

Tumor-specific Cytotoxic Activity and Type of Cell Death Induced by 4-Trifluoromethylimidazoles in Human Oral Squamous Cell Carcinoma Cell Lines

FUMIHIRO TAKEKAWA¹, TATSUHITO NAGUMO², SATORU SHINTANI²,
KEN HASHIMOTO¹, HIROTAKA KIKUCHI³, TADASHI KATAYAMA³,
MARIKO ISHIHARA⁴, OSAMU AMANO⁵, MASAMI KAWASE⁶ and HIROSHI SAKAGAMI¹

*Divisions of ¹Pharmacology, ³Endodontics, ⁴Basic Chemistry and ⁵Anatomy,
Meikai University School of Dentistry, Sakado, Saitama 350-0283;*

²Department of Oral and Maxillofacial Surgery, School of Dentistry, Showa University, Ohta-ku, Tokyo 145-8515;

⁶Faculty of Pharmaceutical Sciences, Matsuyama University, Matsuyama, Ehime 790-8578, Japan

Abstract. Fourteen 4-trifluoromethylimidazole derivatives were investigated for their cytotoxicity against three human normal cells (gingival fibroblast HGF, pulp cell HPC and periodontal ligament fibroblast HPLF) and four human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, HSC-4 and promyelocytic leukemia HL-60). Among these compounds, 4-trifluoromethyl-1,2-diphenylimidazole (**IM5**), 1-benzyl-4-trifluoromethyl-2-phenylimidazole (**IM7**) and 5-[1-ethoxy-2,2,2-trifluoro-1-(trifluoromethyl)ethyl]-1-methyl-2-phenyl-1H-imidazole (**IM12**) showed much higher cytotoxicity and tumor-specificity than the other compounds. **IM5**, the most potent compound, induced different types of cell death depending on the target cells. **IM5** induced DNA fragmentation of oligonucleosomal units (a biochemical hallmark of apoptosis) in the HL-60 cells, but not in such a clear-cut laddering pattern in the HSC-2 cells. On the other hand, **IM5** produced secondary lysosomes digesting broken organelles, without induction of internucleosomal DNA fragmentation and disappearance of cell surface microvilli in the HSC-4 cells, even though the HSC-2 and HSC-4 cells showed comparable sensitivity to **IM5**. These data suggest that the type of cell death is determined by the type of target cells, but not by the drug-sensitivity of the cells.

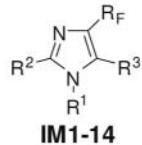
Apoptosis (type I programmed cell death)-inducing activity has been considered as an important feature for antitumor potency of the compounds used by many investigators to date. However, this criterion has to be modified due to the heterogeneity of the type of cell death induced. For example, human malignant glioma and glioblastoma cells lines are easily committed to autophagy (type II programmed cell death) upon treatments with chemotherapeutic agents, radiation and toxicants (1-4). Another factor that may determine the type of cell death is the chemical structure of the inducing agents (5). For example, several α,β -unsaturated ketones, such as 1-trichloroacetyl-3-bromo-2-methoxyazulene, 1-trichloroacetyl-3-chloro-2-ethoxyazulene, 4,4-dimethyl-2-cyclopenten-1-one, α -methylene- γ -butyrolactone, 5,6-dihydro-2H-pyran-2-one, 3,3,3-trifluoro-2-hydroxy-1-phenyl-1-propanone, codeinone and morphinone induced non-apoptotic cell death characterized by the vacuolization and autophagosome formation (5). In order to explore the novel antitumor agents, the tumor-specific cytotoxicity of the test compounds should be first confirmed, before the identification of the type of cell death. Following this strategy, we have compared the cytotoxic activity of hundreds of compounds against both human tumor cell lines and normal cells. We have found that chemotherapeutic agents such as anthracycline antibiotics showed the highest level of tumor selectivity, whereas most of the lower molecular weight polyphenols and antioxidants showed disappointingly lower tumor selectivity (6). As an extension of this series of experiments, an investigation of the tumor-specific cytotoxicity and the type of cell death induced by fourteen 4-trifluoromethylimidazole derivatives (Figure 1) is reported.

