In Vitro Cytotoxicity of Imidazolyl-1,3,5-triazine Derivatives

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We examined *in vitro* cytotoxic activity of imidazolyl-1,3,5-triazine derivatives using human breast cancer cell lines (MCF-7, R-27, T-47D and ZR-75-1) and murine leukemia cell line (P388). The percentage of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Hexamethylmelamine (HMM), a 1,3,5-triazine derivative has previously been recognized as an antitumor agent effective against lung, ovarian and breast cancer, but failed to show a significant cytotoxic activity in the present study. In contrast, four imidazolyl-1,3,5-triazine derivatives, 2-(1-imidazolyl)-4,6-bis(morpholino)-1,3,5-triazine, 2-(1-imidazolyl)-4-morpholino-6-(3-thiazolidinyl)-1,3,5-triazine, 2-(4-cyano-4-phenylpiperidino)-4-(1-imidazolyl)-6-morpholino-1,3,5-triazine and 2-(1-imidazolyl)-4-(*N*-methyl-*N*-phenylamino)-6-morpholino-1,3,5-triazine showed cytotoxic activity for most cell lines, which was significantly greater than the activity of hydroxymethylpentamethylmelamine (HMPMM), a major metabolite of HMM.

Key words imidazolyl-1,3,5-triazine; cytotoxicity; hexamethylmelamine; hydroxymethylpentamethylmelamine

We very recently synthesized imidazolyl-1,3,5-triazine derivatives and examined their effects on activities of human placental aromatase, finding that these derivatives inhibited aromatase activity.¹⁾ Hexamethylmelamine (HMM), a 1,3,5-triazine derivative has previously been recognized as a clinically effective antitumor agent against lung, ovarian and breast cancer.²⁻⁴⁾ It has been reported that HMM is effective on several experimental tumor models *in vivo*⁵⁻⁷⁾ and hydroxymethylpentamethylmelamine (HMPMM), hydroxylated metabolite and a major active form of HMM shows cytotoxic activity *in vitro*.^{8,9)} Furthermore, SAE9, an imidazolyl-1,3,5-triazine derivative was reported to have aromatase inhibitory activity and direct antitumor activity *per se*.¹⁰⁾ Therefore, newly synthesized imidazolyl-1,3,5-triazine derivatives were expected to have direct antitumor activity due to their molecular similarity. Before reaching our final goal of discovering novel antitumor agents which have direct antitumor activity, we first tested *in vitro* cytotoxic activity of imidazolyl-1,3,5-triazine derivatives which have selective aromatase inhibitory activity.

MATERIALS AND METHODS

Chemicals Imidazolyl-1,3,5-triazine derivatives, HMM, HMPMM and CGS16949A (4-{5,6,7,8-tetrahydroimidazo[1,5- α]-pyridin-5-yl}benzonitrile 1/2 oxalate) were synthesized in our laboratory.

Cell Lines The cytotoxic activity was analyzed on four human breast cancer cell lines, MCF-7, R-27, T-47D, ZR-75-1 and a murine leukemia cell line, P388 leukemia (P388). MCF-7, R-27, T-47D and ZR-75-1 were kindly supplied by Dr. T. Kubota, Department of Surgery, School of Medicine, Keio University, Tokyo. P388 was kindly provided by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research.

Cytotoxic Activity Test RPMI1640 medium (GIBCO BRL) supplemented with 10% fetal calf serum (GIBCO BRL), 25 mM HEPES (Dojin), 5 μ M 2-hydroxyethyl

disulfide (Aldrich Chemical Co.) and 100 μ g/ml kanamycin (Dainihon Seiyaku Co., Ltd.) was used as assay medium. Cells were incubated at 37 °C in a humidified atmosphere of 95% air–5% CO₂. Human breast cancer cells (MCF-7, R-27, T-47D and ZR-75-1) in exponential growth phase were harvested by trypsinization using Trypsin–EDTA (GIBCO BRL) and were inoculated into 96 well-microculture plates (TC Micro Well 96F, NUNC) at the density of 2×10^3 cells per well in 100 μ l growth medium. After 1 d incubation, 100 μ l of growth medium containing various concentrations of samples dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd.) was added to each well, and cells were incubated for another 3 (R-27 and T-47D) or 6 (MCF-7 and ZR-75-1) d. Ascitic fluid containing P388 cells was removed from mice 6 or 7 d after intraperitoneal transplantation of P388 cells. P388 cells were separated by centrifugation at $800 \times g$ for a few seconds and were inoculated into 25 cm² tissue culture flasks (TC Flask 25CM2, NUNC) at 2×10^6 cells per flask in 5 ml growth medium. After 1 d incubation, P388 cells were inoculated into 96 well-microculture plates at 10^4 cells per well in 100 μ l growth medium, and 100 μ l of growth medium containing various concentrations of samples dissolved in DMSO was added to culture wells. Cells were incubated for another 2 d.

