Korean J. Toxicol.
Vol. 11, No. 2, pp. 175-180, 1995

Growth-Inhibiting Effect of Bufadienolides on Cultured Vascular Endothelial Cells

Duck Yoon Lee and Hwa Joong Yoon

Department of Veterinary Medicine, College of Animal Husbandry, Kon-Kuk University, 93-1 Mojindong, Kwangjin-Ku, Seoul 143-701, Korea.

(Received October 11, 1995)
(Accepted October 20, 1995)

ABSTRACT : *We found that bufalin, one of the prominent components of the bufadienolides in the Chinese medicine chan'su, has the potent inhibitory effects on growth and proliferation of the cultured bovine aortic endothelial (BAE) and human umbilical vein endothelial (HUVE) cells. All naturally-occuring bufadienolides used in this study inhibited the cell growth in a dose-dependent manner. Particularly, bufalin among the bufadienolides showed the strongest inhibitory activity for the cell growth. The order of growth inhibition by bufadienolides on BAE cells was as follows: bufalin > gamabufotalin > bufotalin > cinobufagin > cinobufotalin > resibufogenin. The IC_{50} values (50% inhibition of cell growth) of bufalin as determined by XTT assay were the range of 1 - 10 nM in BAE and HUVE cells. Bufalin exhibited a higher sensitivity towards cultured bovine aortic endothelial cells than human umbilical vein endothelial cells.*

Key Words : *Bufadienolides, Growth inhibition, Bovine aortic endothelial cells, Human umbilical vein endothelial cells*

I. INTRODUCTION

The Chinese traditional medicine *Senso* or *Chan'su*, a dried material made from toad skin excreta, has been used for the treatment of canker sores, toothache, sinusitis, and local inflammations in China for centuries (Chen and Korarikova, 1967). Bufalin, one of the prominent components of bufadienolides, has particularly been known to have its pharmacological activities such as cardiotonic and anaesthetic action (Okada and Suga, 1962; Chen and Korarikova, 1967). Bufalin was known to exhibit a higher sensitivity towards cultured human and monkey cells than cell lines derived from other species such as mouse, Syrian hamster, and Chinese hamster, and the sensitivity was species-related (Gupta *et al.*, 1986). Recently, it was reported that bufalin has the potent differentiation-inducing activity in four human myeloid leukemia cell lines (K562, U 937, ML1, and HL60) and has selective inhibitory effects in the growth of various human cancer cells (Zhang *et al.*, 1992; Jing *et al.*, 1994). There is no data for the actions of bufadienolides on vascular endothelial cells.

In the present study, we investigated the effects of bufadienolides on growth of bovine aortic endothelial cells and human umbilical vein endothelial cells by XTT-microculture tetrazolium assay.

II. MATERIALS AND METHODS

1. Endothelial Cell Culture

Bovine aortic endothelial (BAE) cells were isolated from the bovine thoracic aorta and cultured in MEM supplemented with 10% FBS, 100 μg/ml penicillin G, 100 U/ml streptomycin, 2 mM L-glutamine as previously described (Gospodarowicz *et al.*, 1976). Human umbilical vein endothelial (HUVE) cells were obtained from the Institute for Fermentation (Osaka, Japan) and cultured in MCDB-107 media supplemented with 10% FBS, 100 μg/ml heparin, 50 μg/ml endothelial cell growth supplement (ECGS) and 30 mM HEPES on gelatin-coated culture flasks. The cells from 3 to 8 passages were used for these experiments.

2. Materials

Bufalin (Bu), bufotalin (Bt), cinobufagin (Cb), cinobufotalin (CBt), gamabufotalin (Gb), and resibufogenin (Rb) were isolated from Chinese toad venom preparation *chan'su* (Komatsu and Okano, 1967) and used for these experiments. N-methyldibenzopyrazine methyl sulfate salt (phenazine methosulfate:PMS) and 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) were purchased from Sigma Chemical Co. (St. Louis, MO).

