in vivo *24*: 39-44 (2010)

Hormetic Response of Cultured Normal and Tumor Cells to 2-Aminotropone Derivatives

HIDETSUGU WAKABAYASHI[1], TAICHI NARITA[1], AKINA SUGA[1] and HIROSHI SAKAGAMI[2]

[1]*Faculty of Science, Josai University, Sakado, Saitama, Japan;*
[2]*Division of Pharmacology, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama, Japan*

Abstract. *We have recently reported that out of twenty benzo[b]cyclohept[e][1,4]oxazines and their S-analogs, and 2-aminotropone derivatives, 7-bromo-2-(4-hydroxyanilino) tropone and 4-isopropyl-2-(2-hydroxyanilino)tropone showed the highest tumor-specificity in human oral squamous cell carcinoma cell lines. To gain more insight into the anti-tumor actions of these compounds, whether they induce the growth stimulation effect observed at low concentrations, known as hormesis, was investigated using a total of ten human normal and tumor cultured cells. The tumor-specificity of both compounds became apparent 48 hours after the start of treatment of the cells with these compounds and reached a maximum level at 72 and 96 hours. On the other hand, their growth stimulatory effects were most prominent at 24 hours, especially in normal skin and lung fibroblasts, but rapidly disappeared with prolonged incubation time (48-96 hours). These data suggest the occurrence of a hormetic response only at restricted times and concentrations as has been previously reported, although the biological significance is yet to be elucidated.*

We have recently reported that 7-bromo-2-(4-hydroxyanilino) tropone **[16]** and 4-isopropyl-2-(2-hydroxyanilino)tropone **[20]** (Figure 1), out of twenty benzo[*b*]cyclohept[*e*][1,4] oxazines and their *S*-analogs and 2-aminotropone derivatives, showed the highest tumor specificity (TS=4.0 and 4.4, respectively), inducing few or no apoptotic markers such as DNA fragmentation and caspase activation in human oral squamous cell carcinoma HSC-2 cells (1). 7-Bromo-2-(4-hydroxyanilino)tropone **[16]** also inhibited the production of pro-inflammatory substances such as nitric oxide (NO) by lipopolysaccharide-activated mouse macrophage-like RAW264.7 cells at concentrations that do not affect the cellular viability (selectivity index=54), and this was mostly due to the inhibition of inducible NO synthase and cyclooxygenase-2 expressions at both protein and mRNA levels (2).

It has been reported that many toxic substances, environmental hormones, inorganic compounds and even irradiation modulate the growth of cultured cells in a biphasic fashion, stimulating or inhibiting the growth at lower and higher concentrations, respectively. The growth-stimulating effect at low concentrations is known as hormesis (3, 4). However, we have recently found that three Chinese herbal extracts (*Drynaria baronii, Angelica sinensis* and *Cornus officinalis* Sieb. et Zucc) failed to induce hormesis in human oral carcinoma cell lines (5). This suggested the possibility that the experimental conditions used may not have been optimal for detecting hormesis. To confirm the generality of the occurrence of hormesis, whether or not these compounds induce hormesis was investigated in seven normal human cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF, skin fibroblasts NB1RGB and 305M and lung fibroblasts WI-38 and MRC-5) and three human oral squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4), after incubating the cells with a wide range of concentrations of these compounds for various lengths of times.

Correspondence to: Dr. Hidetsugu Wakabayashi, Faculty of Science, Josai University, Sakado, Saitama 350-0295, Japan. Tel: +81 0492717959, Fax: +81 0492717985, e-mail: hwaka@josai.ac.jp/ sakagami@dent.meikai.ac.jp

Key Words: 2-Aminotropones, tumor-specificity, hormesis.

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), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich Inc., St. Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Osaka, Japan).

Synthesis of 2-aminotropolones. 7-Bromo-2-(4-hydroxyanilino) tropone **[16]** (6, 7) and 4-isopropyl-2-(2-hydroxyanilino)tropone **[20]** (8, 9) were synthesized, according to the methods previously published.

