

Enhanced Cytotoxicity of Some Triterpenes toward Leukemia L1210 Cells Cultured in Low pH Media: Possibility of a New Mode of Cell Killing

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Several triterpenes were tested for cytotoxicity by our contrived primary screening method using resting or dormant leukemia L1210 cells after 3 d-preculture without medium change. Some triterpenes were found to be more cytotoxic toward the 3 d-precultured resting cells than toward the growing cells in a fresh medium. These triterpenes are distinguished by highly selective cytotoxicity toward the starved resting cells unlike common anticancer agents. The highest selectivity was shown by betulinic acid, the ratio of its IC_{50} values toward the growing *versus* resting cells amounting to 175. It is suggested that this selective cytotoxicity is attributable to low pH (≤ 6.8) of the medium. It is noteworthy that betulinic acid is not cytotoxic at all in media of ordinary pH (≥ 7.0) even after a 48-h exposure. Betulinic acid might be promising as an antitumor agent toward solid tumors because the interior pH of tumor tissues is generally lower than in normal tissues.

Key words resting cell; triterpene; cytotoxicity; pH-dependent; betulinic acid; primary screening method

In the clinical treatment of solid tumors, complete remission could only be achieved by inactivating not only the peripheral growing cell fraction of the tumor tissue but also the dormant cell population inside the tissue where the environment is inadequate for cell proliferation, *i.e.*, under the conditions of low pH, anoxia, nutrient deficiency, *etc.* The present study was undertaken to contrive a random screening method to select agents that would affect dormant tumor cells through a cell cycle phase-specific or environmental condition-specific cytotoxicity, *e.g.*, an apoptosis-like mechanism. This would be of some value as an adjuvant screening method complementary to the known methods using growing cells. We used in this screening method the cultured tumor cells in the plateau growth phase following 3 d-preculture without medium change.¹⁾ These cells will die through an apoptotic mechanism after several more days of culture unless the medium is refreshed.¹⁾ We applied this starved resting cell system to the random screening of a variety of natural and synthetic chemicals for anticancer activity. The present paper describes an interesting feature of the cytotoxicity of some natural triterpenes, which showed a highly selective cytotoxicity toward the starved resting cells unlike common anticancer agents.

Experimental

Materials RPMI 1640 culture medium was purchased from Nissui Pharmaceutical Co. (Tokyo); fetal bovine serum (FBS) from Summit Biotechnology Co., Ltd. (Ft. Collins, CO); kanamycin sulfate from Meiji Seika Co., Ltd. (Tokyo); propidium iodide, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT), phenazine methosulfate (PMS), and RNase A from Sigma Chemical Co. (St. Louis, MO); agarose from Nacalai Tesque, Inc. (Kyoto); proteinase K, Tris-HCl, EDTA, and sodium *N*-lauroyl sarcosinate from Wako Pure Chemical Industries, Ltd. (Osaka); ethidium bromide from Aldrich Chemical Co. (Milwaukee, WI). The phosphate buffered saline (PBS) used was Mg/Ca-free Dulbecco's PBS. The triterpenes tested in the present study are shown in Chart 2. The 5-ring system triterpenes include betulin (1), betulinic acid (2), methyl betulinate (3), methyl nor-betulinate (4) (lupane group); friedelin (5) (friedelane group); oleanolic acid (6), glycyrrhetic acid (7) (oleanane group); and ursolic acid (8), *O*-acetylursolic acid (9), uvaol (10) (ursane group). The 4-ring system triterpenes include fusidic acid (11) (fusidane group); and lanosterol (12), dipterocarpol (13) (lanostane group). The sterols (modified triterpenes) include cholesterol (14), cholic acid (15), progesterone (16), estrone (17), and testosterone (18). Saponins include α -hederin (19) and glycyrrhizin (20). Compounds 1, 2, 6, 8, 10, 11, 13, and 19 were purchased from Aldrich Chemical Co. (Milwaukee, WI), compounds 7, 12, 14, 20 from Tokyo Kasei Industries Inc. (Tokyo), compounds 15—18 from Nacalai Tesque Inc., and other derivatives were synthesized in our laboratory.²⁾ The anticancer agents used in this study were all commercially available and obtained from manufacturers.

