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Tumor-specific Cytotoxicity of 3,5-Dibenzoyl-1,4-dihydropyridines

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Abstract. *In search of compounds which show tumor-specific cytotoxic activity, two 3,5-dibenzoyl-1,4-dihydropyridines (**GB5**, **GB12**) were found to show one or two orders higher cytotoxic activity against human tumor cell lines (squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG, promyelocytic leukemia HL-60) than human normal cells (gingival fibroblast HGF, pulp cells HPC, periodontal ligament fibroblasts HPLF). **GB5** and **GB12** weakly induced several apoptosis-associated properties, such as internucleosomal DNA fragmentation, and activation of caspases -3, -8 and -9, in both HL-60 and HSC-2 cells. Western blot analysis showed that **GB5** and **GB12** transiently increased the expression of both anti-apoptotic protein (Bcl-2) and pro-apoptotic proteins (Bax and Bad) in HL-60 cells. ESR spectroscopy showed these compounds did not produce any detectable amount of radicals, nor scavenged superoxide (generated by hypoxanthine-xanthine oxidase reaction) or nitric oxide (generated by 1-hydroxy-2-oxo-3-(N-3-methyl-3-aminopropyl)-3-methyl-1-triazene), suggesting that the induction of cytotoxic action is not via a radical-mediated reaction. The present study suggests that **GB5** and **GB12** may induce non-apoptotic cell death in tumor cell lines.*

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Cancer chemotherapy has traditionally been based on cytotoxic drugs that damage DNA directly, block its synthesis or interfere with the mechanisms of cell division. While treatment of certain malignancies with chemotherapy has been successful and encouraging, effectiveness has often been limited by side-effects on normal tissues and by drug resistance of tumors. 1,4-Dihydropyridines are well known as Ca^{2+} channel blockers and their role as drugs for the treatment of cardiovascular diseases, including hypertension (1). The 1,4-dihydropyridine heterocyclic ring is a common feature of various bioactive compounds such as vasodilator, bronchodilator, anti-atherosclerotic, neuroprotectant, platelet anti-aggregatory, anti-ischemic, antidiabetic and antitumor agents (2-6). Recent examples are antitubercular agents (7, 8) and multidrug resistance (MDR) modulators (9-12). Among novel 3,5-diacetyl and 3,5-dibenzoyl-1,4-dihydropyridine derivatives, we have found that 4-(2'-trifluoromethylphenyl)- (**GB5**) (MW461) and 4-(3'-chlorophenyl)-3,5-dibenzoyl-2,6-dimethyl-1,4-dihydropyridine (**GB12**) (MW427) (structure shown in Figure 1) showed not only MDR reversal activity, but also markedly higher cytotoxicity against two human oral tumor cell lines than one normal cell (human gingival fibroblast) (11). In this report, we first confirmed the tumor-specificity of **GB5** and **GB12**, using a total of seven human cells, including four tumor cell lines (squamous cell carcinoma HSC-2, HSC-3, submandibular carcinoma HSG, promyelocytic leukemia HL-60) and three normal cells (gingival fibroblast HGF, pulp cells HPC, periodontal ligament fibroblast HPLF). We also investigated whether **GB5** and **GB12** induce apoptotic cell death in HL-60 and HSC-2 cells, using several apoptosis-associated markers, such as internucleosomal DNA

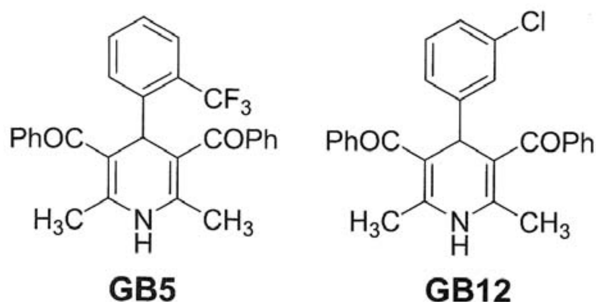

Figure 1. *Structure of **GB5** and **GB12**.*

fragmentation (assayed by agarose gel electrophoresis), activation of caspases -3, -8 and -9 (assayed by substrate cleavage activity) and expression of pro-apoptotic proteins (Bax, Bad) and an anti-apoptotic protein (Bcl-2) (assayed by Western blot analysis). Finally, we investigated whether the cell death is induced by **GB5** and **GB12** *via* radical-mediated reaction, by ESR spectroscopy.

