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Cytotoxic Activity of 2-Aminomethylene-3(2*H*)-benzofuranones against Human Oral Tumor Cell Lines

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Abstract. *A total of 23 newly-synthesized 2-aminomethylene-3(2H)-benzofuranone and structurally-related compounds were compared for their cytotoxic activity against both normal (human gingival fibroblast HGF) and tumor cells (human oral squamous cell lines HSC-2, HSC-3 and human salivary gland tumor cells HSG). There was a significant variability of drug sensitivity among the oral tumor cell lines. In general, HSC-2 cells were the most sensitive, followed by HSG cells, while HSC-3 cells were the most resistant. HGF normal cells were highly resistant to all compounds, suggesting their tumor-specific cytotoxic action. The cytotoxic activity of the compounds with morpholine, 1-methylpiperazine or piperidine structure was generally elevated by the introduction of fluorine, but not chlorine and methoxy functional groups, to the benzofuranone structure, whereas that of compounds attached by 1-phenylpiperazine or 1-(2-pyridyl)piperazine was rather reduced. The most active compounds induced internucleosomal DNA fragmentation in human promyelocytic leukemia HL-60 cells, but not in HSG, further confirming that oral tumor cell lines are resistant to DNase digestion.*

To find effective antitumor agents for treating oral carcinoma, we have adopted the following three strategies: (i) screening of active substances (both natural and synthetic), (ii) mechanism of cytotoxic action (either apoptosis or necrosis), and (iii) interaction with oral environment. We have reported the cytotoxic activity of polyphenols, antioxidants and vitamins (1-3). We have recently found that benzothiepins, benzoxepins and 5-benzoylimidazoles (4-6) showed higher cytotoxic activity against human oral tumor cell lines (human squamous cell carcinoma HSC-2, HSC-3, human salivary gland tumor HSG) than against human normal gingival fibroblast HGF, suggesting their tumor-specific cytotoxic action. Aurone, which has a 3(2*H*)-benzofuranone structure attached by benzene ring, has shown *in vitro* leishmanicidal activity (7) and slightly inhibited the topoisomerase I-catalysed DNA religation (8). Dihydro-benzofuranones have shown antiulcer activity (9, 10).

We have newly synthesized a series of aminomethylene-benzofuranone derivatives which contain the piperidine ring, instead of the benzene ring (11). We investigated here the structure-activity relationship of these compounds, using *in vitro* cytotoxicity assay with both normal and tumor cells.

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Key Words: 2-aminomethylene-3(2*H*)-benzofuranone, cytotoxic activity, oral tumor cells, DNA fragmentation.

Materials and Methods

Materials. The following reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium (Gibco BRL, Gland Island, NY, USA); fetal bovine serum (FBS) (JRH Biosci, Lenexa, KS, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind. Ltd., Osaka, Japan); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chem. Ind., St. Louis, MO, USA); RNase A, proteinase K (Boehringer Mannheim, Germany).

Synthesis of 2-aminomethylene-3(2H)-benzofuranone (general procedure). To a mixture of 3-iodochromone (1 mmol) and K_2CO_3 (1.4 g, 10 mmol) in DMF (10 mL) was added a solution of a secondary amine (1.5 mmol) in DMF (2 mL) at room temperature. After being stirred for 1-3.5 hours, the reaction mixture was diluted with iced-water and extracted with $CHCl_3$ (20 mL x 3). The organic layer was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/AcOEt = 1 : 1 with gradual increase of ethyl acetate) to afford the 2-aminomethylene-3(2*H*)-benzofuranone.

Synthesis of 2-piperidinomethylene-3(2H)-benzofuranone [1a]. According to the general procedure, 3-iodochromone (272 mg, 1 mmol) and piperidine (128 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **1a** (220 mg, 96%).

Synthesis of 5-methoxy-2-piperidinomethylene-3(2H)-benzofuranone [1b]. According to the general procedure, 3-iodo-6-methoxychromone (302 mg, 1 mmol) and piperidine (128 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **1b** (236 mg, 91%).