Assay Method for Cytotoxicity As shown in Chart 1, murine leukemia L1210 cells (1×10^5 cells/ml) were seeded to RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 0.1 mg/ml kanamycin

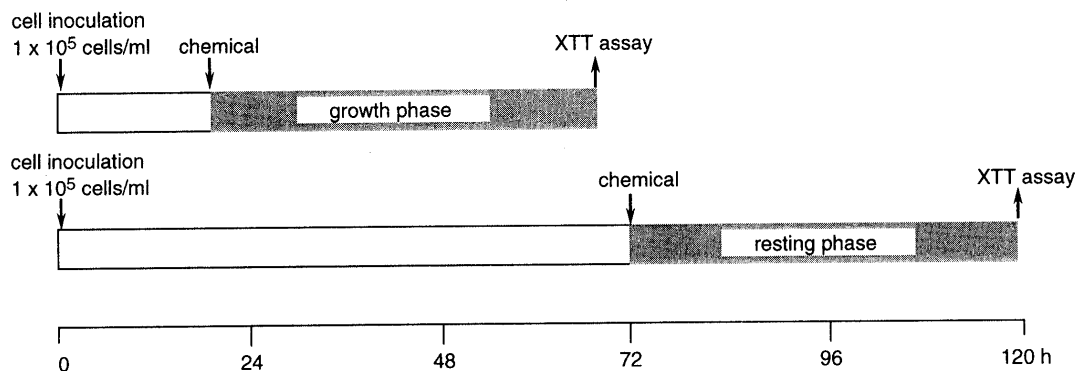


Chart 1. Protocol of Cytotoxicity Assay of 16- and 72-h Precultured Leukemia L1210 Cell Fractions