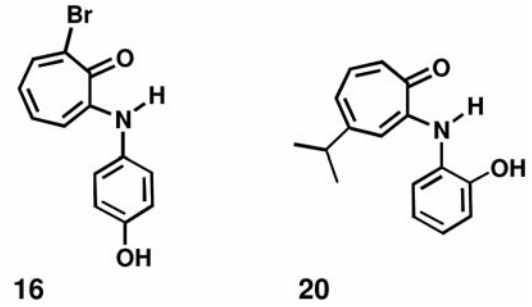

Figure 1. *Chemical structure of 7-bromo-2-(4-hydroxyanilino)tropone* ***[16]*** *and 4-isopropyl-2-(2-hydroxyanilino)tropone* ***[20]***.

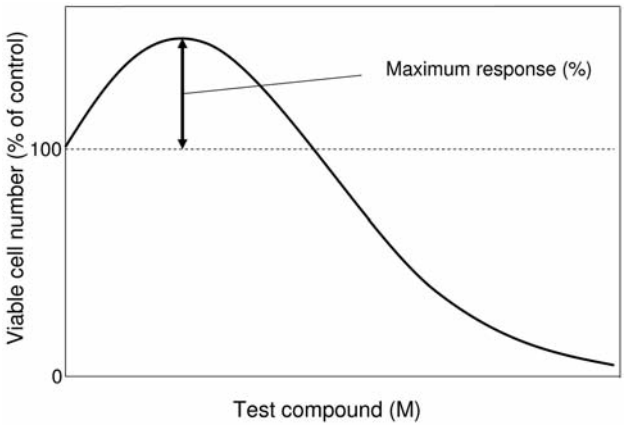

Figure 2. *"Maximum response" used as an index of hormetic growth stimulation at low concentration ranges.*

Cell culture. NB1RGB, 305M, WI-38, MRC-5, HSC-2, HSC-3 and HSC-4 cells were obtained from the Riken Cell Bank (Tsukuba, Japan). HGF, HPC and HPLF cells were prepared from the periodontal tissues, according to the guideline of Institutional Board of Meikai University Ethic Committee (No. 0206), after obtaining informed consent from patients. Since HGF, HPC and HPLF cells have a limited lifespan due to *in vitro* senescence (10), these cells at the population doubling level of 6-10 were used for the present study. All of these adherent cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and subcultured by detaching them with 0.25% trypsin-0.025% EDTA-2Na in PBS (–).

Assay for cytotoxic activity. The cells were inoculated at 1×10^3 cells/0.1 mL in the 60 inside wells (6×10) of a 96-microwell plate (Becton Dickinson Labware, NJ, USA). The surrounding 36 exterior wells were filled with 0.1 mL of PBS (–) to minimize the evaporation of water from the culture medium. After 48 hours, the medium was removed by suction with an aspirator, and replaced with 0.1 mL of fresh medium containing various concentrations of test the compounds. The first well contained a 500 μM sample and was diluted 2-fold sequentially, with triplicate wells for each concentration. The cells were incubated for 24, 48, 72 or 96 hours and the relative viable cell number was then determined by the MTT method. In brief, the treated cells were incubated for another 4 hours in fresh culture medium containing 0.2 mg/mL MTT, then lysed with 0.1 mL of DMSO and the absorbance at 540 nm of the cell lysate was determined using a microplate reader (Biochromatic Labsystem, Helsinki, Finland).

The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve. Tumor specificity (TS) was determined by the following equation: TS = {[CC_{50} (HGF) + CC_{50} (HPC) + CC_{50} (HPLF) + CC_{50} (NB1RGB) + CC_{50} (305M) + CC_{50} (WI-38) + CC_{50} (MRC-5)] / [CC_{50} (HSC-2) + CC_{50} (HSC-3) + CC_{50} (HSC-4)]} × (3/7).

Assay for hormesis. The hormetic response was evaluated by the maximum response in each dose-response curve (Figure 2), as described previously (3, 4).

Results

Tumor specificity. The cytotoxic activity of 7-bromo-2-(4-hydroxyanilino)tropone **[16]** and 4-isopropyl-2-(2-hydroxyanilino)tropone **[20]** against normal human oral cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF, Figure 3A), human skin fibroblast (NB1RGB, 305M, Figure 3B), human lung fibroblasts (WI-38, MRC-5, Figure 3C) and human oral squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4, Figure 3D) increased with incubation time (24 → 48 → 72 → 96 hours), as reflected by the decrease in the CC_{50} value with each of these compounds. With prolonged incubation time, the tumor cells became more sensitive to compounds **[16]** and **[20]**, as compared with the normal cells, resulting in the elevation of the (>1.2 → 3.3 → 3.7 → 3.8 for [16]; >1.2 → 3.9 → 5.1 for **[20]**) (Table I). The TS values for **[16]** and **[20]** were comparable with those we reported recently for these compounds (1).