Materials and Methods

Materials. 4-Trifluoromethylimidazole derivatives (**IM1-12**) were prepared according to the previously published procedure (7, 8). The following chemicals and reagents were obtained from the indicated

Correspondence to: Hiroshi Sakagami, Division of Pharmacology, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: +81 49 279 2758, 2769, Fax: +81 49 285 5171, e-mail: sakagami@dent.meikai.ac.jp

Key Words: 4-Trifluoromethylimidazoles, oral squamous cell carcinoma, non-apoptotic cell death, DNA fragmentation, electron microscopy.



R ¹	R ²	R ³	R _F
IM1	CH ₃	C ₆ H ₅	CF ₃
IM2	CH ₃	C ₆ H ₅ CH ₂	CF ₃
IM3	CH ₃	tert-Butyl	CF ₃
IM4	C ₆ H ₅	CH ₃	CF ₃
IM5	C ₆ H ₅	C ₆ H ₅	CF ₃
IM6	C ₆ H ₅ CH ₂	CH ₃	CF ₃
IM7	C ₆ H ₅ CH ₂	C ₆ H ₅	CF ₃
IM8	C ₆ H ₅ CH ₂	OH	C ₆ H ₅ CH ₂
IM9	CH ₃	C ₆ H ₅	C ₂ F ₅
IM10	CH ₃	C ₆ H ₅	C ₃ F ₇
IM11	CH ₃	C ₆ H ₅	CHO
IM12	CH ₃	C ₆ H ₅	C(OEt)(CF ₃) ₂
IM13	C ₆ H ₅	H	H
IM14	H	C ₆ H ₅	CH ₃

Figure 1. Structure of 4-trifluoromethylimidazoles.

companies: 1-phenylimidazole (**IM13**) and 4-methyl-2-phenylimidazole (**IM14**) (Aldrich Chemical Co., Wyoming, IL, USA); Dulbeccos's modified Eagle's medium (DMEM), RPMI 1640 (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH, Bioscience, Lenexa, KS, USA), dimethyl sulfoxide (DMSO) (Wako Pure Chem, Ind, Ltd, Osaka, Japan); 3-(4,5-dimethylthiazol-2-yl)-2,2-diphenyltetrazolium bromide (MTT), melphalan (Sigma Chem Co., St. Louis, MO, USA); 5-fluorouracil (5-FU) (Kyowa, Tokyo, Japan).

Cell culture. Human oral normal cells (gingival fibroblast HGF, pulp cell HPC and periodontal ligament fibroblast HPLF) were prepared from periodontal tissues, according to the guidelines of the Intramural Board of Ethic Committee (No. 0206) after obtaining the informed consent from the patients. Since these normal cells have a limited lifespan due to the *in vitro* senescence (9), cells at the 5-8 population doubling levels were used for the present study. These normal cells and human oral squamous cell carcinoma cell lines [HSC-2, HSC-4 (supplied by Prof. M. Nagumo, Showa University), HSC-3 (supplied by Prof. Y. Ohmori, Meikai University)] were cultured in DMEM supplemented with 10% heat-inactivated FBS. Human promyelocytic leukemic HL-60 cells (provided by Prof. Nakaya, Show University) were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, as described previously (10).

Assay for cytotoxic activity. The cells (other than the HL-60 cells) were inoculated at 5x10³ cells/well in 96-microwell plates (Becton Dickinson Labware, NJ, USA), unless otherwise stated. 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 compounds. The first well contained a 400 μM sample, which was diluted 2-fold sequentially, with 3 replicate wells for each concentration. The cells were incubated for another 48

hours, and the relative viable cell number was then determined by the MTT method (10). The HL-60 cells were inoculated at 7.5x10⁴/0.1 mL in 96-microwell plates, and various concentrations of test compounds were added. After incubation for 48 hours, the viable cell number was determined by Trypan blue exclusion, under a light microscope. The 50% cytotoxic concentration (CC₅₀) was determined from the dose-response curve. Tumor-specificity (TS) was determined by the following equation:

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

Assay for DNA fragmentation. The cells were washed once with phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS (-)), lysed, digested with RNase A and proteinase K. The DNA was then prepared and subjected to 2% agarose gel electrophoresis, as described previously (10). A DNA molecular marker (Bayou Biolabs, Harahan, LA, USA) was run in parallel. 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).

Electron microscopy. The cells were harvested by trypsinization, washed, fixed, postfixed, dehydrated and embedded in Araldite 502, as described previously (10). Fine sections were stained with uranyl acetate and lead citrate, and then observed under a JEM-1210 electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 100 KV (10).