Materials and Methods

Materials. The following reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Biosci, Lenexa, KS, USA); 3-[4, 5-dimetylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT), hypoxanthine (HX), xanthine oxidase (XOD), diethylenetriaminepentaacetic acid (DETAPAC), phenylmethylsulfonyl fluoride (PMSF) (Sigma Chem. Ind., St. Louis, MO, USA); RNase A, proteinase K (Boehringer, Mannheim, Germany); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Osaka, Japan); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy1-3-oxide (carboxy-PTIO) (a spin trap agent), 1-hydroxy-2-oxo-3-(*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) (NO-generator), superoxide dismutase (SOD) from bovine erythrocytes (Dojin, Kumamoto, Japan); DNA size marker (100 bp DNA Ladder, Bayou Biolabs, Harahan, LA, USA); DEVD-*p*NA (*p*-nitroanilide), IETD-*p*NA, LEHD-*p*NA (MBL, Nagoya, Japan); anti-Bcl-2 antibody, anti-Bax antibody, anti-Bad antibody (Santa Cruz, Delaware, Cam, USA); horseradish peroxidase (HRP)-linked anti-rabbit IgG, HRP-linked anti-mouse IgG (Amersham, Bioscienes Corp., NJ, USA); RNase A, proteinase K, anti-Actin antibody (Sigma); HL-60, HSC-2, HSC-3 (Japanese Collection of Research Bioresources (JCRB), Cell Bank, Japan); HSG (supplied by Dr. Atsumi, Meikai University, Japan); HGF, HPC, HPLF (supplied by Dr. Kikuchi, Meikai University, Japan).

*Synthesis of **GB5** and **GB12**.* **GB5** and **GB12** (structure shown in Figure 1) were synthesized as described previously (11).

Cell culture. HSC-2, HSC-3, HSG, HGF, HPC and HPLF cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Normal cells were prepared from periodontal tissues, following the guidelines of Meikai University Ethics Committee, Japan (No. 0206), after obtaining informed consent from the patients. Since normal cells (HGF, HPC, HPLF) have a limited lifespan, all of them ceasing proliferation at the 20 population doubling level (PDL), these cells were used at 5-9 PDL in the present study. HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% FBS.

Assay for cytotoxic activity. Near confluent cells grown in 96-microwell plate (Falcon, flat bottom, treated polystyrene, Becton Dickinson, Franklin Lakes, NJ, USA) were incubated for 24 hours with various concentrations of samples. The cells were washed once with phosphate-buffered saline (PBS), and incubated for 4 hours with fresh culture medium containing 0.2 mg/mL MTT. After removing the medium, the cells were lysed with 100 μL DMSO and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate, using the Labsystem Multiskan^R (Biochromatic, Helsinki, Finland). The HL-60 viable cell number was determined by trypan blue exclusion. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve. Tumor-specific cytotoxic activity (TS) was measured by the following equation:

$$TS= [CC_{50}\,(HGF) + CC_{50}\,(HPC) + CC_{50}\,(HPLF)] / [CC_{50}\,(HSC\text{-}2) + CC_{50}\,(HSC\text{-}3) + CC_{50}\,(HSG) + CC_{50}\,(HL\text{-}60)] \times (4/3)$$

Assay for DNA fragmentation. The cells were pelleted, lysed and digested with RNaseA and proteinase K. DNA was isolated and assayed for DNA fragmentation by 2% agarose gel electrophoresis (13). The DNA isolated from the UV-irradiated HL-60 cells was run in parallel, as a marker of oligonucleosomal DNA fragments (13).