Hormetic response. At 24 hours, the human lung fibroblasts showed the highest hormetic response to both **[16]** and **[20]** (maximum response: 74.3±23.1%, n=4), followed by skin fibroblast (42.5±27.3%, n=4), oral squamous cell carcinoma (26.5±10.8%, n=6) and then normal oral cells (5.6±3.8%, n=4). With prolonged incubation time (48→72→96 hours), their hormetic response declined: 3.2±3.1 → 2.6±4.5 → 5.4±6.3 % (n=20, Table II).

Discussion

The present study demonstrated that hormetic responses occured only at the early stages (within 24 hours) after treatment with either 7-bromo-2-(4-hydroxyanilino)tropone **[16]** or 4-isopropyl-2-(2-hydroxyanilino)tropone **[20]**. It seems unlikely that this transient occurrence of hormesis was an experimental artifact, since the exterior wells were not used for cell inoculation to minimize bacterial contamination and evaporation of water during the culture. It should be noted that the viable cell number increased with increasing concentrations of **[16]** and **[20]** and then abruptly declined,

Table I. *Cytotoxicity and tumor specificity of 7-bromo-2-(4-hydroxyanilino)tropone **[16]** and 4-isopropyl-2-(2-hydroxyanilino)tropone **[20]**, as a function of incubation time.*

	CC_{50} (μM)							
	[16]				**[20]**			
Cells	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Normal cells								
Oral cells								
HGF	397	325	265	271	409	318	275	268
HPC	366	345	148	194	408	274	182	183
HPLF	388	299	250	244	408	296	254	260
Skin fibroblasts								
NB1RGB	494	375	284	242	500	346	232	284
305M	>500	396	292	227	>500	>500	250	125
Lung fibroblasts								
WI-38	>500	339	224	175	>500	333	301	222
MRC-5	>500	313	193	163	>500	305	152	118
Tumor cells								
Oral squamous cell carcinoma cells								
HSC-2	289	84	62	61	375	63	31	37
HSC-3	450	102	69	63	409	103	52	48
HSC-4	345	124	63	49	354	94	56	50
TS value	>1.2	3.3	3.7	3.8	>1.2	>3.9	5.1	4.6

The CC_{50} values were determined from the dose-response experiments in Figure 3. Each value represents the mean of two independent experiments, which were conducted in triplicate.

Table II. *Hormetic response to 7-bromo-2-(4-hydroxyanilino)tropone **[16]** and 4-isopropyl-2-(2-hydroxyanilino)tropone **[20]**.*

	Maximum response (%)							
	[16]				**[20]**			
Cells	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Normal cells								
Oral cells								
HPC	9.4	1.9	0.0	0.0	9.8	0.0	0.0	0.0
HGF	0.7	3.3	0.0	1.7	3.3	2.9	1.3	0.0
HPLF	2.7	1.3	0.6	1.9	7.4	0.0	0.0	3.0
Skin fibroblasts								
NB1RGB	71.5	4.5	2.8	3.9	59.9	5.9	2.7	23.9
305M	22.6	10.0	17.8	3.9	16.0	7.8	11.7	2.8
Lung fibroblasts								
WI-38	57.8	3.5	1.2	5.4	51.3	1.9	4.4	10.1
MRC-5	97.3	5.1	5.0	6.4	90.9	3.4	0.7	7.4
Tumor cells								
Oral squamous cell carcinoma								
HSC-2	29.8	0.0	0.0	2.5	33.4	0.0	0.0	0.6
HSC-3	18.0	1.5	0.0	14.8	12.4	0.7	0.6	16.1
HSC-4	42.0	9.3	0.0	4.3	23.2	1.1	2.8	0.0

The values for maximum response were determined from the dose-response experiments in Figure 3. Each value represents the mean of two independent experiments, which were conducted in triplicate.