MTT assay Cytotoxic activity was determined by the MTT assay.¹¹⁾ Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) was prepared at 5 mg/ml in saline and 20 μ l of MTT solution was added to microculture wells at the end of the incubation. After an additional 4 h incubation, the supernatant was removed and the cell-generated MTT formazan was solubilized by the addition of 150 μ l of isopropanol (Wako Pure Chemical Industries, Ltd.) supplemented with 0.04 N HCl and sonication. Absorption-spectra of formazan were measured using a microplate spectrophotometer (MTP-32, CORONA) at 550 nm. The percentage of cell growth inhibition was calculated as follows: percent of cell growth

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Table 1. Cytotoxic Activity (IC₅₀) of 1,3,5-Triazines (below) and CGS16949A

Compound	R ¹	R ²	R ³	IC ₅₀ (M)				
				MCF-7	R-27	T-47D	ZR-75-1	P388
1				> 2.0 × 10 ⁻⁴	1.1 × 10 ⁻⁴	> 2.0 × 10 ⁻⁴	> 2.0 × 10 ⁻⁴	1.0 × 10 ⁻⁴
2				7.3 × 10 ⁻⁶	1.9 × 10 ⁻⁵	1.9 × 10 ⁻⁵	5.5 × 10 ⁻⁶	3.0 × 10 ⁻⁵
3				2.6 × 10 ⁻⁵	3.0 × 10 ⁻⁵	4.3 × 10 ⁻⁵	1.1 × 10 ⁻⁵	6.4 × 10 ⁻⁵
4				1.3 × 10 ⁻⁵	2.1 × 10 ⁻⁵	1.2 × 10 ⁻⁵	4.8 × 10 ⁻⁵	1.6 × 10 ⁻⁵
5				1.3 × 10 ⁻⁵	2.0 × 10 ⁻⁵	2.4 × 10 ⁻⁵	7.8 × 10 ⁻⁶	2.9 × 10 ⁻⁵
HMM	-NMe ₂	-NMe ₂	-NMe ₂	> 2.0 × 10 ⁻⁴	> 2.0 × 10 ⁻⁴	> 2.0 × 10 ⁻⁴	1.5 × 10 ⁻⁴	> 2.0 × 10 ⁻⁴
HMPMM	-NMe ₂	-N(Me)CH ₂ OH	-NMe ₂	> 2.0 × 10 ⁻⁴	1.2 × 10 ⁻⁴	1.4 × 10 ⁻⁴	1.5 × 10 ⁻⁴	4.9 × 10 ⁻⁵
SAE 9	-NMe ₂			4.7 × 10 ⁻⁵	7.7 × 10 ⁻⁵	7.7 × 10 ⁻⁵	4.4 × 10 ⁻⁵	5.1 × 10 ⁻⁵
CGS16949A				1.4 × 10 ⁻⁴	> 2.0 × 10 ⁻⁴	> 2.0 × 10 ⁻⁴	2.0 × 10 ⁻⁴	> 2.0 × 10 ⁻⁴

Compounds **1**–**5** and SAE9 are imidazolyl-1,3,5-triazine derivatives. Cells were incubated with various concentrations of compounds for either 2 (P388), 3 (R-27 and T-47D) or 6 (MCF-7 and ZR-75-1) d. IC₅₀, 50% inhibitory concentration determined by MTT assay.

inhibition = (1 – T/C) × 100, where C is the mean OD₅₅₀ of the control group and T is that of treated group. Means were obtained by 6 determinations. IC₅₀ value was determined graphically from the dose–response curve.

RESULTS AND DISCUSSION

The cytotoxic effects of 1,3,5-triazine derivatives and CGS16949A on human breast cancer cell lines and murine P388 leukemia cell line are shown in the table in terms of IC₅₀ (M). HMPMM showed a moderate cytotoxic activity, but HMM showed none, except a moderate activity for ZR-75-1 cells. These results are not inconsistent with the report that HMM requires microsomal metabolism to HMPMM for its cytotoxicity.^{8,12} 2-(1-Imidazolyl)-4,6-bis(4-tetrahydro-4H-1,4-thiazinyl)-1,3,5-triazine (compound **1**) which has two tetrahydro-4H-1,4-thiazinyl (thiomorpholinyl) moieties showed a meager cytotoxic activity, while 2-(1-imidazolyl)-4,6-bis(morpholino)-1,3,5-triazine (compound **2**), 2-(1-imidazolyl)-4-morpholino-6-(3-thiazolidinyl)-1,3,5-triazine (compound **3**), 2-(4-cyano-4-phenylpiperidino)-4-(1-imidazolyl)-6-morpholino-1,3,5-triazine (compound **4**) and 2-(1-imidazolyl)-4-(N-methyl-N-phenylamino)-6-morpholino-1,3,5-triazine (compound **5**) containing one or two morpholino moieties showed stronger cytotoxic activities than HMM, HMPMM, SAE9 and CGS16949A in most cell lines. Recently, we reported that compounds **1**–**5** inhibited strongly aromatase activity with little inhibition of P450SCC activity.¹ There is a report showing that CGS16949A has a strong aromatase inhibitory activity and inhibits testosterone-induced growth of MCF-7 cultivated in estrogen free medium,¹³ and the compound has been used clinically for estrogen-

dependent breast cancer. In the present study, CGS16949A and compound **1** showed only weak cytotoxic activity against human breast cancer cell lines and murine P388 leukemia cell line. The growth inhibitory effect of compounds **2**–**5** could not be attributed to their aromatase inhibitory activity, because estrogens were not removed from the culture medium supplemented with 10% fetal calf serum. These results suggest that effects of compounds **2**–**5** were due to their direct cytotoxicity which was not related to their aromatase inhibitory activity. Compounds **2**–**5** are thought to be candidates for unique antitumor agents bearing both direct cytotoxicity and selective aromatase inhibitory activity. Experiments are under way to evaluate whether these compounds can be useful against estrogen-dependent breast cancer.

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