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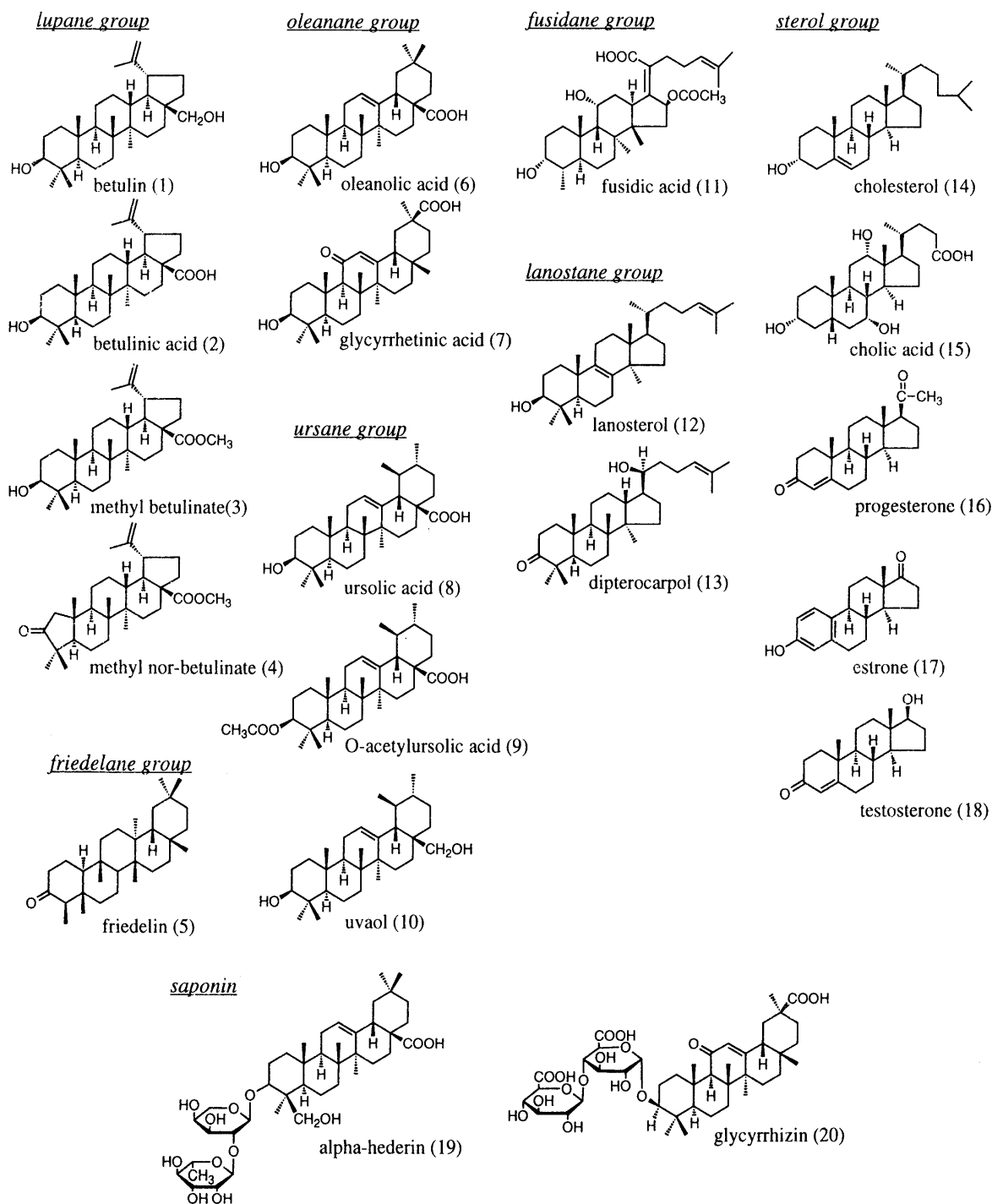


Chart 2. Structures of Triterpenes Examined

at 37°C in a humidified atmosphere containing 5% CO₂ for the indicated periods of time (16, 24, 36, 48, or 72 h). Test compounds were added to this culture and incubated at 37°C for another 48 h without medium change. Then, the cell viability was evaluated by the XTT-reducing test and compared with that of the control culture where the cells were treated without added test compounds.

DNA Electrophoresis DNA fragmentation was monitored by agarose gel electrophoresis.³⁾ Cells (1×10^6) in question were washed with 1 ml of cold PBS, centrifuged at 4°C, and resuspended in 20 μ l of 50 mM Tris-HCl (pH 8.0) containing 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl sarcosinate, and 0.5 mg/ml proteinase K. After 1 h incubation at 50°C, 10 μ l of 0.5 mg/ml RNase A was added. Incubation at 50°C was continued for one additional hour followed by heating to 70°C. The cell suspension was then mixed with 10 μ l of 10 mM EDTA (pH 8.0) containing 1% (w/v) low-melting-temperature agarose, 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose, and loaded into wells prepared on a 2% (w/v) high-melting-temperature agarose gel plate. Electrophoresis was

carried out at a voltage of 100 V in 2 mM EDTA-89 mM Tris-borate (pH 8.0) until the marker dye had migrated a distance of 3–4 cm. The agarose gel was then stained with 0.1 μ g/ml ethidium bromide for 10 min.