Assay for caspase activation. The cells were washed with PBS and lysed in lysis solution (MBL, Nagoya, Japan). After standing for 10 minutes on ice and centrifugation for 5 minutes at 10,000 xg, the supernatant was collected. The lysate (50 μL, equivalent to 200 μg protein) was mixed with 50 μL 2x reaction buffer (MBL) containing substrates for caspase-3 (DEVD-*p*NA (*p*-nitroanilide)), caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*NA). After incubation for 4 hours at 37˚C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by plate reader.

Western blotting. The cells were lysed with 100 μL of lysis buffer (10 mM Tris-HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA and 2 mM PMSF) for 10 minutes in ice-water, and then incubated for 50 minutes at 4˚C with ROTATOR RT-5 (Titec, Saitama, Japan). The cell lysates were centrifuged at 16,000 xg for 20 minutes at 4˚C to remove insoluble materials and the supernatant was collected. The protein concentration of the supernatant was determined by Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). The cell lysates (containing 25 μg protein) were mixed with an equal volume of 2 x sodium dodecyl sulfate (SDS)-sample buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromphenol blue, 1.2% 2-mercaptoethanol), boiled for 5 minutes and applied to the SDS-12% polyacrylamide gel electrophoresis, before being transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in PBS plus 0.05% Tween 20 for 1.5 hours and incubated with anti-Bcl-2 (1:1,000), anti-Bax antibody (1:1,000), anti-Bad antibody (1:1,000) or anti-Actin antibody (1:1,000) for 1.5 hours at room temperature or overnight at 4˚C,

Table I. *Tumor-specific cytotoxic action of two 3,5-dibenzoyl-1,4-dihydropyridines.*

	CC_{50} (μM)							TS
	Normal cells			Tumor cell lines				
	HGF	HPC	HPLF	HSC-2	HSC-3	HSG	HL-60	
GB5	>1870	>2164	>1859	111	72	44	13	>33
GB12	>2300	>2110	>2300	39	113	12	5	>53

Each value represents the mean from two independent experiments.

and then incubated with HRP-linked anti-rabbit IgG or anti-mouse IgG for 1 hour at room temperature. Immunoblots were detected by Western Lightning™ Chemiluminescence Reagent *Plus* (Perkin Elmer Life Sciences, Boston, MA, USA).

Assay for radical intensity. The radical intensity of the test samples in 0.1 M Tris-HCl (pH 7.4, 8.0), in 0.1 M $Na_2CO_3/NaHCO_3$ (pH 9, 10) or in 0.1 M KOH (pH 12.5) was determined at 25˚C, using ESR spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency). Instrument settings; center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT: gain, 500 or 630, time constant, 0.03 or 0.1 seconds; scanning time, 2 or 4 minutes.

For determination of superoxide anion radical (O_2^-), produced by hypoxanthine (HX) and xanthine oxidase (XOD) reaction (total volume : 200 μL) [2 mM HX in 0.1 M phosphate buffer (pH 7.4) (PB) 50 μL, 0.5 mM DETAPAC 20 μL, 8% DMPO 30 μL, sample (in DMSO) 40 μL, H_2O 30 μL, XOD (0.5 U/ml in PB) 30 μL] , the gain, time constant and scanning time were changed to 500, 0.1 seconds and 2 minutes, respectively. The radical intensity was determined by ESR spectroscopy 1 minute after mixing.

The radical intensity of NO, produced from the reaction mixture of 50 μM NOC-7 (NO-generator) and 20 μM carboxy-PTIO, was determined in 0.1 M phosphate buffer, pH 7.4 in the presence of 30% DMSO (microwave power, 5 mW; modulation amplitude, 5 mT; gain, 250; time constant, 0.1 seconds). NO radical intensity was defined as the ratio of the radical intensity of the first peak of C-PTI (indicated by asterisks in Figure 5C) to that of MnO. Samples were added to the carboxy-PTIO/NOC-7 system to evaluate the NO radical scavenging activity.

Results

Tumor-specific cytotoxic action. **GB5** and **GB12** showed higher cytotoxicity against the tumor cell lines (HSC-2, HSC-3, HSG, HL-60) than normal cells (HGF, HPC, HPLF), yielding a TS value of >33 and >53, respectively (Table I). The tumor-specific cytotoxic action of **GB5** and **GB12** was thus confirmed.

