Cytotoxic Effects of Several Hopenoids on Mouse Leukemia L1210 and P388 Cells

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The cytotoxic effects of hopenoids, including bacteriohopen-32, 33, 34, 35-tetrol (Tetrol), bacteriohopen-32-ol (Monol), diplotene, diploterol and acetylated Monol (Ac-O-Monol) isolated from Acetobacter aceti, were tested against two leukemia cell lines. Tetrol and Monol have been shown to be toxic to mouse L1210 and P388 compared to the other hopenoids. By measuring the ESR spectra of the spin labeled membranes of these cells, it was shown that the incorporation of Monol resulted in a decrease in the fluidity of the membranes. The decrease in membrane fluidity may correlate, in part, with the cytotoxicity of hopenoids against the two cell lines.

Key words hopenoid; ESR spectrum; cytotoxicity; L1210; P388

Hopenoids are a class of pentacyclic triterpenoids (Fig. 1) and are considered to maintain the hydrophobic barrier properties in prokaryotes as cholesterol does in eukaryotes.1–5) Bacteriohopen-32-ol (Monol), a semisynthetic molecule derived from hopenepolyols, has been shown to be efficient in modulating the fluidity and stability of artificial membranes.6,7) In the process of testing its usefulness as a membrane material, we preliminarily showed that Monol has a cytotoxic effect against two leukemia cell lines.8) As its mechanism, we proposed that the incorporation of Monol into cell membranes resulted in changes in both the fluidity and/or permeability of the cell membranes and thus disturbed the transport of biologically essential molecules through the cell membranes.9) This paper describes the cytotoxicity of several hopenoids against two mouse leukemia cell lines using the methyltetrazolium (MTT) method. We also examined the relationship between the incorporation of hopenoids and the change in membrane properties using the ESR technique.

MATERIALS AND METHODS

Materials MTT was purchased from Tokyo Chemical Industry Co., Ltd. RPMI 1640 was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo). Fetal bovine serum (FBS) was a product of Irvine Scientific (Santa Ana, CA). Dipalmitoylphosphatidylcholine (DPPC) was from Nippon Oil & Fat Co., Ltd. A stearic acid spin label, 16-doxyl stearic acid (16-SASL), was obtained from Aldrich Chemical Co., Ltd.

The hopenoids were isolated by the method of Rohmer et al.1,9) from dry cells of Acetobacter aceti, which was kindly provided by Del Monte Co., Ltd. Liposomal hopenoid was prepared by incorporating hopenoid into small unilamellar vesicle (SUV) membranes composed of DPPC, as reported previously.7)

Cell Lines DBA/2 mouse leukemia L1210 and P388

Fig. 1. Structures of Hopenoids Used in the Present Study
(a) hopane skeleton, (b) diplotene, (c) diploterol, (d) bacteriohopen-32,33,34,35-tetrol (Tetrol), (e) bacteriohopen-32-ol (Monol), (f) acetylated Monol (Ac-O-Monol).

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cells were cultured in RPMI 1640 medium containing 10% FBS, an antibiotic (penicillin, 100 IU/ml; kanamycin, 60 μg/ml) and 10^{-5} M 2-mercaptoethanol.

Cytotoxicity The cytotoxic potencies of hopenoids were determined by the MTT tetrazolium dye reduction assay. Briefly, 100 μl of cell suspension (2 × 10^5/ml) was seeded into 96-well plastic plates (Nunc), and 4 μl of hopenoid solutions in tetrahydrofuran (THF)/MeOH were added. The cells were incubated for 72 h at 37°C in an incubator with 5% CO2 in air. Then, 20 μl of MTT solution (5 mg/ml in phosphate buffered saline (PBS)) was added to each well, and the absorbance was measured at 590 nm. Cytotoxicities were expressed as percentages of absorbance in the presence of hopenoids to reagent blank.

Membrane Fluidity Determination For spin labeling of cells, 60 μl (1.2 nmol) of 16-SASL in ethanol was placed in the bottom of a small glass test tube and evaporated to form a thin film on the wall of the tube. Thirty μl of the cell suspension (1 × 10^5 cells/ml), treated with hopenoids for 6 h, 3 μl of K3Fe(CN)6 and K3Cr(C2O4)3 (at final concentrations 1 and 30 mM, respectively), were added to the above test tube. After gentle vortex-mixing for 10 s, the cell suspensions were used for ESR measurement. ESR spectra were recorded at 20°C with a JEOL JES-FE spectrometer kept at 4 mW of microwave in a field intensity of 3280 G (100 G field sweep), and membrane fluidities were calculated from the spectra.