Single Cell Gel Electrophoresis (Comet Assay) The comet assay was carried out using a modification of the method reported by McKelvey-Martin *et al.*⁴⁾ and Higami *et al.*⁵⁾ L1210 cells (5×10^5 cells) exposed to betulinic acid and/or cycloheximide were collected by centrifugation. After they were washed with PBS, they were resuspended in 500 μ l of PBS. A 20 μ l aliquot of the cell suspension was mixed with 140 μ l of low melting agarose (Nacalai Tesque Inc.). One hundred μ l of this mixture was rapidly placed onto a frosted microscope slide and allowed to gel at 4°C for 10 min. The slide was then immersed in the lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl buffer (pH 10), 1% sodium *N*-lauroyl sarcosinate with 1% Triton X-100 and 10% dimethyl sulfoxide (DMSO)) at 4°C for 1 h, and left in a high pH electrophoresis buffer (1 mM EDTA, 300 mM NaOH) at 4°C for 20 min before electrophoresis. Electrophoresis was conducted at 4°C for 20 min

at 25 V, 300 mA. The slide was then immersed 3 times in 0.4 M Tris-HCl buffer (pH 7.5) for 5 min each time, stained with 4',6-diamidino-2-phenylindole (DAPI) (1 µg/ml), and covered with a cover slip. Comet images were observed at 200× magnification with a fluorescence microscope attached to a video camera (CCD X-2, Shimadzu) connected to a display screen. Fifty images were randomly selected from each sample and their lengths (diameter of the nucleus plus migrating DNA) were measured on the screen. The actual comet lengths in µm units were calculated using a calibration scale.

Results

Cytotoxicities toward Growing Cells after 16-h Preculture and Resting Cells after 72-h Preculture Leukemia L1210 cells in an early log-phase (1 × 10⁵ cells/ml) were precultured at 37 °C for 16 or 72 h in RPMI 1640 medium supplemented with 10% FBS. Then, the 16-h precultured growing cells and the 72-h precultured resting cells were treated with triterpenes at 37 °C without medium change for a further 48 h. The IC₅₀ values, evaluated by the XTT-reducing ability, are shown in Table 1, in which the ratio of IC₅₀ toward the growing cells *versus* IC₅₀ toward the resting cells is termed “selectivity” for each chemical. The highest cytotoxicity and selectivity were observed with betulinic acid. A dose–response curve for betulinic acid is shown in Fig. 1. Table 2 lists the IC₅₀ values and selectivities of common anticancer agents, all of which showed cytotoxic preference toward the growing cells when

the XTT-reducing ability is used as the criterion of cell viability.

Dependence of Cytotoxicity of Betulinic Acid upon Preculture Period Immediately after the log-phase cells (1 × 10⁵ cells/ml) were seeded to the medium (adjusted to pH 7.8 with NaHCO₃), they started growing in a logarithmic manner. The number of viable cells, evaluated by the trypan blue dye exclusion method, was 6.8 × 10⁵ cells/ml at 24 h (an early log-phase), 1.8 × 10⁶ cells/ml at 36 h, 2.8 × 10⁶ cells/ml at 48 h (a late log-phase), and 3.6 × 10⁶ cells/ml at 72 h (an early plateau phase) after cell-seeding, as shown in Fig. 2.⁶⁾ In the course of cultivation at 37 °C in a CO₂-incubator, pH of the medium gradually decreased to 7.8 at 16 h and 24 h; 7.4 at 36 h; 7.1 at 42 h; and 6.8 at 72 h and 120 h. Betulinic acid was

Table 1. Cytotoxicity of Triterpenes toward 16- and 72-h Precultured Leukemia L1210 Cells