Apoptosis induction. **GB5** and **GB12** dose-dependently, but very faintly, induced internucleosomal DNA fragmentation in HL-60 cells (Figure 2), whereas they failed to induce DNA fragmentation in HSC-2 cells (data not shown). **GB5** and **GB12** also activated three representative caspases (caspases -3, -8 and -9), to an extent significantly lower than that attained by actinomycin D (a positive control) in HL-60 cells (Figure 3A, B), and only at higher concentration (160 μM) in HSC-2 cells (Figure 3C, D). Western blot analysis showed that **GB5** (Figure 4A) and **GB12** (Figure 4B) transiently increased the expression of both the anti-apoptotic protein (Bcl-2) and pro-apoptotic proteins (Bax, Bad) at lower concentrations (2.5-40 μM), and reduced their expression at higher concentrations in HL-60 cells.

Radical generation and scavenging activity. **GB5** (5 mM) and **GB12** (5 mM) did not produce any detectable radical in 0.1 M Tris-HCl (pH 7.4, 8), 0.1 M $Na_2CO_3/NaHCO_3$ buffer (pH 9, 10) or in 0.1 M KOH (pH 12.5) (radical intensity <0.03) (Figure 5A).

GB5 (2 mM) and **GB12** (2 mM) did not significantly scavenge O_2^- (measured as DMPO-OOH adduct) generated by hypoxanthine and xanthine oxidase reaction, in the presence of DMPO, a spin trap agent. The intensity of DMPO-OOH in the reaction buffer without (control) or with **GB5** or **GB12** (1 mM), was 2.89, 3.30 and 2.97, respectively (Figure 5B).

We next investigated the NO radical scavenging activity of **GB5** and **GB12**. When NOC-7 (NO generator) and carboxy-PTIO (a spin trap agent) were mixed, NO was oxidized to NO_2 and carboxy-PTI. Carboxy-PTIO and carboxy-PTI were not overlapped with each other, so it was easy to distinguish the signals of carboxy-PTIO from that of carboxy-PTI. NO radical intensity was defined as the ratio of signal intensity of the first peak of carboxy-PTI (indicated by asterisks) (Figure 5C) to that of MnO. The radical intensity in the absence (control) or presence of **GB5** or **GB12** (1 mM) was 2.13, 1.94 and 2.21, respectively (Figure 5C). This indicates that **GB5** and **GB12** did not scavenge the NO radical.