3. Growth Inhibition Assay

XTT-microculture tetrazolium assay for the growth inhibition was performed. In brief, BAE and HUVE cells were dispensed within 96-well culture plates by the numbers of 5×10^3 cells/well in 100 μl medium. After overnight incubation at 37°C in a 5% CO_2 condition, each 50 μl of culture medium containing different concentrations of bufadienolides (Bu, Bt, Cb, CBt, Gb, and Rb) was dispensed within appropriate wells. The culture plates were incubated for 3 days prior to the addition of tetrazolium reagent. XTT was prepared at 1 mg/ml in prewarmed (37°C) medium without serum. PMS was prepared at 5 mM (1.53 mg/ml) in PBS. For a 0.025 mM PMS-XTT solution, 25 μl of the stock 5 mM PMS was added per 5 ml of XTT (1 mg/ml). Fifty μl of this mixture was dispensed to each well after the 4-day incubation. After a 2-hr incubation at 37°C, the plates were measured at 492 nm absorbance wavelength using a microplate reader (Navapath Mini Reader, Biorad, Japan). For a morphological examination, the cultures were photographed with a phase contrast microscope (IMT 2, Olympus, Japan) on day 3 after treatment of bufadienolides.

III. RESULTS AND DISCUSSION

The effect of bufadienolides on the growth of the bovine aortic endothelial cells and human umbilical vein endothelial cells was determined (Fig. 1 and Fig. 2). In BAE cells, bufadienolides inhibited the cell growth in a dose-dependent manner (Fig. 1). Bufalin, particularly, was shown to have the strongest activity for the cell growth inhibition among the bufadienolides.

Bufalin has not only the antileukemic activity on leukemia cell lines such as K562, U937, ML1, and HL60 but also the growth inhibitory effects on human solid tumour cells (Zhang *et al.*, 1992; Zhang *et al.*, 1992; Jing *et al.*, 1994). It was also reported that bufalin exhibited a specifically higher sen-

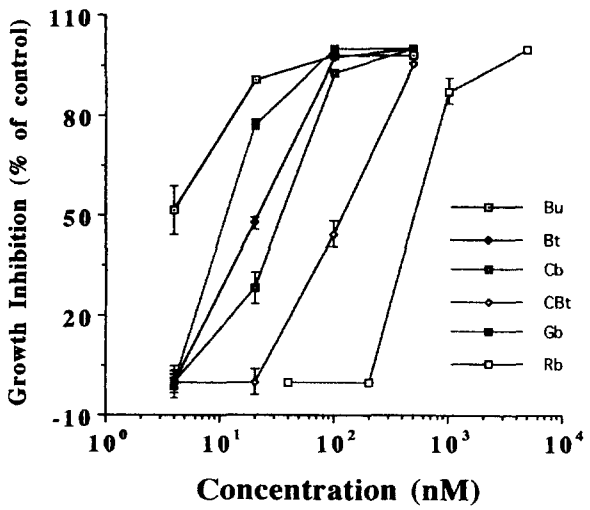

Fig. 1. Effects of bufadienolides on the growth of bovine aortic endothelial cells. The cells were seeded at 5×10^3 cells/well in 96-well culture plates, and incubated with the bufadienolides at various concentrations for 3 days. Cell growth was determined by XTT assay as described in materials and methods. Each value is the mean±SD of triplicate determinations.

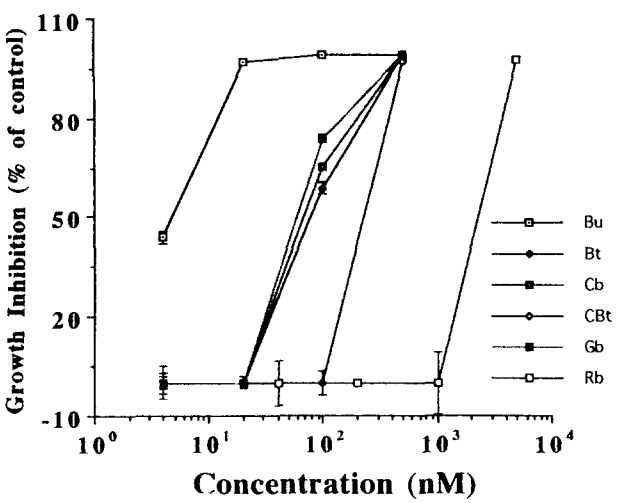

Fig. 2. Effects of bufadienolides on the growth of human umbilical vein endothelial cells. The cells were seeded at 5×10^3 cells/well in 96-well culture plates, and incubated with the bufadienolides at various concentrations for 3 days. Cell growth was determined by XTT assay as described in materials and methods. Each value is the mean±SD of triplicate determinations.

sitivity towards cultured human and monkey cells than cell lines derived from other species such as mice, Syrian hamsters, and Chinese hamsters, and the sensitivity was species-related (Gupta *et al.*, 1986).