Synthesis of 6-methoxy-2-piperidinomethylene-3(2H)-benzofuranone [1c].

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According to the general procedure, 3-iodo-7-methoxychromone (302 mg, 1 mmol) and piperidine (128 mg, 1.5 mmol) were treated with K_2CO_3 for 2 hours to give **1c** (243 mg, 84%).

Synthesis of 5-fluoro-2-piperidinomethylene-3(2H)-benzofuranone [1d]. According to the general procedure, 6-fluoro-3-iodochromone (290 mg, 1 mmol) and piperidine (128 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **1d** (207 mg, 84%).

Synthesis of 2-pyrrolidinomethylene-3(2H)-benzofuranone [2a]. According to the general procedure, 3-iodochromone (272 mg, 1 mmol) and pyrrolidine (107 mg, 1.5 mmol) were treated with K_2CO_3 for 2 hours to give **2a** (189 mg, 88%).

Synthesis of 2-hexamethyleneiminomethylene-3(2H)-benzofuranone [3a]. According to the general procedure, 3-iodochromone (272 mg, 1 mmol) and hexamethyleneimine (149 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **3a** (231 mg, 95%).

Synthesis of 2-morpholinomethylene-3(2H)-benzofuranone [4a]. According to the general procedure, 3-iodochromone (272 mg, 1 mmol) and morpholine (131 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **4a** (203 mg, 88%).

Synthesis of 5-methoxy-2-morpholinomethylene-3(2H)-benzofuranone [4b]. According to the general procedure, 3-iodo-6-methoxychromone (302 mg, 1 mmol) and morpholine (131 mg, 1.5 mmol) were treated with K_2CO_3 for 2 hours to give **4b** (238 mg, 91%).

Synthesis of 6-methoxy-2-morpholinomethylene-3(2H)-benzofuranone [4c]. According to the general procedure, 3-iodo-7-methoxychromone (302 mg, 1 mmol) and morpholine (131 mg, 1.5 mmol) were treated with K_2CO_3 for 3.5 hours to give **4c** (243 mg, 93%).

Synthesis of 5-fluoro-2-morpholinomethylene-3(2H)-benzofuranone [4d]. According to the general procedure, 6-fluoro-3-iodochromone (290 mg, 1 mmol) and morpholine (131 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **4d** (224 mg, 90%).

Synthesis of 2-thiomorpholinomethylene-3(2H)-benzofuranone [5a]. According to the general procedure, 3-iodochromone (272 mg, 1 mmol) and thiomorpholine (155 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **5a** (217 mg, 88%).

Synthesis of 2-[(4-methylpiperazino)methylene]-3(2H)-benzofuranone [6a]. According to the general procedure, 3-iodochromone (272 mg, 1 mmol) and 1-methylpiperazine (150 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **6a** (220 mg, 90%).

Synthesis of 5-methoxy-2-[(4-methylpiperazino)methylene]-3(2H)-benzofuranone [6b]. According to the general procedure, 3-iodo-6-methoxychromone (302 mg, 1 mmol) and 1-methylpiperazine (150 mg, 1.5 mmol) were treated with K_2CO_3 for 2 hours to give **6b** (238 mg, 87%).

Synthesis of 6-methoxy-2-[(4-methylpiperazino)methylene]-3(2H)-benzofuranone [6c]. According to the general procedure, 3-iodo-7-methoxychromone (302 mg, 1 mmol) and 1-methylpiperazine (150 mg, 1.5 mmol) were treated with K_2CO_3 for 2.5 hours to give **6c** (241 mg, 88%).

Synthesis of 5-fluoro-2-[(4-methylpiperazino)methylene]-3(2H)-benzofuranone [6d]. According to the general procedure, 6-fluoro-3-iodochromone (290 mg, 1 mmol) and 1-methylpiperazine (150 mg, 1.5 mmol) were treated with K_2CO_3 for 1.5 hours to give **6d** (246 mg, 94%).