Results

Cytotoxicity. Among the fourteen 4-trifluoromethylimidazole derivatives, 4-trifluoromethyl-1,2-diphenylimidazole (**IM5**) showed the highest cytotoxicity, followed by 5-[1-ethoxy-2,2,2-trifluoro-1-(trifluoromethyl)ethyl]-1-methyl-2-phenyl-1*H*-imidazole (**IM12**) and 1-benzyl-4-trifluoromethyl-2-phenylimidazole (**IM7**), whereas the other compounds showed much lower cytotoxicity (Table I). Out of these three compounds, **IM5** showed the highest tumor-specificity (TS=4.2), followed by **IM12** (TS>2.9) and then **IM7** (>2.7).

Drug sensitivity. A slight difference in drug-sensitivity was found between the cell lines. The sensitivity to **IM5** was in the order: HL-60 (CC₅₀=3 μM) (most sensitive) > HSC-3 (38 μM) > HSC-4 (43 μM) > HSC-2 (70 μM) > HPC (134 μM) > HGF (147 μM) > HPLF (>221 μM) (most resistant) (Table I). The sensitivity to **IM12** was in the order: HL-60 (CC₅₀=11 μM) (most sensitive) > HSC-4 (67 μM) > HSC-2 (77 μM) > HSC-3 (83 μM) > HPC (118 μM) > HGF (182 μM) > HPLF (>233 μM) (most resistant). The sensitivity to **IM7** was in the order: HL-60 (8 μM) (most sensitive) > HSC-3 (86 μM) > HSC-2, HSC-4 (115 μM) > HPC (153 μM) > HGF (196 μM) > HPLF (>251 μM) (most resistant) (Table I). The sensitivity of the HSC-2 and HSC-4 cells to **IM5**, **IM7** and **IM12** was found to be comparable with each other.

Table I. Cytotoxic activity of 4-trifluoromethylimidazoles against human tumor and normal cells.

	CC ₅₀ (μM)							
	Human tumor cell lines				Human normal cells			
	HSC-2	HSC-3	HSC-4	HL-60	HGF	HPC	HPLF	TS
IM1	>395±9.2	>400	>381±32	255±42	>400	>371±30	>310±148	><1.0±0.1
IM2	>389±12	>400	>383±30	149±54	368±19	344±21	319±86	<1.0±0.1
IM3	>400	>400	>400	>354±79	>400	>400	>370±51	><1.0±0.0
IM4	325±16	294±5.5	>341±61	31±18	323±3.1	303±22	310±24	<1.3±0.1
IM5	70±9.7	38±4.4	43±6.5	3.0±2.9	147±30	134±6.1	>221±155	4.3±1.2
IM6	>354±41	>399±2.3	>380±35	31±27	>389±19	328±31	299±145	><1.2±0.2
IM7	115±38	86±12	115±25	8.1±6.6	196±60	153±31	>251±130	>2.5±0.9
IM8	251±37	262±10	276±169	145±63	285±15	256±20	302±38	1.4±0.3
IM9	325±66	320±30	>310±156	102±36	>393±13	357±37	>350±87	><1.5±0.3
IM10	241±20	215±30	>236±150	27±19	338±25	293±19	331±29	1.9±0.4
IM11	>400	>400	>400	122±37	>400	>400	>305±165	><1.2±0.1
IM12	77±5	83±13	67±34	11±5	182±48	118±13	>233±145	>3.0±1.5
IM13	>371±41	230±90	>281±119	29±16	361±22	305±26	374±44	<1.6±0.2
IM14	>400	>400	>400	>367±58	>400	>400	>400	><1.0±0.1
Melphalan	10±5.7	31±5.5	40±3.2	<2.6±0.9	176±23	>322±135	>236±46	>12±1.7
5-FU	<118±151	<57±29	<156±144	<24±7.6	>4000	>4000	>4000	>49±16

Each value represents mean±S.D. from three independent experiments.

