphatidylcholine could distinguish between apoptosis and necrosis. Neither apoptosis nor necrosis was observed for hybrid liposomes of DPPC/10 mol % $C_{12}(EO)_{23}$.

Furthermore, apoptotic or necrotic rate of HL60 cells after the treatment of hybrid liposomes were examined

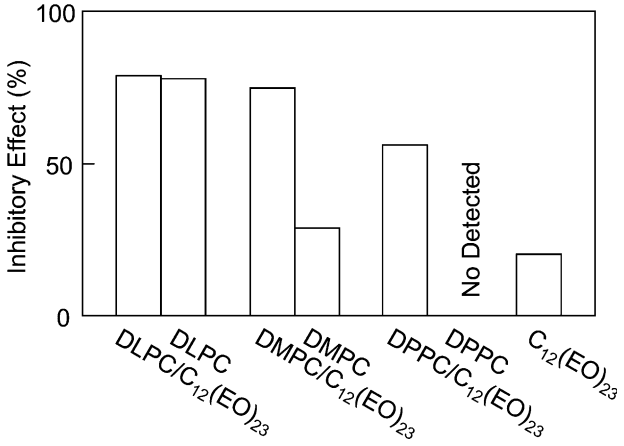


Figure 1. Inhibitory effects of hybrid liposomes on the growth of HL-60 cells. [Phospholipid] = 300 μM, [$C_{12}(EO)_{23}$] = 33 μM.

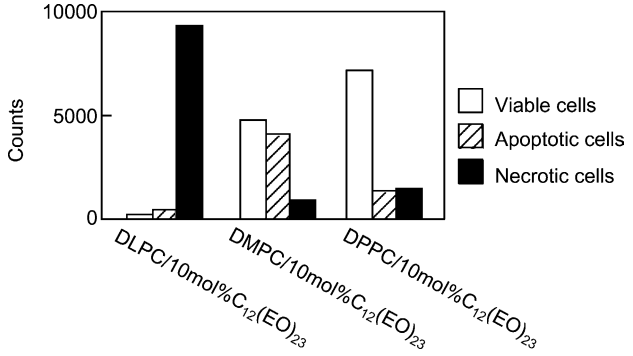


Figure 3. Apoptotic and necrotic rate for HL60 cells treated with hybrid liposomes. [Phospholipid] = 300μM, [$C_{12}(EO)_{23}$] = 33 μM.

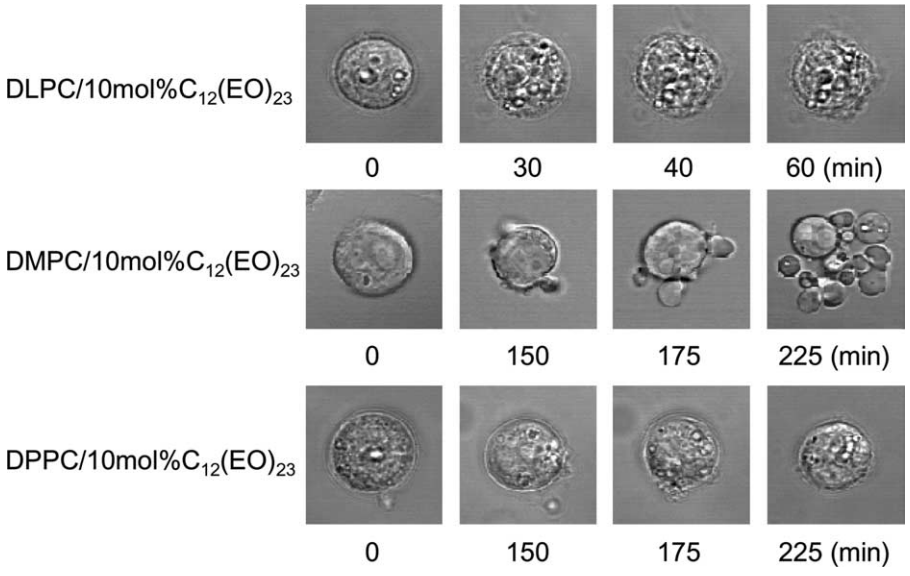


Figure 2. Morphological changes in HL60 cells treated with hybrid liposomes. Photographs taken 80× magnification under a confocal laser microscope. [Phospholipid] = 300 μM, [$C_{12}(EO)_{23}$] = 33 μM.

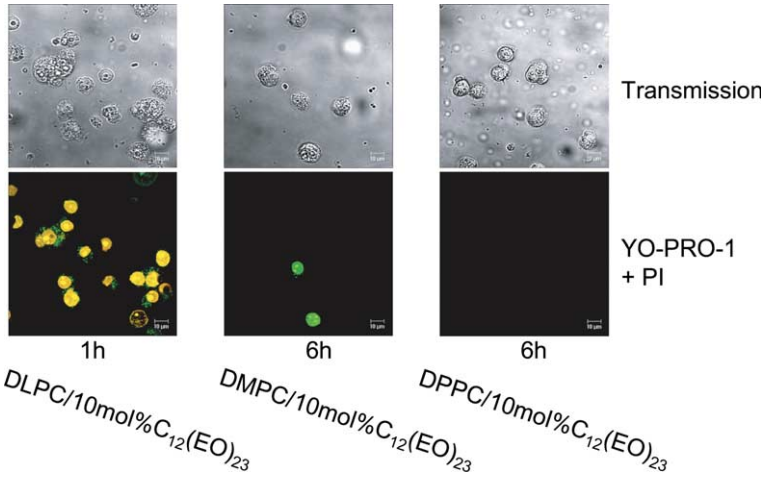


Figure 4. Fluorescence micrographs of HL60 cells treated with hybrid liposomes. [Phospholipid] = 300 μM, [$C_{12}(EO)_{23}$] = 33 μM.

using flow cytometry.[16] The results are shown in Figure 3. Interestingly, almost the same large rates of apoptotic and viable cells were obtained using DMPC/$C_{12}(EO)_{23}$ hybrid liposomes. On the other hand, a large ratio of necrotic and almost no viable cells was obtained for HL60 cells after the treatment with DLPC/$C_{12}(EO)_{23}$ hybrid liposomes. In the case of DPPC/$C_{12}(EO)_{23}$ hybrid liposomes, a small ratio of apoptotic and necrotic cells was obtained along with the large ratio of viable cells.