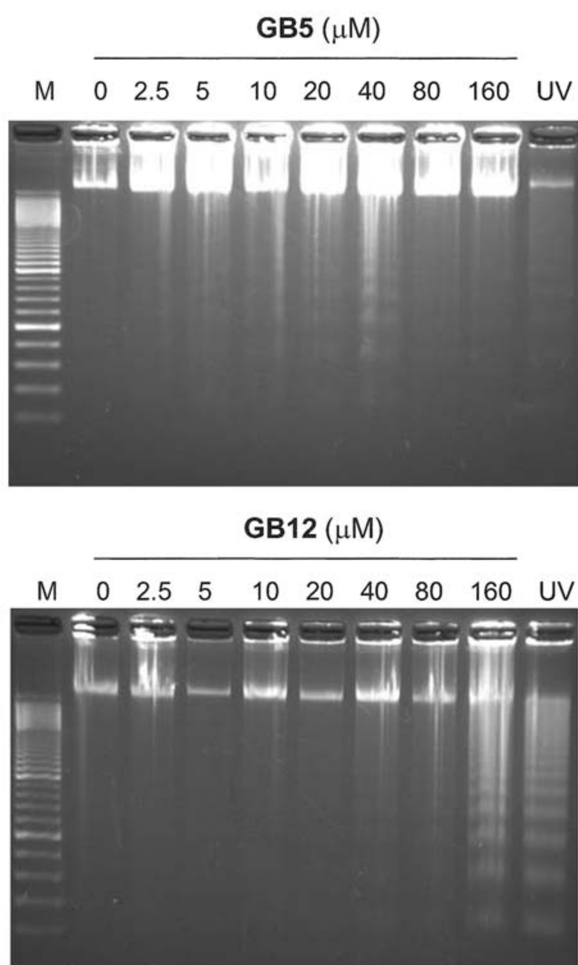

Figure 2. *Induction of internucleosomal DNA fragmentation by **GB5** and **GB12**. HL-60 cells were incubated for 6 hours with the indicated concentrations of **GB5** or **GB12**. The DNA was then isolated and applied to agarose gel electrophoresis. M, DNA ladder marker; UV, UV-irradiation.*

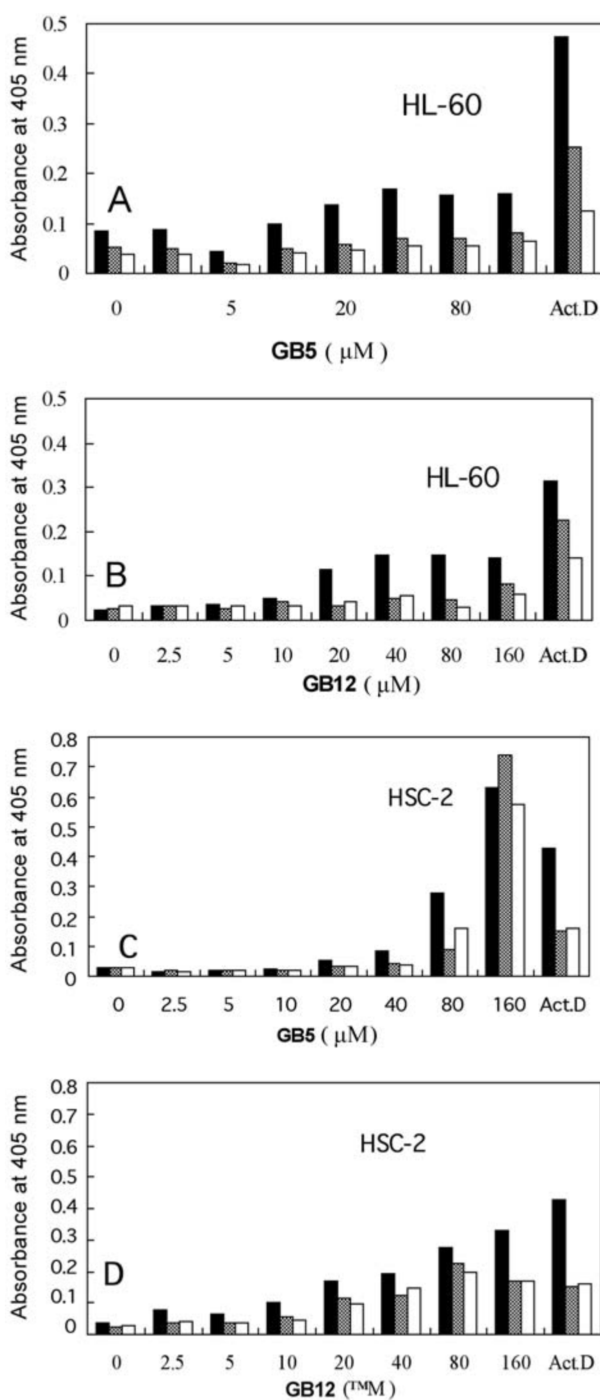

Figure 3. *Activation of caspases -3, -8 and -9 by **GB5** and **GB12** in HL-60 and HSC-2 cells. HL-60 or HSC-2 cells were incubated for 4 hours with the indicated concentrations of **GB5** or **GB12**, or actinomycin D (1 μg/mL), and caspases -3 (black bar), -8 (dotted bar) and -9 (white bar) activities were determined by cleavage of the respective substrates.*