In our study, as shown in Fig. 1 and 2, both vascular endothelial cells derived from human beings and bovines showed very high sensitivities to bufalin at a concentration as low as 10 nM, and bufalin exhibited a higher sensitivity towards cultured bovine aortic endothelial cells than human umbilical vein endothelial cells. The order of growth inhibition activity on BAE cells by bufadienolides was Bu > Gb > Bt > Cb > CBt > Rb. As shown in Table 1, then, each IC_{50} was as follows: 4, 15, 24, 47, 143, and 720 nM, respectively. Bufalin exhibited very high sensitivity towards vascular endothelial cells in contrast to cinobufotalin and resibufogenin. This difference was thought to be related to the chemical structure of bufadienolides. Bufalin has been known to inhibit Na^+/K^+-ATPase, an enzyme that catalyzes active transport of Na^+ and K^+ across the plasma membrane, thereby regulating intracellular Na^+, K^+, ATP, and Ca^{2+} concentrations (Bernstein *et al.*, 1976; Schwartz *et al.*, 1975; Reeves, 1992; Numazawa *et al.*, 1994). Bufalin exhibited variant activities for the cell cycle depending on cell lines (Zhang *et al.*, 1992), the growth inhibition of BAE cells following the bufalin treatment resulted from the arrest in the G2-M phases of the cell cycle (results not shown). In HUVE cells, bufalin also exhibited the strongest activity for the growth inhibition among the bufadienolides. Other bufadienolides except bufalin (Gb, Bt, Cb, CBt and Rb) were shown to have less effects on HUVE cells in comparison with the effects on BAE cells (Fig. 2). The order of growth inhibition activity on HUVE cells was Bu > Gb > Cb > Bt > CBt > Rb, and then the IC_{50} was 6, 75, 84, 90, 320, and 3100 nM, respectively (Table 1). On day 3 after treatment of bufadienolides, we examined the morphological changes. As shown in Fig. 3, non-treated control BAE cells showed the typical cobblestone-like appearance when reached a confluence. Bufalin-treated BAE cells (Fig. 4A, 4B and 4C) exhibited a slight toxic effect at a concentration of 20 nM, but at a concentration of 20

Table 1. Fifty % inhibition of cell growth (IC_{50}) of bufadienolides on bovine and human-derived vascular endothelial cells (Unit; nM)

Bufadienolides	BAECs	HUVECs
Bufalin	4	6
Bufotalin	24	90
Cinobufagin	47	84
Cinobufotalin	143	320
Gamabufotalin	15	75
Resibufogenin	720	3,100

BAE and HUVE cells were cultured in 96-well culture plates for 3 days after treatment of different concentrations of bufadienolides. Cell growth was determined by XTT assay. BAECs: bovine aortic endothelial cells, HUVECs: human umbilical vein endothelial cells.

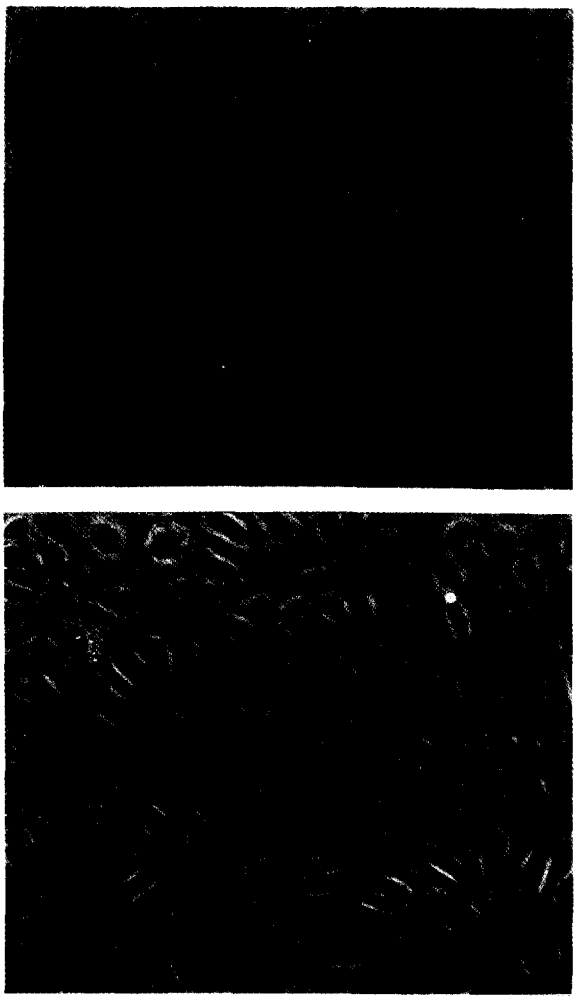

Fig. 3. Morphological appearance of normal BAE cells. BAE cells were inoculated into 96-well culture plates as the number of 5×10^3 cells/well, and photographed under phase contrast microscope after 4-day incubation. (A) $\times 100$; (B) $\times 200$.