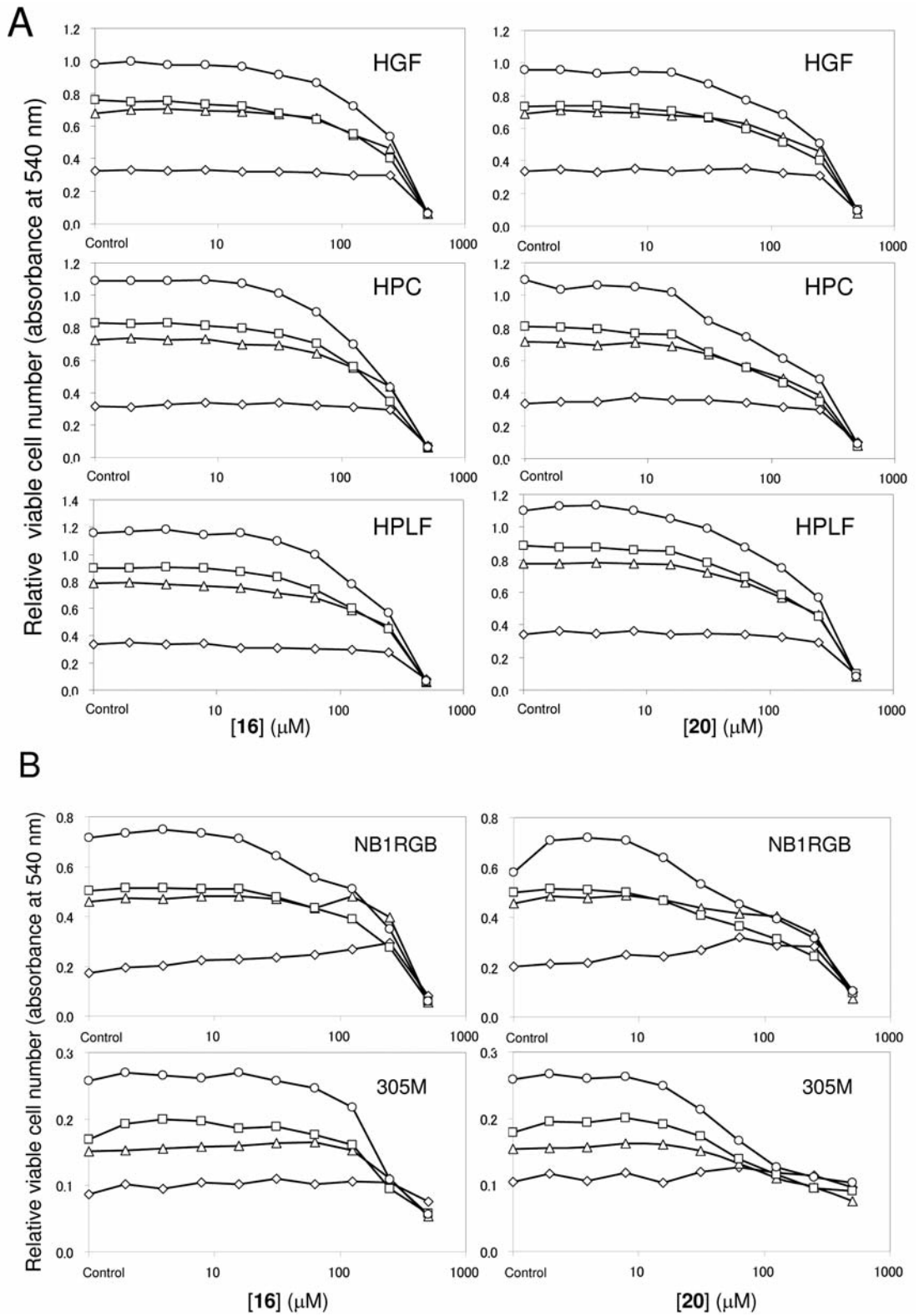

Figure 3. *A-D. Cytotoxic activity of 7-bromo-2-(4-hydroxyanilino)tropone **[16]** (left panel) and 4-isopropyl-2-(2-hydroxyanilino)tropone **[20]** (right panel) after 24 (◇), 48 (△), 72 (□) or 96 (○) hours incubation. A: Human normal oral cells, B: human normal skin fibroblasts, C: human normal lung fibroblasts, D: human oral squamous cell carcinoma cell lines. Viable cell number (absorbance at 540 nm) was determined by MTT method. Control values are indicated on the intersection of x- and y-axes. Each value represents the mean of two independent experiments, which were conducted in triplicate.*