Type of cell death. Since **IM5** showed the highest cytotoxicity and tumor-specificity, the type of cell death induced by **IM5** was investigated. Six hours treatment with 12 μM (four times higher concentration than CC₅₀) of **IM5** induced internucleosomal DNA fragmentation in the HL-60 cells (Figure 2A). When the treatment time was prolonged to 24 hours, an even lower concentration (3 μM) of **IM5** induced DNA fragmentation (Figure 2B). **IM5** (70-280 μM) induced DNA fragmentation in the HSC-2 cells only after 24 hours, without a clear-cut laddering pattern of DNA fragmentation (Figure 2B). On the other hand, **IM5** did not induce any trace of DNA fragmentation in the HSC-4 cells. Transmission electron microscope analysis demonstrated various profiles of lysosomes in the cells cultured with **IM5** for 6 and 24 hours (Figure 3). The number of lysosomes increased from 6 to 24 hours of culture in the cells incubated with 43 and 86 μM of **IM5**. The lysosomes observed in the cells cultured with **IM5** contained small vesicular and/or lamellar structures suggesting the digestion of organelles (Figures 3J and 3K) (11). In these cells, enlarged mitochondria accompanying electron-lucent matrices were also found with the lysosomes. Moreover, rod-shaped lysosomes were observed surrounding small vesicles and/or lucent materials (Figure 3K, arrowheads). Therefore, these lysosomes could be identified as secondary lysosomes digesting broken organelles. It should be noted that the disappearance of cell surface microvilli, one of the morphological markers of apoptosis, was not observed in the **IM5**-treated cells.

Discussion

Compounds with imidazole ring systems have many pharmacological properties and play important roles in biochemical processes (12). Many substituted imidazoles are known as inhibitors of p38 mitogen-activated protein (MAP) kinase, anti-inflammatory agents, angiotensin II receptor antagonists, fungicides and herbicides (13). Some imidazole derivatives have been reported to induce apoptosis in Ehrlich ascites tumor cells (14). Vicinal diaryl-substituted five-membered heteroaromatic rings including imidazole, oxazole, isoxazole, thiazole, pyrazole and tetrazole have been considered to be the bioisosteric replacement of the olefinic double bond of combretastatin A-4, a potent antimitotic agent isolated from the stem wood of the South African tree *Combretum caffrum* (15, 16).

Cytotoxicity of 1,2-diphenylimidazole (**IM5**) was much more than that of 1-methyl-2-phenylimidazole (**IM1**) and 2-methyl-1-phenylimidazole (**IM4**), suggesting that the presence of two aromatic rings at both the N-1 and C-2 positions was beneficial. 1-Benzyl-2-phenylimidazole (**IM7**) also showed potent cytotoxicity against tumor cells. These findings suggested that the 1,2-diphenylimidazole framework of **IM5** might serve as the basis for development of a new series of more tumor-specific cytotoxic compounds.

IM5 induced different types of cell death in the HSC-2, HSC-4 and HL-60 cells. It induced apoptotic internucleosomal DNA fragmentation in the HL-60 cells.

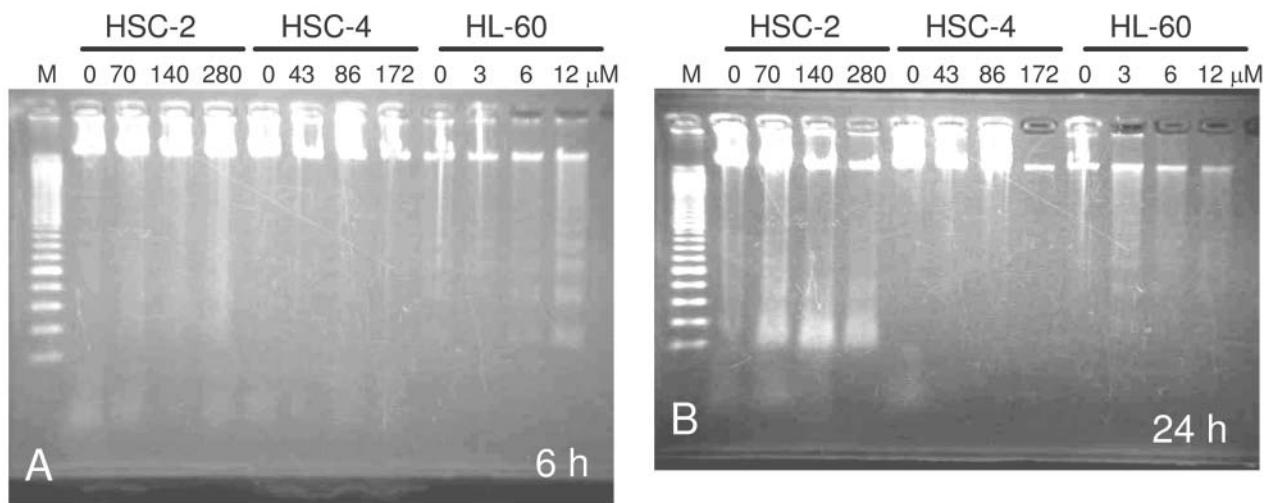


Figure 2. Effect of 4-trifluoromethylimidazoles on DNA fragmentation. Near confluent HSC-2, HSC-4 and HL-60 cells were incubated for 6 (A) or 24 (B) hours with the indicated concentration of IM5, and DNA was prepared for agarose gel electrophoresis. M, DNA size marker.

