Tumor-specificity and Type of Cell Death Induced by Phenoxazines

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Abstract. Phenoxazines have shown diverse biological activities, but tumor-specific cytotoxic activity has not been investigated. A total of 24 phenoxazine derivatives (WM1-24) was investigated for their relative cytotoxicity against human tumor cell lines vs. normal cells. WM7 and WM8 showed the highest tumor-specificity index of 4.3 and 4.8, respectively. Considerable difference in drug-sensitivity was found among these tumor cell lines. Human promyelocytic leukemia HL-60 cells showed the highest sensitivity to both WM7 and WM8, followed by human oral squamous cell carcinoma (HSC-2, HSC-3, HSC-4), and human gingival fibroblast (HGF), pulp cell (HPC) and periodontal ligament fibroblast (HPLF) were the most resistant. WM7 and WM8 induced little or no internucleosomal DNA fragmentation, and activated caspase-3 in HSC-2, HSC-4 and human glioblastoma T98G cells. These compounds failed to induce autophagic cell death, as judged by acridine orange and microtubule-associated protein 1 light chain 3 (LC3)-GFP assays. These results suggested that the higher cytotoxicity of WM7 and WM8 are derived from the positively-charged quaternary nitrogen substituents on the phenoxazine ring and the electron density of nitrogen at N12, and that inhibition of autophagy is not always coupled with apoptosis induction.

Actinomycin D has been clinically used for the treatment of many carcinomas and is known to be a DNA intercalator. The structure of actinomycin D is based on a phenoxazine ring bound to two cyclic pentapeptides (1). The presence of the phenoxazine ring in the structure of actinomycin D suggests that phenoxazine derivatives may possess anticancer activity. Phenoxazine derivatives are known to be effective multidrug resistance (MDR) modulators in cancer cells (2), potent inhibitors of Akt signaling in cells (3), inhibitors of human plasma cholinesterase (4), and photochemotherapeutic agents in cancer cells (5).

Recent studies have also shown that the relatively water soluble phenoxazines, such as 2-amino-4,4·-dihydro-4·,7-dimethyl-2H-phenoxazine-3-one and 2-aminophenoxazine-3-one exert antitumor effects on various cancer cells in vitro and in vivo (6).

We have recently investigated hundreds of natural and synthetic compounds for their cytotoxicity against both human oral squamous cell carcinoma cell lines and human normal cells, and found that most of the synthetic compounds have shown much lower tumor-specific cytotoxicity as compared with anthracycline antibiotics, cyclic α,β-unsaturated ketone and nocoactins (7). To search for more potent antitumor compounds, 24 phenoxazines were investigated for their cytotoxicity against four human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, HSC-4 and promyelocytic leukemia HL-60) and three human normal oral cells (gingival fibroblast HGF, pulp cell HPC and periodontal ligament fibroblast HPLF). There are at least three types of cell death, apoptosis (type I programmed cell death), autophagy (type II programmed cell death) and necrosis, and the type of cell death induced by chemicals is determined by various factors such as the chemical structure of the inducers and the type of target cells (8). Therefore, the type of cell death induced by WM7 and WM8, that were found to express the highest tumor-specificity and cytotoxicity, was also investigated.

Key Words: Phenoxazines, cytotoxicity, cell death, caspase, type of cell death.

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Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH, Bioscience, Lenexa, KS, USA), RPMI1640, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., Ind., St. Louis, MO, USA) and dimethylsulfoxide (DMSO)(Wako Pure Chemical Ind., Osaka, Japan).

Synthesis of phenoxazines. The phenoxazines were synthesized as described previously (2, 9). Their structures are shown in Figure 1.

Cell culture. Human oral normal cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF) were prepared from the periodontal tissues, according to the guideline of Intramural Board of Ethic Committee (No. 0206) after obtaining the informed consent from the patients. Since these normal cells have the limited lifespan due to the in vitro senescence, the cells at the 8-12 population doubling levels were used for the present study. These normal cells, human oral squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4) and human glioblastoma cell line T98G were cultured in DMEM medium supplemented with 10% heat-inactivated FBS in a humidified 5% CO2 atmosphere. The human promyelocytic leukemia HL-60 cells were cultured in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, as described previously (10).