Chemicals (No.)	IC ₅₀ (µg/ml)		Selectivity ^{a)}
	Growing cells	Resting cells	
5-Ring triterpenes			
(Lupane group)			
Betulin (1)	~10	2.3	~5
Betulinic acid (2)	~40	0.23	~174
Methyl betulinate (3)	12.3	9.6	1.3
Methyl nor-betulinate (4)	27.4	4.2	6.5
(Friedelane group)			
Friedelin (5)	>20	>20	—
(Oleanane group)			
Oleanolic acid (6)	12.6	0.40	32
Glycyrrhetic acid (7)	17.2	18.3	0.9
(Ursane group)			
Ursolic acid (8)	4.0	>20	<0.5
O-Acetylursolic acid (9)	>40	2.7	>15
Uvaol (10)	5.0	2.3	2.2
4-Ring triterpenes			
(Fusidane group)			
Fusidic acid (11)	33.6	>40	<0.8
(Lanostane group)			
Lanosterol (12)	>40	>40	—
Dipterocarpol (13)	6.3	1.4	4.4
Others			
(Sterol group)			
Cholesterol (14)	>40	>40	—
Cholic acid (15)	>40	>40	—
Progesterone (16)	>40	13.1	>3.1
Estrone (17)	>40	>40	—
Testosterone (18)	>40	>40	—
(Saponins)			
α-hederin (19)	6.3	5.2	1.2
Glycyrrhizin (20)	>40	>40	—

a) Selectivity = $\frac{IC_{50} \text{ toward growing cells}}{IC_{50} \text{ toward resting cells}}$

Table 2. Cytotoxicity of Anticancer Agents toward 16- and 72-h Precultured Leukemia L1210 Cells

Chemicals	IC ₅₀ (µg/ml)		Selectivity
	Growing cells	Resting cells	
Antimetabolites			
5FU	0.5	>130	0.0
MTX	0.001	>500	0.0
6MP	0.2	>17	0.0
HU	18.1	>760	0.0
Alkylating agents			
ACNU	5.8	>80	<<0.1
BCNU	11.0	>50	<<0.2
NM	0.2	0.7	0.2
Antibiotics			
ACD	0.1	0.4	0.2
ADR	0.7	12	0.1
BLM	18.8	150	0.1
MMC	0.6	4.8	0.1
Plant's alkaloids			
CPT	0.03	1.0	0.03
VCR	0.1	2.5	0.04
Miscellaneous			
CDDP	1.0	4.8	0.2

Abbreviations: 5FU, 5-fluorouracil; MTX, methotrexate; HU, hydroxyurea; ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; NM, nitrogen mustard; ACD, actinomycin D; ADR, adriamycin; BLM, bleomycin; MMC, mitomycin C; CPT, camptothecin; VCR, vincristine; CDDP, *cis*-dichlorodiammine-platinum(II).

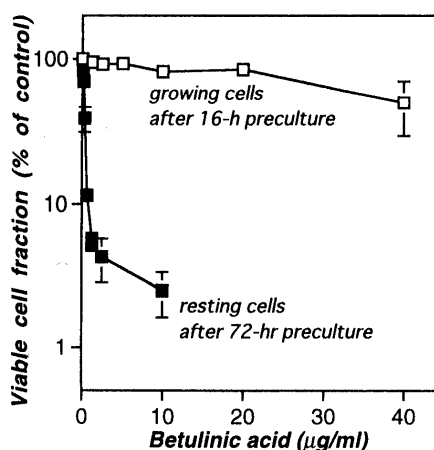


Fig. 1. Cytotoxicity of Betulinic Acid toward the 16-h Precultured Growing Cells and the 72-h Precultured Resting Cells

The cells were treated with betulinic acid at 37 °C for 48 h.

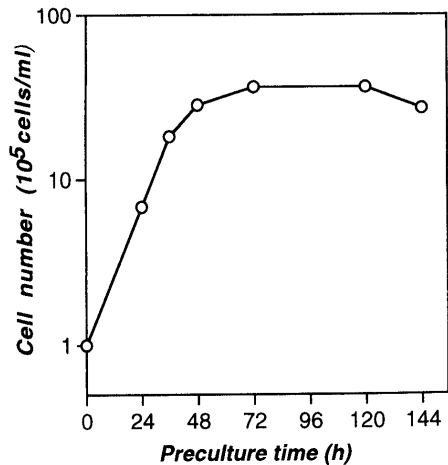


Fig. 2. Growth Rate of Leukemia L1210 Cells

L1210 cells (1×10^5 cells/ml) in an early log-phase were seeded in RPMI 1640 medium supplemented with 10% FBS adjusted to pH 7.4 with NaHCO_3 and incubated at 37°C in a CO_2 -incubator.