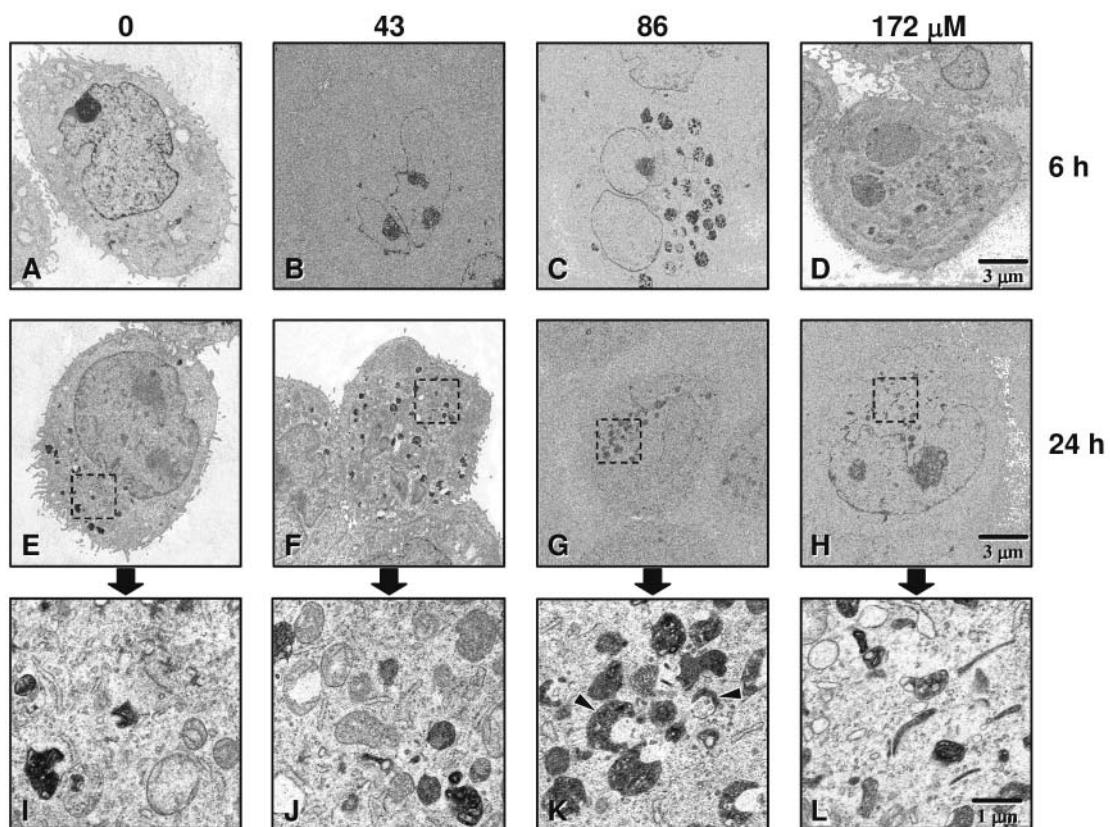


Figure 3. Electron microscopy of control and IM5-treated HSC-4 cells. Near confluent HSC-4 cells were incubated for 6 (A-D) or 24 (E-L) hours without (control) (A, E, I), or with 43 (B, F, J), 86 (C, G, K) or 172 μM (D, H, L) of IM5, and processed for electron microscopy. The areas surrounded by dotted lines (E, F, G, H) were magnified in I, J, K and L, respectively. Arrowhead indicates rod-shaped lysosomes surrounding small vesicles and/or lucent materials.

IM5 also induced DNA fragmentation, but without a clear-cut laddering pattern in the HSC-2 cells. On the other hand, **IM5** induced the formation of secondary lysosomes without the induction of apoptosis markers (such as DNA fragmentation and disappearance of cell surface microvilli) in the HSC-4 cells, even though HSC-2 and HSC-4 cells showed comparable sensitivity to **IM5**. This suggested that the type of cell death may be determined by the type of target cells, but not the drug-sensitivity of the cells.