Assay for cytotoxic activity. Near-confluent cells were treated for the indicated times with various concentrations of test samples, and the relative viable cell number of adherent cells was then determined by the MTT method, as described previously (10). The viable cell number of HL-60 cells was determined by hemocytometer after staining with trypan blue. The 50% cytotoxic concentration (CC50) was determined from the dose-response curve. The tumor-specificity index (TS) was measured by the following equation: TS = [CC50 (HGF) + CC50 (HPC) + CC50 (HPLF)] / [CC50 (HSC-2) + CC50 (HSC-3) + CC50 (HSC-4) + CC50 (H-L-60)] x (4/3) (Table I).

Assay for DNA fragmentation. The HSC-2, HSC-4 and T98G cells
(collected by scraping with a rubber policeman) and HL-60 cells (in suspension) that had been treated for 6 hours without or with test samples, were pelleted and washed once with phosphate-buffered saline without Ca$^{2+}$ and Mg$^{2+}$ (PBS(–)). They were then lysed, digested with RNase A and proteinase K. The DNA was extracted and subjected to 2% agarose gel electrophoresis, as described previously (10). A DNA molecular weight marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic cells induced by treatment with actinomycin D (1 ìg/mL, 6 hours) were run in parallel as positive controls. After staining with ethidium bromide, the DNA was visualized by UV irradiation, and photographed by CCD camera (Bio Doc-It, UVP, Inc., Upland, CA, USA).

**Assay for caspase activation.** The cells were washed, lysed and incubated for 4 hours at 37°C with substrates for caspase-3 (DEVD-pNA (p-nitroanilide)), caspase-8 (IETD-pNA) or caspase-9 (LEHD-pNA). The absorbance at 405 nm of the liberated chromophore pNA was then measured by plate reader, as described previously (10).

**Detection of acidic vesicular organelles with acridine orange staining.** Acidic vesicular organelles were stained with acridine orange (Sigma Chemical Co., St. Louis, MO, USA) as described previously (10). The HSC-4 or T98G cells were treated for 2 or 20 hours without (control), or with test samples and then stained with 1 µg/mL acridine orange for 20 minutes. After washing once with PBS(–), the samples were then examined under a Laser Scanning Microscope LSM510, using the following filter: excitation filter 488 nm, emission filter 505-530 nm and >650 nm.

**Results**

**Cytotoxicity.** Among the 24 phenoxazine derivatives, WM4 showed the highest cytotoxicity, followed by WM5>WM10>WM7>WM8, whereas the other 19 phenoxazines showed much less cytotoxic activity (Table I). In particular, WM8 showed the highest tumor-specific index (TS=4.8), followed by WM7 (TS=4.3)>WM10 (TS=3.4)>WM4 (TS=2.3)>WM5 (TS=1.2). The TS values of the other 19 phenoxazines were 1.0 ~2.6. WM23 and WM24 showed a cytostatic effect, but did not show a cytotoxic effect (data not shown). Since WM7 and WM8 showed the highest tumor-specific cytotoxic activity, these phenoxazines were investigated in more detail.

**Drug sensitivity.** A considerable difference in drug-sensitivity was found among these cell lines. The sensitivity to WM7 was in the order: HL-60 (CC$_{50}<1.6$ µM) (most sensitive) >HSC-
3 (3.8 μM) > HSC-2 (25 μM) > HSC-4 (28 μM) > HPLF (37 μM) > HPC (75 μM) = (77 μM) (most resistant) (Table I).

Similarly, the sensitivity to WM8 was in the order: HL-60 (CC50 < 1.6 μM) (most sensitive) > HSC-4 (17 μM) > HSC-2 (18 μM) > HSC-3 (31 μM) > HPLF (44 μM) > HGF (67 μM) > HPC (133 μM) (most resistant) (Table I).