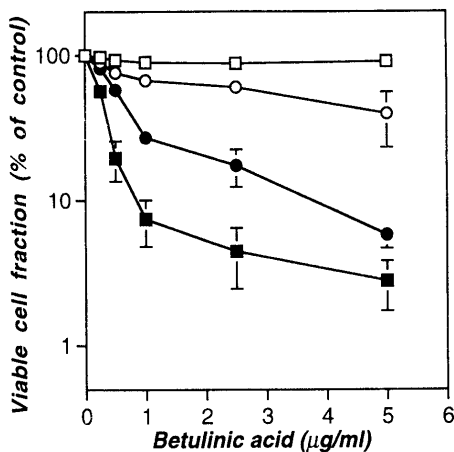


Fig. 3. Cell Viability after Treatment of Precultured Cells with Betulinic Acid for 48 h

Preculture periods: \square —, 24 h; \circ —, 36 h; \bullet —, 48 h; \blacksquare —, 72 h.

added to these cell cultures 24, 36, 48, and 72 h after cell-seeding. The cells were incubated for another 48 h without medium change and tested for the XTT-reducing ability. As shown in Fig. 3, betulinic acid inactivated the 72-h precultured cells to a marked extent, but did not affect the 24-h precultured cells at all.

pH Dependent Cytotoxicity of Betulinic Acid in Depleted Medium (Used for Preculture) During the 48-h treatment of each cell culture with betulinic acid after the specified preculture periods, pH of the medium further decreased as follows: from 7.8 to 6.8 for the 24-h precultured cells; from 7.3 to 6.8 for the 36-h precultured cells; from 7.1 to 6.8 for the 48-h precultured cells, and 6.8 remained for the 72-h precultured cells. To examine the role of pH of the medium for selective cytotoxicity, we treated the 72-h precultured cells with betulinic acid for 48 h in two ways: pH of the depleted medium was not adjusted (pH 6.8) or was adjusted to 7.4 by addition of NaHCO_3 prior to the treatment with betulinic acid. As shown in Fig. 4, little cytotoxicity was demonstrated in the pH-adjusted (pH 7.4) depleted medium.

pH-Dependent Cytotoxicity of Betulinic Acid in Fresh Media at Various pH's The 72-h precultured resting cells

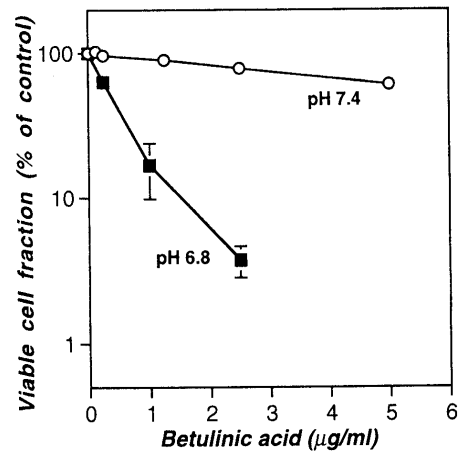


Fig. 4. Cell Viability after Treatment of the 72-h Precultured Resting Cells with Betulinic Acid in the Depleted Media (Used for Preculture) at pH 6.8 and 7.4 for 48 h

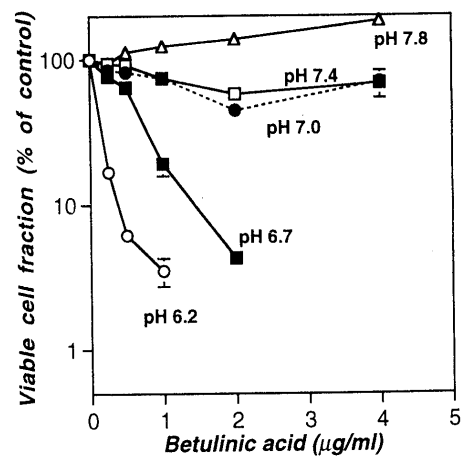


Fig. 5. Cell Viability after Treatment of the 72-h Precultured Resting Cells in Fresh Media at Various pH's for 48 h

No cell growth was observed at any pH because of the high cell density (3.6×10^6 cells/ml).