Synthesis of 2-[(4-phenylpiperazino)methylene]-3(2H)-benzofuranone [7a]. According to the general procedure, 3-iodochromone (272 mg, 1 mmol) and 1-phenylpiperazine (243 mg, 1.5 mmol) were treated with K_2CO_3 for 2 hours to give **7a** (285 mg, 93%).

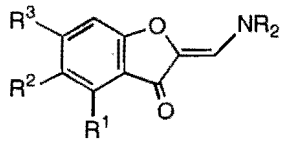
Synthesis of 5-fluoro-2-[(4-phenylpiperazino)methylene]-3(2H)-benzofuranone [7d]. According to the general procedure, 6-fluoro-3-iodochromone (290 mg, 1 mmol) and 1-phenylpiperazine (243 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **7d** (214 mg, 66%).

Table I. *Cytotoxic activity and tumor specificity of 2-aminomethylene-3(2H)-benzofuranones.*

Compound	Cytotoxic activity (CC_{50}: μg/mL) HSC-2 (A)	HSC-3	HSG	HGF (B)	Tumor specificity B/A
Exp.I					
1a	310	359	104	369	1.19
1b	227	244	123	360	1.59
1c	173	230	111	350	2.02
1d	125	120	148	347	2.78
2a	348	373	200	391	1.12
3a	144	176	135	281	1.95
4a	498	461	224	>500	>1.00
4b	490	490	236	448	0.91
4c	231	>500	232	>500	>2.16
4d	244	>500	222	>500	>2.05
5a	261	238	186	332	1.27
6a	>500	>500	433	>500	><1.00
6b	>500	>500	383	>500	><1.00
6c	348	>500	390	>500	>1.44
6d	125	307	292	>500	>4.00
7a	46	93	104	250	5.43
8a	73	139	85	184	2.52
Exp . II					
7a	78	442	156	153	1.96
7d	144	455	163	219	1.52
7e	295	>500	417	365	1.24
7f	188	439	356	293	1.56
8a	121	176	94	146	1.21
8d	399	>500	304	383	0.96
8e	179	>500	452	393	2.20
8f	38	386	167	346	9.11

Near confluent cells were incubated for 24 hours with various concentrations of each compound. The relative viable cell number was then determined by MTT method. Control A_{540} of HSC-2, HSC-3, HSG and HGF cells was 1.22, 2.00, 0.89 and 0.31, respectively. Each value represents the mean from 2 independent experiments which were done in duplicate.

2-aminomethylene-3(2*H*)-benzofuranone



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a: $R^1=R^2=R^3=H$
b: $R^1=R^3=H$, $R^2=OMe$
c: $R^1=R^2=H$, $R^3=OMe$
d: $R^1=R^3=H$, $R^2=F$
e: $R^1=R^3=H$, $R^2=Cl$
f: $R^1=R^3=OMe$, $R^2=H$

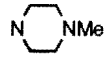
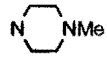
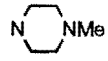
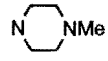
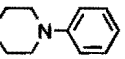
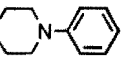
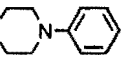
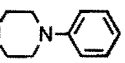
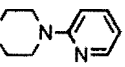
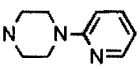
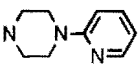
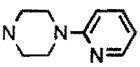
Compd. No.	NR_2	R^1	R^2	R^3	Compd. No.	NR_2	R^1	R^2	R^3
1a		H	H	H	**6a**		H	H	H
1b		H	OMe	H	**6b**		H	OMe	H
1c		H	H	OMe	**6c**		H	H	OMe
1d		H	F	H	**6d**		H	F	H
2a		H	H	H	**7a**		H	H	H
3a		H	H	H	**7d**		H	F	H
4a		H	H	H	**7e**		H	Cl	H
4b		H	OMe	H	**7f**		OMe	H	OMe
4c		H	H	OMe	**8a**		H	H	H
4d		H	F	H	**8d**		H	F	H
5a		H	H	H	**8e**		H	Cl	H
					8f		OMe	H	OMe