nM resulted in a almost complete cell death. Bufotalin had no toxic effect at a concentration of 20 nM, and resulted in a cell death at a concentration of 100 nM (Fig. 5A and 5B). Cinobufagin-treated BAE cells showed a slight toxic effect at a concentration of 100 nM (Fig. 6A and 6B). As shown in Fig. 7, cinobufotalin exhibited a growth inhibition but showed no toxic effect at a concentration of 100 nM. Gamabufotalin-treated BAE cells showed almost complete cell death at a concentration of 100 nM (Fig 8A and 8B). Resibufogenin at a concentration of 20 nM showed no growth inhibition, and there was no toxic effect on BAE cells at a concentration of 100 nM (Fig. 9A and 9B). Bufadienolides exhibited less growth inhibition and cytotoxicity towards HUVE cells than BAE cells. Then, 100 nM bufotalin and gamabufotalin showed growth inhibition but did not result in the cell death.

In conclusion, this study has revealed that bufalin has a potent growth-inhibitory activity on bovine- and human-derived endothelial cells, and bovine-derived endothelial cells showed more higher sensitivity than the cells derived from human beings. The futher detailed study for the mechanism on growth inhibition of vascular endothelial cells has being undertaken by us.

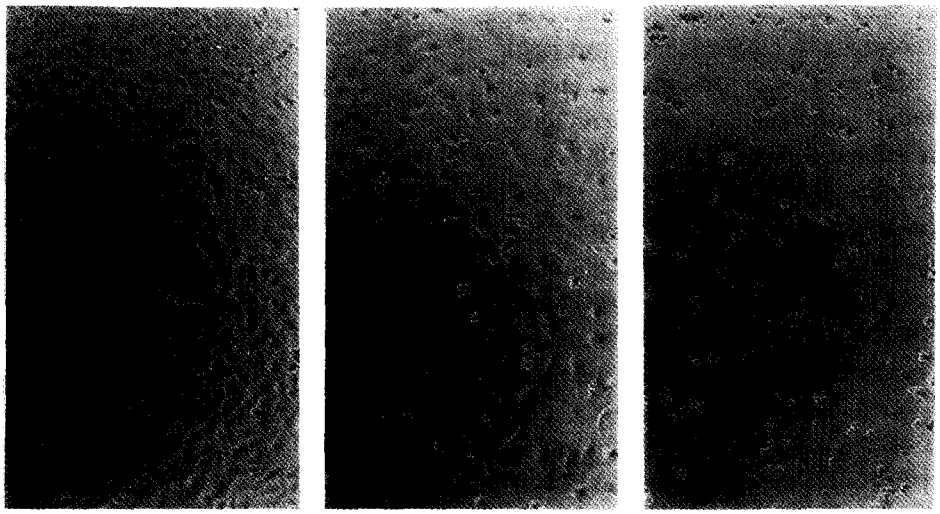

Fig. 4. Morphological appearance of bufalin-treated BAE cells. BAE cells were inoculated into 96-well culture plates as the number of 5×10^3 cells/well. After the overnight incubation, the cells were cultured for 3 days in the presence of 4 nM (A), 20 nM (B), and 100 nM bufalin. $\times$100.