Discussion

The present study confirmed that **GB5** and **GB12** showed tumor-specific cytotoxic activity against 4 human tumor cell lines, as compared with that against 3 normal human cells. It was unexpected that these compounds did not strongly induce apoptosis-associated characteristics such as DNA fragmentation, caspase activation and expression of several apoptosis-related proteins such as Bcl-2, Bax and Bad. It is generally accepted that the expression of the anti-apoptotic protein (Bcl-2) goes down while that of pro-apoptotic proteins (Bax, Bad) is elevated during apoptosis (14). Contrary to our expectation, the expression of all these proteins was increased and then decreased, with an increase in the concentrations of **GB5** or **GB12**. These bimodal concentration-dependent changes in the expression of these proteins may be linked to "hormesis", *i.e.*, beneficial effects at lower concentrations and adverse effects at higher concentrations (15). From these data, we conclude that **GB5** and **GB12** do not induce apoptotic cell

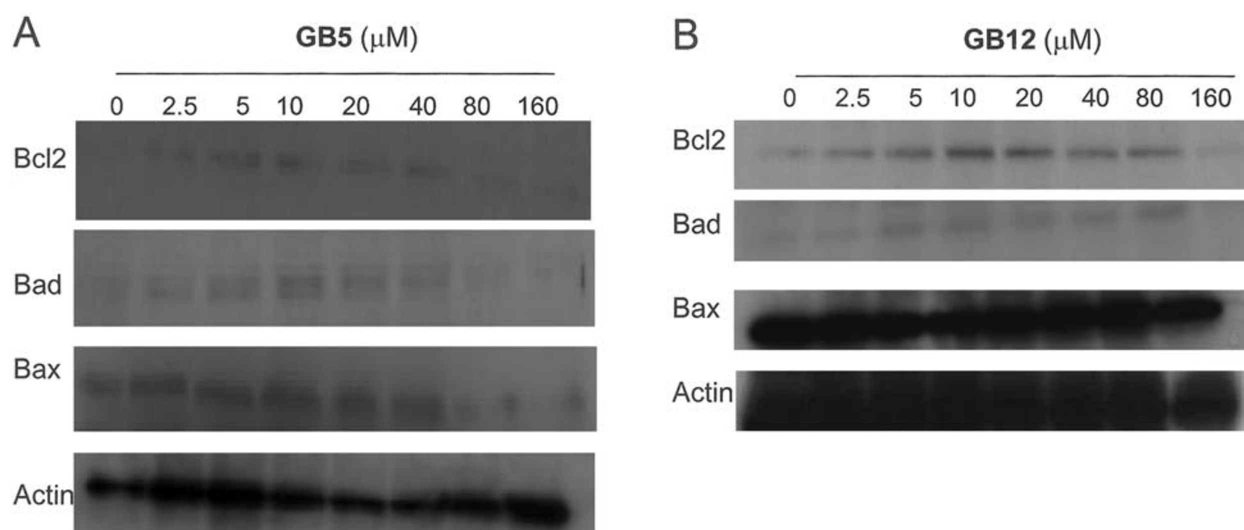

Figure 4. *Effect of **GB5** and **GB12** on the intracellular concentration of Bcl-2, Bax, Bad and Actin in HL-60 cells (Western blot analysis). HL-60 cells were incubated for 4 hours with the indicated concentrations of **GB5** (A) or **GB12** (B).*