Figure 1. *Structure of 2-aminomethylene-3(2H)-benzofuranone.*

Synthesis of 5-chloro-2-[(4-phenylpiperazino)methylene]-3(2H)-benzofuranone [7e]. According to the general procedure, 6-chloro-3-iodochromone (307 mg, 1 mmol) and 1-phenylpiperazine (243 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **7e** (100 mg, 29%).

Synthesis of 4,6-dimethoxy-2-[(4-phenylpiperazino)methylene]-3(2H)-benzofuranone [7f]. According to the general procedure, 3-iodo-5,7-dimethoxychromone (332 mg, 1 mmol) and 1-phenylpiperazine (243 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **7f** (301 mg, 82%).

Synthesis of 2-{[4-(2-pyridyl)piperazino]methylene}-3(2H)-benzofuranone [8a]. According to the general procedure, 3-iodochromone (272 mg, 1 mmol) and 1-(2-pyridyl)piperazine (245 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **8a** (279 mg, 91%).

Synthesis of 5-fluoro-2-{[4-(2-pyridyl)piperazino]methylene}-3(2H)-benzofuranone [8d]. According to the general procedure, 6-fluoro-3-iodochromone (290 mg, 1 mmol) and 1-(2-pyridyl)piperazine (245 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **8d** (207 mg, 64%).

Synthesis of 5-chloro-2-{[4-(2-pyridyl)piperazino]methylene}-3(2H)-benzofuranone [8e]. According to the general procedure, 6-chloro-3-iodo-