(3.6×10^6 cells/ml) were treated with betulinic acid in fresh media adjusted with NaHCO_3 at pH 6.2, 6.8, 7.0, 7.4, and 7.8. As shown in Fig. 5, cytotoxicity was not appreciably demonstrated in the media at pH's above 7. Similar results were obtained when the growing cells in a log-phase were treated with betulinic acid in fresh media adjusted with NaHCO_3 at various pH's. (data not shown). These results also indicate that the cytotoxicity of betulinic acid depends mainly on the pH of medium, regardless of the growth phase of the cells, resting or growing.

Dependence of Cytotoxicity of Betulinic Acid upon Exposure Time To define the exposure time required for the severe cytotoxicity of betulinic acid in low pH media, we treated the 72-h precultured cells with betulinic acid in the depleted medium (pH 6.8) for 8, 24, or 48 h. The cells were collected by centrifugation and resuspended in a fresh culture medium at pH 7.4 at a density of 2×10^5 cells/ml. Each cell culture was incubated for a total of 48 h after betulinic acid was added. As shown in Fig. 6, the 8 h exposure to betulinic acid did not affect the cell viability at all; the 24-h exposure was still not enough for inactivating the cells. The 48 h exposure greatly deprived the cells of the abilities of XTT reduction and trypan blue

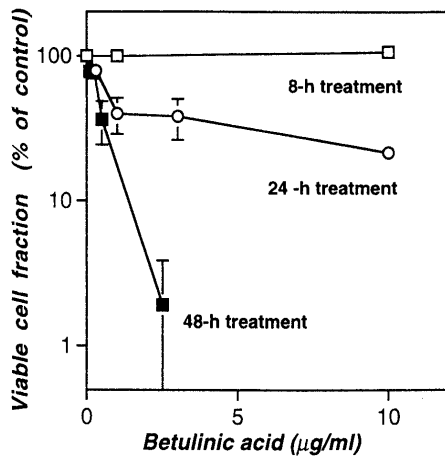


Fig. 6. Cell Viability after Treatment of the 72-h Precultured Cells with Betulinic Acid in the Depleted Medium for Indicated Times

The treated cells were resuspended in a fresh medium at a cell density of 2×10^5 cells/ml and allowed to grow by successive incubation for a total of 48 h.

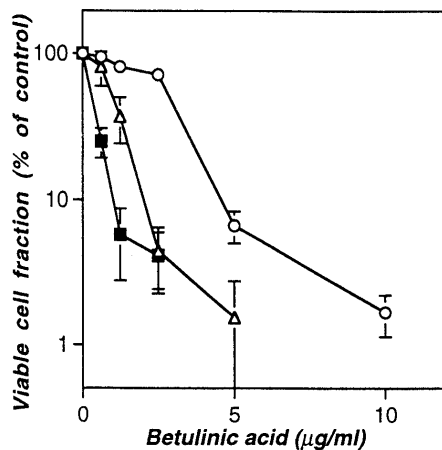


Fig. 7. Cell Viability after Treatment of the 72-h Precultured Cells with Betulinic Acid in the Depleted Medium for Indicated Times, Followed by Successive Incubation without Medium Change for a Total of 48 h

Treatment periods: —○—, 2 h; —△—, 8 h; —■—, 48 h.