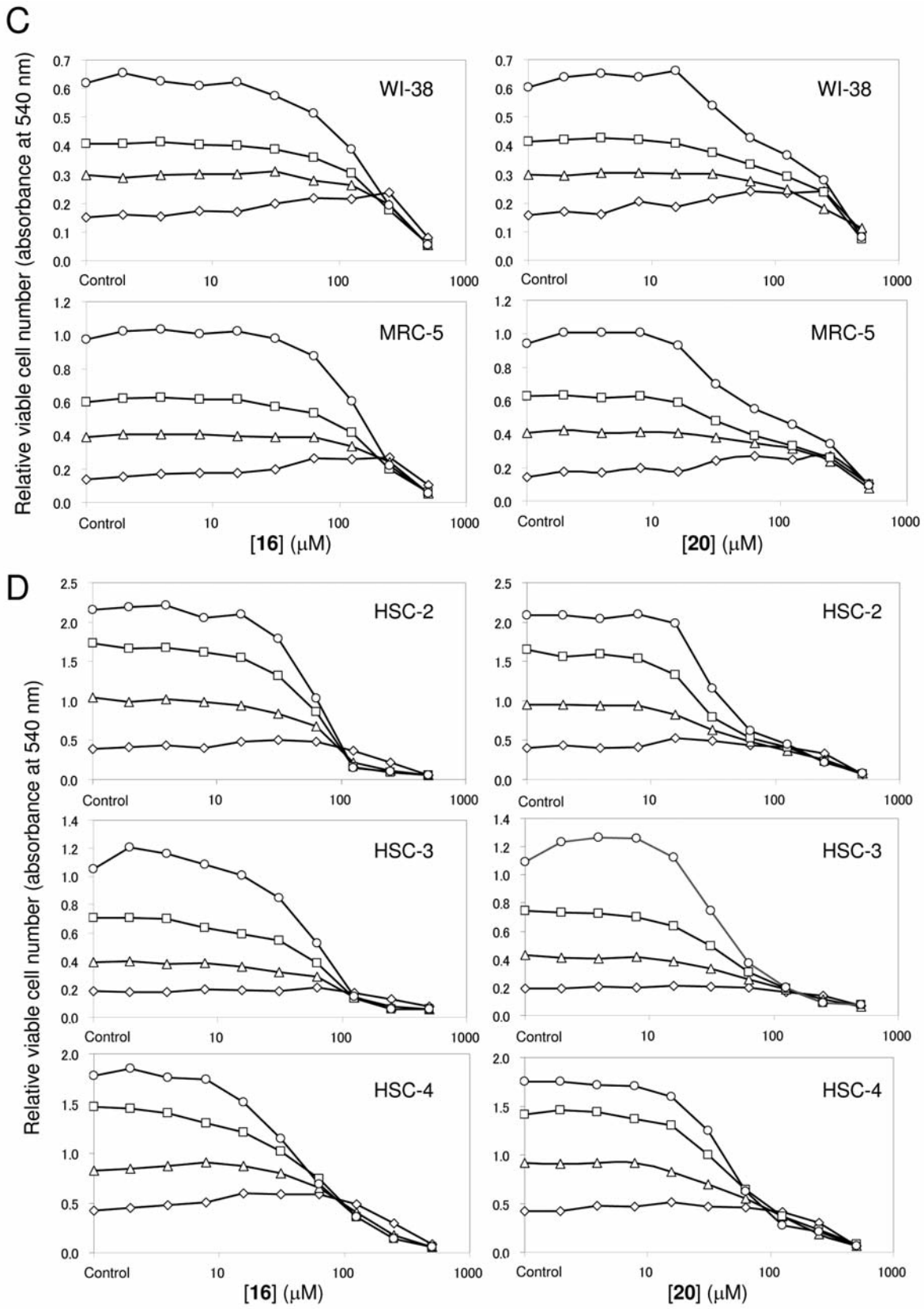

Figure 3. *continued*

indicating a continuity of growth stimulation and inhibition. This suggested that hormesis may be the mechanism by which cells escape cell death. However, there was no apparent correlation between the hormetic response and drug resistancy, since the normal oral cells, which were more resistant to **[16]** and **[20]** than the oral squamous cell carcinoma cells, showed much weaker hormetic responses.