In conclusion, the 1,2-diphenylimidazole scaffold thus appears to be appropriate for the development of more potent anticancer agents with tumor-specific cytotoxicity. The semiempirical method can be applicable to estimate the biological activity of novel imidazole derivatives (17).

Acknowledgements

We thank Drs. Yumiko Kanda and Shiro Kunii for their excellent technical support in undertaking the electron microscopy. 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 Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D and Yahalom J: A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* 61: 439-444, 2001.
- 2 Kanzawa T, Kondo Y, Ito H, Kondo S and Germano I: Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. *Cancer Res* 63: 2103-2108, 2003.
- 3 Daido S, Yamamoto A, Fujisawa K, Sawaya R, Kondo S and Kondo Y: Inhibition of the DNA-dependent protein kinase catalytic subunit radiosensitizes malignant glioma cells by inducing autophagy. *Cancer Res* 65: 4368-4375, 2005.
- 4 Broker LE, Krut FA and Giaccone G: Cell death independent of caspase: A review. *Clin Cancer Res* 11: 3155-3162, 2005.
- 5 Sakagami H, Kawase M, Wakabayashi H and Kurihara T: Factors that affect the type of cell death induced by chemicals. *Autophagy* 3: 493-495, 2007
- 6 Sakagami H, Kobayashi M, Chien C-H, Kanegae H and Kawase M: Selective toxicity and type of cell death induced by various natural and synthetic compounds in oral squamous cell carcinoma. *In Vivo* 21: 311-320, 2007.
- 7 Kawase M, Saito S and Kurihara T: Convenient synthesis of 4-trifluoromethyl-substituted imidazole derivatives. *Chem Pharm Bull* 49: 461-464, 2001.
- 8 Kawase M and Saito S: Convenient synthesis of 5-trifluoroacetylated imidazoles by ring transformation of mesoionic 1,3-oxazolium-5-olates. *Chem Pharm Bull* 48: 410-414, 2000.
- 9 Satoh R, Kishino K, Morshed SRM, Takayama F, Otsuki S, Suzuki F, Hashimoto K, Kikuchi H, Nishikawa H, Yasui T and Sakagami H: Change in fluoride sensitivity during *in vitro* senescence of human normal oral cells. *Anticancer Res* 25: 2085-2090, 2005.
- 10 Sekine T, Takahashi J, Nishishiro M, Arai A, Wakabayashi H, Kurihara T, Kobayashi M, Hashimoto K, Kikuchi H, Katayama T, Kanda Y, Kunii S, Motohashi N and Sakagami H: Tumor-specificity and type of cell death induced by trihaloacetylazulenes in human tumor cell lines. *Anticancer Res* 27: 133-144, 2007.
- 11 Fawcett DW: A Textbook of Histology, 12th ed. Chapman and Hall, New York, 1994.
- 12 Grimmett MR: Comprehensive Heterocyclic Chemistry, vol. 5. Potts KT (ed.). Pergamon, Oxford, pp. 345-498, 1984.
- 13 Bellina F, Cauteruccio S and Rossi R: Synthesis and biological activity of vicinal diaryl-substituted *1H*-imidazoles. *Tetrahedron* 63: 4571-4624, 2007.
- 14 Anil KC, Jayarama SB, Salimath BP and Rangappa KS: Pro-apoptotic activity of imidazole derivatives mediated by up-regulation of Bax and activation of CAD in Ehrlich ascites tumor cells. *Invest New Drugs* 25: 343-350, 2007.
- 15 Tron GC, Pirali T, Sorba G, Pagliai F, Busacca S and Genazzani AA: Medicinal chemistry of combretastatin A4: present and future directions. *J Med Chem* 49: 3033-3044, 2006.
- 16 Bellina F, Cauteruccio S, Monti S and Rossi R: Novel imidazole-based combretastatin A-4 analogues: evaluation of their *in vitro* antitumor activity and molecular modelling study of their binding to the colchicine site of tubulin. *Bioorg Med Chem Lett* 16: 5757-5762, 2006.
- 17 Ishihara M, Kawase M and Sakagami H: Quantitative structure-activity relationship analysis of 4-trifluoromethylimidazole derivatives with the concept of absolute hardness. *Anticancer Res* 27: 4045-4050, 2007.

Received July 26, 2007

Revised October 9, 2007

Accepted October 22, 2007