Type of cell death. WM7 (10-80 μM) did not induce internucleosomal DNA fragmentation in three tumor cell lines (HSC-2, HSC-4 and T98G), in contrast with the apoptosis induced by actinomycin D in HL-60 cells (Figure 2). T98G cells were used, since this cell line was resistant, and easily committed to autophagy. Actinomycin D induced only traces of DNA fragmentation in the HSC-2, HSC-4 and T98G cells. Even when treatment time was prolonged to 24 hours, WM7 and WM8 did not induce DNA fragmentation in the HSC-2, HSC-4 and T98G cells (Figure 2B).

Both WM7 and WM8 enhanced the caspase-3, caspase-8 and caspase-9 activity but only at the higher concentrations (40 or 80 μM) in the HSC-2, HSC-4 and T98G cells.
However, the extent of caspase-3 activation by these compounds was much less than that induced by actinomycin D in HL-60 cells (positive control) (Figure 3).

The little or no apoptosis-inducing activity of WM7 and WM8 urged us to investigate whether these compounds can induce autophagy, another type of cell death. The formation of an acidic organelle that is the secondary lysosome engulfing broken organelles was used as a maker of autophagy. Treatment with WM7 considerably reduced the incidence of acid organelles detected by acridine orange staining in HSC-4 cells (Figure 4). Similarly, WM7 did not affect the accumulation of microtubule-associated protein 1 light chain 3 (LC3)-GFP fusion protein in the autophagosome, a popular marker of autophagy (data not shown). No apparent effect of WM8 on autophagosome formation detected by acridine orange and LC3-GFP was found (data not shown). These data demonstrated that WM7 and WM8 failed to induced autophagy in the HSC-4 cells.

**Discussion**

The twenty-four phenoxazine derivatives showed low to moderate tumor-specific cytotoxicity (TS=1.0-4.8), which was highest for WM7 and WM8. These compounds induced little or no apoptotic characteristics such as internucleosomal DNA fragmentation and caspase activation, and rather inhibited autophagosome formation. This finding is not consistent with the recent report that the inhibition of autophagy by 3-methyladenine enhanced the oridonin-induced apoptosis in HeLa cells (11). It therefore seems likely that the inhibition of autophagy is not always coupled with the apoptosis induction.

Four cationic phenoxazine dyes (WM4-7) were found to be cytotoxic against both tumor and normal cells. The presence of positively-charged quaternary nitrogen substituents on the phenoxazine ring may be beneficial to the cytotoxicity.

Of the three benzophenoxazines WM8, 9 and 10, WM8 and 10 were cytotoxic against the tumor cells. The difference in chemical structure between WM9 and 10 is the position of the hydroxyl group in ring D. The presence of the hydroxyl group at C-1 in WM9 was not beneficial to the cytotoxic activity, presumably due to the presence of the intramolecular hydrogen bond between N12 and the 1-hydroxyl group. It is therefore speculated that the electron density of the nitrogen at the 12 position played an important role in determining the activity of the phenoxazine derivatives, because the hydrogen bond reduces the density of the nitrogen and could possibly reduce their ability to interact with other molecules.

Among the compounds WM14-24, cytostatic WM23 and 24 have a tertiary amine group at the end of the side-chain. The difference in chemical structure between WM14-22 and WM23/24 is the presence or absence of the quaternized nitrogen at the side chain. Thus, it is suggested that the presence of positively-charged quaternary nitrogen on the side chain substituent of the ring was favourable to the cytostatic activity. It should be noticed, however, that the above conclusions may be limited to the relatively small set of compounds investigated in the present study. To establish whether these conclusions are valid, further studies would be required in which larger sets of phenoxazines were used.

Chemical structure also affects the type of cell death (8). We have recently found that α,β-unsaturated ketones, such as 4,4-dimethyl-2-cyclopenten-1-one, α-methylene-γ-butyrolactone, 5,6-dihydro-2H-pyran-2-one (12), codeinone (13) and morphinone (14), and α-hydroxyketone such as 3,3,3-trifluoro-2-hydroxy-1-phenyl-1-propanone, induced caspase-independent cell death (15), induced vacuolization or autophagosome formation engulfing organelles, but without
induction of apoptosis markers. WM8 has an α,β-unsaturated ketone structure, whereas WM7 has an imine structure. Further structure and activity study is required to determine whether this structure is required for non-apoptotic cell death.

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References


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