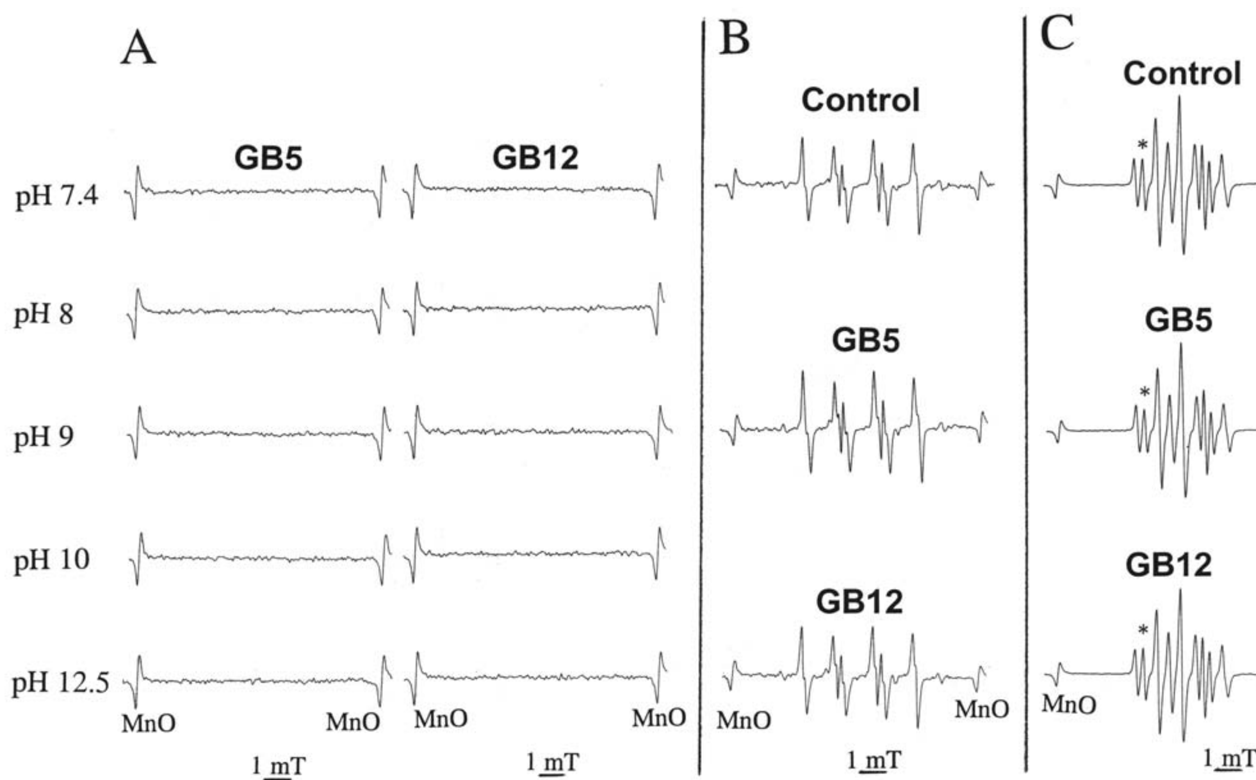

Figure 5. *Radical intensity (A), and O_2^- (B) and NO (C) scavenging activity of **GB5** and **GB12**.*

death, but possibly induce another type of cell death such as necrosis or autophagy (classified as type II programmed cell death) (16). We have recently suggested that tumor-specificity and apoptosis-inducing activity are not always coupled with each other (17). Therefore, for future clinical application, it is important to determine the type of induced cell death and investigate the signaling pathway triggered by newly-discovered tumor-specific cytotoxic substances.

The present study also demonstrated that **GB5** and **GB12** did not produce any detectable amount of radical under

alkaline conditions (pH 7.4-12.5), nor scavenged O_2^- and NO radicals. Therefore, the tumor-specific cytotoxicity of **GB5** and **GB12** seems not to be induced by radical-mediated reaction.

Since 3,5-dibenzoyl-1,4-dihydropyridines, such as **GB5** and **GB12**, showed both MDR reversal activity (1) and tumor-specific cytotoxicity (found in this study), these compounds may be applicable as possible antitumor candidates.