It has recently been reported that repeated mild heat stress induced wound healing capacity in human skin fibroblasts and enhanced the angiogenic ability of endothelial cells, suggesting the applicability of mild stress-induced hormesis for the modulation, intervention and prevention of aging (11). Mild exposure to heat shock has been reported to reduce initial mortality in a worm (12). Tai Chi exercise has been reported to stimulate endogenous antioxidant enzymes and reduce oxidative damage markers (13). The present study demonstrated that human normal cells, especially lung and skin fibroblasts, showed some hormetic response to compounds **[16]** and **[20]** at the restricted (24-hour) period of cell growth. It has previously been reported that human diploid lung fibroblasts are destined for cell death after a limited number of cell division (14). It remains to be investigated whether the extents of hormetic responses are different between the logarithmic and confluent phases of cell growth in aging lung and skin fibroblasts.

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (Sakagami, No. 19592156).

References

1 Narita T, Suga A, Kobayashi M, Hashimoto K, Sakagami H, Motohashi N, Kurihara T and Wakabayashi H: Tumor-specific cytotoxicity and type of cell death induced by benzo[*b*] cyclohept[*e*][1,4]oxazine and 2-aminotropone derivatives. Anticancer Res *29*: 1123-1130, 2009.

2 Suga A, Narita T, Zhou L, Sakagami H, Satoh K and Wakabayashi H: Inhibition of NO production in LPS-stimulated mouse macrophage-like cells by benzo[*b*]cyclohept[*e*][1,4] oxazine and 2-aminotropone derivatives. In Vivo *23*: 691-698, 2009.

3 Calabrese EJ: Paradigm lost, paradigm found: the re-emergence of hormesis as a fundamental dose-response model in the toxicological sciences. Environ Pollut *138*: 379-412, 2005.

4 Cook RC and Calabrese EJ: The importance of hormesis to public health. Environ Health Perspectives *114*: 1631-1635, 2006.

5 Chu Q, Kobayashi M, Hashimoto K, Satoh K, Kanamoto T, Terakubo S, Nakashima H, Wang Q and Sakagami H: Antitumor potential of three herbal extracts against human oral squamous cell lines. Anticancer Res *29*: 3211-3220, 2009.

6 Wakabayashi H, Ishikawa S, Okai H and Nozoe T: Intermolecular bromine transfer of 2-amino-7-bromotropone derivatives catalyzed by strong acid. Bull Chem Soc Jpn *58*: 2840-2843, 1985.

7 Nozoe T, Okai H, Wakabayashi H and Ishikawa S: Cyclohepta[*b*][1,4]benzoxazines 3. The reaction of 6-bromocyclohepta [*b*][1,4]benzoxazine with *o*-aminophenol. Bull Chem Soc Jpn *62*: 2307-2314, 1989.

8 Nozoe T: Cyclohepta[*b*][1,4]benzoxazine and its related compounds. Some novel aspects in heterocyclic chemistry. Heterocycles *30*: 1263-1306, 1990.

9 Nozoe T and Someya T: Reactive troponoids and *o*-aminophenol. II. The formation of cyclohepta[*b*][1,4] benzoxazine and 11*H*-cyclohepta[*b*][1,4]benzoxazin-10-one derivatives from isomeric isopropyl-2-chlorotropones. Bull Chem Soc Jpn *51*: 3316-3319, 1978.

10 Satoh R, Kishino K, Morshed SRM, Takayama F, Otsuki S, Suzuki F, Hashimoto K, Kikuchi H, Nishikawa H, Yasui T and Sakagami H: Changes in fluoride sensitivity during *in vitro* senescence of human normal oral cells. Anticancer Res *25*: 2085-2090, 2005.

11 Rattan SI, Fernandes RA, Dermirovic D, Dymek B and Lima CF: Heat stress and hormetin-induced hormesis in human cells: effects on aging, wound healing, angiogenesis, and differentiation. Dose Response *7*: 90-103, 2009.

12 Wu D, Cypser JR, Yashin AI and Johnson TE: The U-shaped response of intial mortality in *Caenorhabditis elegans* to mild heat shock: does it explain recent trends in human mortality? J Gerontol A Biol Sci Med Sci *37*: 660-668, 2008.

13 Goon JA, Aini AH, Musalmah M, Anum MY, Nazaimoon WM and Ngah WZ: Effect of Tai Chi exercise on DNA damage, antioxidant enzymes, and oxidative stress in middle-age adults. J Phys Act Health *6*: 43-54, 2009.

14 Hayflick L: The limited *in vitro* lifetime of human diploid cell strains. Exp Cell Res *37*: 614-636, 1965.

Received May 26, 2009
Revised October 20, 2009
Accepted October 27, 2009