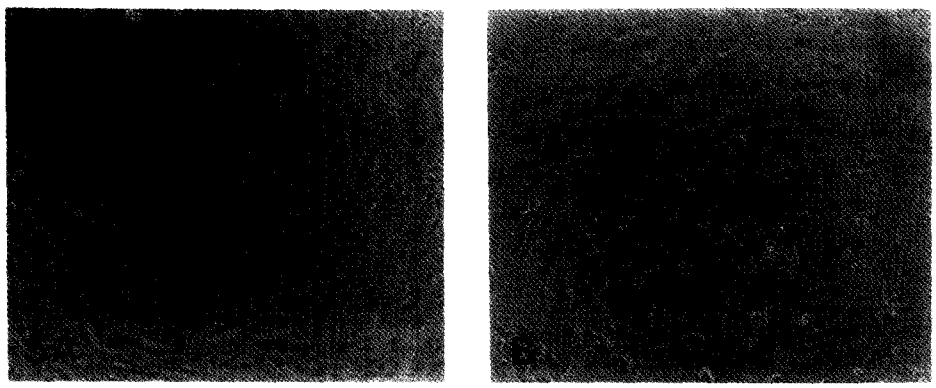

Fig. 5. Morphological appearance of bufotalin-treated BAE cells. BAE cells were inoculated into 96-well culture plates as the number of 5×10^3 cells/well. After the overnight incubation, the cells were cultured for 3 days in the presence of 20 nM (A) and 100 nM (B) bufotalin. $\times$100.