Fluorescence micrograph is shown in Figure 4.[18] Apoptotic and necrotic cells were dyed in green (YO-PRO-1) and red or orange color (propidium iodide), respectively. Interestingly, cells were dyed in green after adding DMPC/$C_{12}(EO)_{23}$ hybrid liposomes, indicating that those liposomes could induce apoptosis. DNA fragmentation characteristic of apoptosis by DMPC/$C_{12}(EO)_{23}$ hybrid liposomes toward HL-60 cells was also observed using DNA agarose gel electrophoresis (data not shown). In the case of DLPC/$C_{12}(EO)_{23}$ hybrid liposomes, cells were dyed in orange, indicating necrosis. On the other hand, cells were not dyed using DPPC/$C_{12}(EO)_{23}$ hybrid liposomes, indicating that neither apoptosis nor necrosis could be induced by those liposomes.

In conclusion, marked inhibitory effects of DMPC/$C_{12}(EO)_{23}$ hybrid liposomes on the growth of HL60 and induction of apoptosis were obtained without any antitumor agents. It is noteworthy that two methylene groups in phospholipids could distinguish between apoptosis and necrosis for the first time in human leukemia cells. The selection of hydrophobic alkyl chain in lipids should be most important for the clinical application to patients with carcinoma in the future.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Science Research from the Ministry of Education, Science and Culture of Japan (No. 17360403, 17500323).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.11.034.

References and notes

1. Bangham, D. A.; Sandish, M. M.; Watkins, C. J. *J. Mol. Biol.* **1965**, *13*, 238.
2. Graybill, R. J.; Craven, C. P.; Taylor, L. R.; Williams, M. D.; Magee, E. W. *J. Infect. Dis.* **1982**, *145*, 748.
3. Gabizon, A.; Catane, R.; Uziely, B.; Kaufman, B.; Safra, T.; Cohen, R.; Martin, F.; Huang, A.; Barenholz, Y. *Cancer Res.* **1994**, *54*, 987.
4. Ueoka, R.; Matsumoto, Y.; Moss, R. A.; Swarup, S.; Sugii, A.; Harada, K.; Kikuchi, J.; Murakami, Y. *J. Am. Chem. Soc.* **1988**, *110*, 1588.
5. Kitamura, I.; Kochi, M.; Matsumoto, Y.; Ueoka, R.; Kuratsu, J.; Ushio, Y. *Cancer Res.* **1996**, *56*, 3986.
6. Matsumoto, Y.; Imamura, C.; Ito, T.; Taniguchi, C.; Ueoka, R. *Biol. Pharm. Bull.* **1995**, *18*, 1456.
7. Imamura, C.; Kemura, Y.; Matsumoto, Y.; Ueoka, R. *Biol. Pharm. Bull.* **1997**, *20*, 1119.
8. Matsumoto, Y.; Kato, T.; Iseki, S.; Suzuki, H.; Nakano, K.; Iwahara, M.; Ueoka, R. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1937.
9. Nakano, K.; Iwamoto, Y.; Takata, W.; Matsumoto, Y.; Ueoka, R. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3251.
10. Ueoka, R.; Matsumoto, Y.; Kanno, A.; Ichihara, H. *Biol. Pharm. Bull.* **2000**, *23*, 1262.
11. Kanno, A.; Tsuzaki, K.; Miyagi, M.; Matsumoto, Y.; Ueoka, R. *Biol. Pharm. Bull.* **1999**, *22*, 1013.
12. Kanno, A.; Terada, Y.; Tsuzaki, K.; Matsumoto, Y.; Ueoka, R. *Drug Deliv. Syst.* **1999**, *14*, 37.
13. Matsumoto, Y.; Kato, T.; Kemura, Y.; Tsuchiya, M.; Yamamoto, M.; Ueoka, R. *Chem. Lett.* **1999**, *28*, 53.
14. Ishiyama, M.; Shiga, M.; Sasamoto, K.; Mizoguchi, M. *Chem. Pharm. Bull.* **1993**, *41*, 1118.
15. The tumor cells were incubated in a humidified 5% CO_2 incubator at 37 °C. The cells were cultured for 48 h after adding the hybrid liposome and the inhibitory effects of hybrid liposomes on the growth of tumor cells were evaluated by inhibitory effect = $(1 - A_{Mean}/A_{Control}) \times 100(\%)$, where A_{Mean} and $A_{Control}$ denote the absorbance

of water-soluble formazan at 450 nm in the presence and absence of hybrid liposomes, respectively.
16. The double staining was carried out using Vybrant Apoptosis Assay Kit #4 (molecular probes)[17] and the number of cells after being dyed with YO-PRO-1 (apoptotic cells) or propidium iodide (necrotic cells) was measured using a flow cytometer (Epics XL, Beckman Coulter).
17. Daly, J. M.; Jannot, C. B.; Beerli, R. R.; Graus-Porta, D.; Maurer, F. G.; Hynes, N. E. *Cancer Res.* **1997**, *57*, 3804.
18. Fluorescent micrographs were taken using a confocal laser microscope (TCS, Leica).