exclusion and also of the proliferative ability when they were further cultivated in a fresh medium. It is worth emphasizing here that the cytotoxicity is apparently manifested after only several hours' exposure to betulinic acid whenever the treated cells are allowed to remain in a low pH medium for several-dozen hours. Thus, the 72-h precultured cells were treated with betulinic acid for 2, 8, or 48 h in the depleted medium and then collected by centrifugation. They were then resuspended in a depleted medium separately prepared and incubated for a total incubation time of 48 h including the period of treatment with betulinic acid, *i.e.*, a further 46, 40, and 0 h, respectively. As shown in Fig. 7, the several hours treatment with betulinic acid was enough for an appreciable cytotoxicity when the treated cells were continuously maintained in a low pH medium.

DNA Damage Induced by Betulinic Acid in Low pH Media The 16-h precultured growing cells and the 72-h precultured resting cells were treated with betulinic acid and subjected to the electrophoretic analysis for DNA strand breakage. Smear-like tailings (without ladder patterns) were observed on the electrophoretic gel only

with the 72-h precultured resting cells treated with betulinic acid at its IC_{50} and $10 \times IC_{50}$, but not at all with the 16-h precultured growing cells similarly treated (data not shown). In addition, single cell gel-electrophoresis, known as the comet assay, showed severe DNA breakage only in the resting cells. The selective cytotoxicity of betulinic acid might possibly be related to the induction of DNA breakage although it is not certain whether the DNA damage is the cause or consequence of cell death.

Discussion

Much attention has been paid to diverse biological activities of saponins, which are defined as glycosides of triterpenes and sterols,⁷⁾ however, only a few reports have appeared on the cytotoxicity of triterpenes of plant origin.⁸⁻¹²⁾ It was rather surprising that some of the triterpenes were potently cytotoxic toward the cells even in a resting phase when pH of the medium reached 6.8 after 3-d cultivation of the cells. Among the chemicals so far tested in our laboratory, cytotoxic materials such as anticancer agents were much less cytotoxic toward the resting cells in the depleted medium than toward the growing cells when the viability was evaluated by the mitochondrial activity to reduce XTT.¹³⁾ Thus, these triterpenes are distinguished from common cytotoxic chemicals by selective cytotoxicity toward the resting cells in depleted medium. Among them, betulinic acid showed an IC_{50} value which was 1/175 of its IC_{50} toward the cells in a log-phase. It seems that the difference in the cytotoxicity arises mainly from a decrease in pH of the medium to below 7. It might be possible that such triterpenes as betulinic acid can exert an environmental condition-specific cytotoxicity toward the cell population inside the solid tumor tissue, since it is widely recognized that pH of the inner tumor tissues is lower than those of normal tissues, probably due to proficient glycolysis in an anoxic environment. The preliminary study by gel-electrophoresis suggests that cell inactivation might be related to DNA strand breakage, but not through an apoptotic mechanism. Details are now being investigated.

Pisha *et al.* recently reported¹²⁾ that betulinic acid inhibited growth of human melanoma in both *in vitro* and *in vivo* assay systems. This triterpene has been predicted to be ineffective by the criteria established by the National Cancer Institute of U.S.A.¹⁴⁾ Thus, no appreciable inhibitory effect was observed on either cell lines of A431 (squamous cells), BC-1 (breast), COL-2 (colon), HT-1080 (sarcoma), KB and LNCaP (prostate), LU-1 (lung), or U373 (glioma). Betulinic acid was reported to be a melanoma-specific cytotoxic agent (cell lines MEL-1, -2, -3, and -4) and also to be an *in vivo* therapeutic agent which completely inhibited tumor growth in athymic mice carrying human melanomas. In their report, Pisha *et al.* stated that, as judged by a variety of cellular responses, the cytotoxicity was mediated by induction of apoptosis. It is worth examining whether there might be a mechanistic correlation between the melanoma-specific cytotoxicity and the medium condition-specific cytotoxicity described in the present study.

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