RESULTS AND DISCUSSION

Figure 2 shows the effect of Monol on respiratory inhibition against L1210 and P388 cells after 72 h incubation. The approximate IC_{50} values are 23 μM for L1210 and 27 μM for P388. These are consistent with our previous results. When Monol solutions in THF/MeOH were diluted with the culturing medium, the solution became turbid at concentrations higher than 40 μM. In an effort to investigate the cytotoxicity at higher drug concentrations, Monol was incorporated into liposomal membrane composed of DPPC. DPPC was not toxic to the two cell lines, even at 500 μM. As shown in Fig. 2b, Monol added as a liposomal suspension also had a cytotoxic effect in a dose-dependent manner. IC_{50} is determined to be 25 μM for both cell lines. Monol is an amphiphilic molecule with a hydrophobic ring system and a hydrophilic polar head (Fig. 1), and is probably incorporated into the membrane of cells. We modified the amphiphilic property of Monol by acetyling the polar hydroxy group, and tested its cytotoxicity. Figure 3a clearly shows that AcO-Monol has less toxic effects than that of Monol. In the presence of 10 μM of AcO-Monol, respiratory activity is 60% of that of control. Even at higher concentrations, further inhibition was not observed. We have also investigated the cytotoxicity of other hopenoids such as diploptene (Fig. 3b), diploptol (Fig. 3c) and bacteriohptane-32,33,34,35-tetrol (Tetrol, Fig. 3d). Diploptene, having less polarity, showed no cytotoxic effects against L1210 and P388 cells. In the presence of 15 μM of diploptol, the activity was reduced to about 40% and the inhibitory effect was slightly increased at higher concentrations. This mode of action is somewhat similar to that of AcO-Monol. Tetrol has a maximum inhibitory effect among these hopenoids, and its IC_{50} is 14 μM for L1210 and 18 μM for P388, respectively. The different cytotoxic effects shown in Fig. 3 suggest that the side chains play an important role in the cytotoxicity of hopenoids. In an attempt to understand the mechanism of the cytotoxicity of these compounds, we examined the effects of hopenoid treatment on the fluidity of cellular membranes of L1210 cells. The cellular respiratory activity was maintained at >90% of the control after incubating the cells with hopenoids for 24 h. Therefore, the fluidity of cell membranes was estimated 24 h after the addition

Fig. 2. Cytotoxicity of Monol Added as a Solution (a) and as a Liposomal Suspension (b) against L1210 (○) and P388 (●) Cells
Cells were incubated for 72 h at 37°C and cytotoxicity was determined by MTT assay as described in Materials and Methods. Each point represents the mean ± S.D. obtained from three measurements.

Fig. 3. Cytotoxicity of AcO-Monol (a), Diploptene (b), Diploptol (c) and Tetrol (d) against L1210 (○) and P388 (●) Cells
Cells were incubated for 72 h at 37°C and cytotoxicity was determined by MTT assay as described in Materials and Methods. Each point represents the mean ± S.D. obtained from three measurements.
Fig. 4. Representative ESR Spectrum of L1210 Cells Labeled with 16-SASL.

Cells were labeled with the spin probe, 16-SASL, as described under Materials and Methods. The rotational correlation time, $\tau_c$, was determined from the line width parameters shown in the spectra (Results and Discussion).

Fig. 5. Changes in $\tau_c$ for 16-SASL Incorporated into L1210 Cells Membranes after Incubation with Various Concentrations of Monol (O) or AcO-Monol (●) (a), Diploptene (b), Diplopterol (c) and Tetrold (d) for 24 h.

Each point represents the mean ± S.D. obtained from three measurements.

of hopenoids. The spectra of 16-SASL incorporated into the membranes of L1210 cell lines reflect an isotropic motion of the interior acyl chains of phospholipid molecules (Fig. 4). A rotational correlation time, $\tau_c$, can be estimated from the linear term of the line width parameters in the ESR spectra of 16-SASL:

$$\tau_c = 6.5 \times 10^{-10} \Delta H_0 ((h_0/h_{+1})^{1/2} + (h_0/h_{-1})^{1/2} - 2)$$

where $\Delta H_0$ is the peak-to-peak width of the central line in gauss and $h_{+1}$, $h_0$ and $h_{-1}$ are the heights of the low, central and high field peaks, respectively. $\tau_c$ increases as the motion of the phospholipid acyl chains decrease. Figure 5 shows the changes in $\tau_c$ after incubation with various concentrations of hopenoids. As can be seen from Fig. 5a, $\tau_c$ increased in a dose-dependent manner for Monol, indicating a dose-dependent decrease in the fluidity of the cell membranes. Compared with Monol, AcO-Monol slightly decreased membrane fluidity (Fig. 5a). On the other hand, diploptene had no influence on the $\tau_c$ values (Fig. 5b), whereas the addition of both diplopterol (Fig. 5c) and Tetrod (Fig. 5d) increased the $\tau_c$ values, indicating the fluidity of cell membranes was decreased by the incorporation of diplopterol or Tetrod. This result suggests the correlation between a decrease in membrane fluidity and the cytotoxicity of hopenoids. We postulate, based on the present observation, that the cytotoxic effects of these hopenoids against L1210 cells may result from the cell membrane perturbation induced by hopenoids.

REFERENCES