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References

1 Bossert F, Meyer H and Wehinger E: 4-Aryldihydropyridine, a new class of highly active calcium antagonists. Angew Chem Int Ed Engl *20*: 762-769, 1981.
2 Godfraid T, Miller R and Wibo M: Calcium antagonism and calcium entry blockade. Pharmacol Rev *38*: 321-416, 1986.
3 Mager PP, Coburn RA, Solo AJ, Triggle DJ and Rothe H: QSAR, diagnostic statistics and molecular modelling of 1,4-dihydropyridine calcium antagonists: a difficult road ahead. Drug Design Discovery *8*: 273-289, 1992.
4 Ogawa AK, Willoughby CA, Bergeron R, Ellsworth KP, Geissler WM, Myers RW, Yao J, Harris G and Chapman KT: Glucose-lowering in a *db/db* mouse model by dihydropyridine diacid glycogen phosphorylase inhibitors. Bioorg Med Chem Lett *13*: 3405-3408, 2003.
5 Sabitha G, Reddy GSKK, Reddy CS and Yadav JS: A novel TMSI-mediated synthesis of Hantzsch 1,4-dihydropyridines at ambient temperature. Tetrahedron Lett *44*: 4129-4131, 2003.
6 Tewari N, Dwivedi N and Tripathi RP: Tetrabutylammonium hydrogen sulfate catalyzed eco-friendly and efficient synthesis of glycosyl 1,4-dihydropyridines. Tetrahedron Lett *45*: 9011-9014, 2004.
7 Desai B, Sureja D, Naliapara Y, Shah A and Saxena A: Synthesis and QSAR studies of 4-substituted phenyl-2,6-dimethyl-3,5-bis-*N*-(substituted phenyl)carbamoyl-1,4-dihydropyridines as potential antitubercular agents. Bioorg Med Chem *9*: 1993-1998, 2001.
8 Kharkar PS, Desai B, Gaveria H, Varu B, Loriya R, Naliapara Y, Shah A and Kulkarni VM: Three-dimensional quantitative structure-activity relationship of 1,4-dihydropyridines as antitubercular agents. J Med Chem *45*: 4858-4867, 2002.
9 Shah A, Gaveriya H, Motohashi N, Kawase M, Saito S, Sakagami H, Satoh K, Tada Y, Solymosi A, Walfard K and Molnar J: 3,5-Diacetyl-1,4-dihydropyridines: synthesis and MDR reversal in tumor cells. Anticancer Res *20*: 373-378, 2000.
10 Tasaka S, Ohmori H, Gomi N, Iino M, Machida T, Kiue A, Naito S and Kuwano M: Synthesis and structure-activity analysis of novel dihydropyridine derivatives to overcome multidrug resistance. Bioorg Med Chem Lett *11*: 275-277, 2001.
11 Kawase M, Shah A, Gaveriya H, Motohashi N, Sakagami H, Varga A and Molnar J: 3,5-Dibenzoyl-1,4-dihydropyridines: synthesis and MDR reversal in tumor cells. Bioorg Med Chem *10*: 1051-1055, 2002.
12 Saponara S, Kawase M, Shah A, Motohashi N, Molnar, Ugocsai K, Sgaragli G and Fusi F: 3,5-Dibenzoyl-4-(3-phenoxyphenyl)-1,4-dihydro-2,6-dimethylpyridine (DP7) as a new multidrug resistance reverting agent devoid of effects on vascular smooth muscle contractility. Br J Pharmacol *141*: 415-422, 2004.
13 Yanagisawa-Shiota F, Sakagami H, Kuribayashi N, Iida M, Sakagami T and Takeda M: Endonuclease activity and induction of DNA fragmentation in human myelogenous leukemic cell lines. Anticancer Res *15*: 259-266, 1995.
14 Schwall R, Sinicropi D and Ashkenazi A: Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. Nature Med *8*: 274-281, 2002.
15 Kaiser J: Sipping from a poisoned chalice. Science *302*: 376-379, 2003.
16 Shintani T and Klionsky DJ: Autophagy in health and disease: a double-edged sword. Review. Science *306*: 990-995, 2004.
17 Sakagami H, Chowdhury SA, Suzuki F, Hashimoto K, Hatano H, Takekawa H, Ishihara M, Kikuchi H, Nishikawa H, Taniguchi S, Ito H, Hatano T, Yoshida T, Fukai T, Shirataki Y, Kawase M, Watanabe K, Mimaki Y, Itoh K, Horiuchi A, Chai W, Horiuchi A and Motohashi N: Tumor-specific cytotoxic activity of polyphenols, terpenoids, ketones and other synthetic compounds. (Review) Functional Polyphenols with Antioxidative Action, a review book series of "*Chem. Pharmaceutical Sciences* (CPS), ed., Motohashi" ("REFLASH", India) (ed.), in press.

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