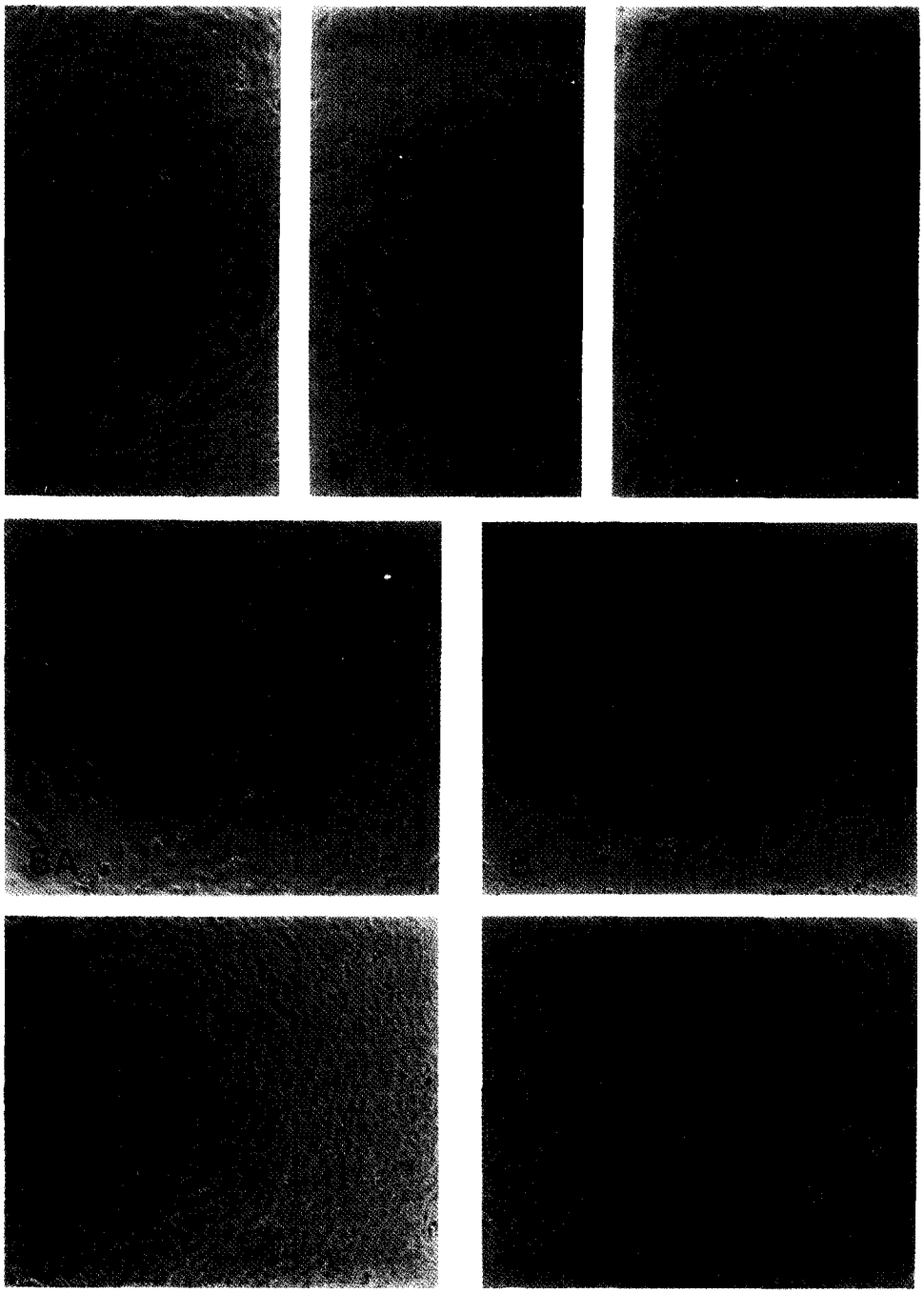

Fig. 6. Morphological appearance of cinobufagin-treated BAE cells. BAE cells were inoculated into 96-well culture plates as the number of 5×10^3 cells/well. After the overnight incubation, the cells were cultured for 3 days in the presence of 20 nM (A) and 100 nM (B) cinobufagin. $\times 100$.

Fig. 7. Morphological appearance of cinobufotalin-treated BAE cells. BAE cells were inoculated into 96-well culture plates as the number of 5×10^3 cells/well. After the overnight incubation, the cells were cultured for 3 days in the presence of 100 nM cinobufotalin. $\times 100$.

Fig. 8. Morphological appearance of gamabufotalin-treated BAE cells. BAE cells were inoculated into 96-well culture plates as the number of 5×10^3 cells/well. After the overnight incubation, the cells were cultured for 3 days in the presence of 20 nM (A) and 100 nM (B) gamabufotalin. $\times 100$.

Fig. 9. Morphological appearance of resibufogenin-treated BAE cells. BAE cells were inoculated into 96-well culture plates as the number of 5×10^3 cells/well. After the overnight incubation, the cells were cultured for 3 days in the presence of 20 nM (A) and 100 nM (B) resibufogenin. $\times 100$.

REFERENCES

Bernstein, A., Hunt, D.M., Crichley, V., and Mak, T.W. (1976): Induction by ouabain of hemoglobin synthesis in cultured friend erythroleukemia cells. Cell **9**, 375-381.

Chen, K.K. and Korarikova, A. (1967): Pharmacology and toxicology of toad venom. J. Pharm. Sci. **56**, 1535-1541.

Gospodarowicz, D., Moran, J., Braun, D., birdwell, C. (1976): Clonal growth of bovine vascular endothelial cells: Fibroblast growth factor as a survival agent. Proc. Natl. Acad. Sci. USA **73**, 4120-4124.

Gupta, R.S., Chopra, A., and Stetsko, D.K. (1986): Cellular basis for the species differences in sensitivity to cardiac glucosides (digitalis). J. Cell. Physiol. **127**, 197-206.

Jing, Y., Ohizumi, H., Kawazoe, N., Hashimoto, S., Masuda, Y., Nakajo, S., Yoshida, T., Kuroiwa, Y., and Nakaya, K. (1994): Selective inhibitory effect of bufalin on growth of human tumor cells *in vitro*: Association with the induction of apoptosis in leukemia HL-60 cells. Jpn. J. Cancer Res. **85**, 645-651.

Komatsu, M. and Okano, T. (1967): Separation of bufadienolides by column chromatography on silica gel. Yakugaku Zasshi **87**, 712-718.

Numazawa, S., Shinoki, M., Ito, H., Yoshida, T., and Kuroiwa, Y. (1994): Involvement of Na^+, K^+-ATPase inhibition in K562 cell differentiation induced by bufalin. J. Cell. Physiol. **160**, 113-120.

Okada, M. and Suga, T. (1962): Pharmacology of the principles isolated from senso (*chan'su*), the dried venom of the Chinese toad. Asian Med. J. **5**, 353-361.

Reeves, J.P. (1992): Molecular aspects of sodium-calcium exchange. Arch. Biochem. Biophys. **292**, 329-334.

Schwartz, A., Lindenmayer, G.E., and Allen, J.C. (1975): The sodium-potassium adenosine triphosphatase: Pharmacological, physiological and biochemical aspects. Pharmacol. Rev. **27**, 3-134.

Zhang, L., Nakaya, K., Yoshida, T., and Kuroiwa, Y. (1992): Bufalin as a potent inducer of differentiation of human myeloid leukemia cells. Biochem. Biophys. Res. Commun. **178**, 686-693.

Zhang, L., Nakaya, K., Yoshida, T., and Kuroiwa, Y. (1992): Induction by bufalin of differentiation of human leukemia cells HL60, U937, and ML1 toward macrophage/monocyte-like cells and its potent synergistic effect on the differentiation of human leukemia cells in combination with other inducers. Cancer Research **52**, 4634-4641.