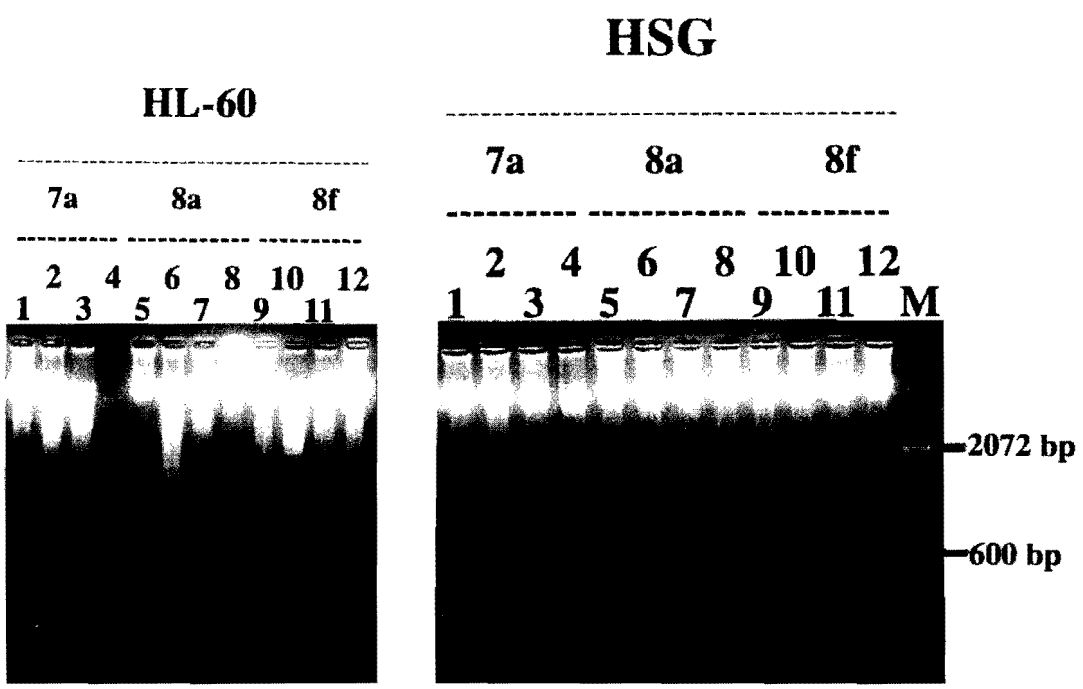


Figure 2. *Induction of DNA fragmentation by compounds* ***7a, 8a*** *and* ***8f****. Near confluent HL-60 and HSG cells were incubated for 6 hours with 0 (control) (lane 1, 5, 9), 100 (lanes 2, 6, 10), 200 (lanes 3, 7, 11), or 400 (lanes 4, 8, 12) μg/mL of compound* ***7a*** *(lanes 1-4),* ***8a*** *(lanes 5-8) or* ***8f*** *(lanes 9-12). DNA was then extracted and subjected to 2% agarose gel electrophoresis. M, marker DNA.*

chromone (307 mg, 1 mmol) and 1-(2-pyridyl)piperazine (245 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **8e** (124 mg, 36%).

Synthesis of 4,6-dimethoxy-2-{[4-(2-pyridyl)piperazino]methylene}-3(2H)-benzofuranone [8f]. According to the general procedure, 3-iodo-5,7-dimethoxychromone (332 mg, 1 mmol) and 1-(2-pyridyl)piperazine (245 mg, 1.5 mmol) were treated with K_2CO_3 for 1 hour to give **8f** (302 mg, 82%).

Cell culture. Human oral squamous cell carcinoma (HSC-2, HSC-3) and human salivary gland tumor (HSG) cells and human gingival fibroblast (HGF) (7-9th passage) were cultured in DMEM medium supplemented with 10 % heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Human promyelocytic leukemia HL-60 cells were cultured in RPMI1640 medium supplemented with 10% FBS (4-6).

Assay for cytotoxic activity. Near confluent cells grown in 96-microwell plate (Falcon, flat bottom, treated polystyrene, Becton Dickinson) were incubated for 24 hours with various concentrations of samples. The cells were washed 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 with the Labsystem Multiskan^R (Biochromatic) with a Star/DOT Matrix Printer JL-10. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve (4-6).

Assay for DNA fragmentation. The cells were pelleted, lysed and digested with RNase A and proteinase K. DNA was isolated and assayed for DNA fragmentation by 2% agarose gel electrophoresis (5, 10). The DNA size marker (GIBCO BRL) (Cat. No. 10380-012) was run in parallel.

Results and Discussion

Twenty-three 2-aminomethylene-3(2*H*)-benzofuranone and structurally-related compounds (Figure 1) were compared for their cytotoxic activity against both normal (human gingival fibroblast HGF) and tumor cells (human oral squamous cell lines HSC-2, HSC-3 and human salivary gland tumor cells HSG). There was a significant variability of drug sensitivity among oral tumor cell lines. In general, HSC-2 cells were the most sensitive, followed by HSG cells, while HSC-3 cells were the most resistant. HGF cells were highly resistant to all compounds, suggesting their tumor-specific cytotoxic action. The cytotoxic activity of benzofuranones was relatively weak, as compared with benzothiepins [4] and benzoylimidazoles [5]. Since we have found the synergistic action of benzothiepins and anticancer drugs, it might be beneficial to investigate such combination effects of benzofuranones and anticancer drugs. The cytotoxic activity of benzofuranones, which have piperidine **[1a, 1b, 1c, 1d]**, pyrrolidine **[2a]**, hexamethyleneimine **[3a]**, morpholine **[4a, 4b, 4c, 4d]**, thiomorpholine **[5a]** or 1-methylpiperadine **[6a, 6b, 6c, 6d]** structures, were generally enhanced by the introduction of the fluorine functional group, whereas that of compounds which have 1-phenylpiperazine **[7a, 7d, 7e, 7f]** or 1-(2-pyridyl) piperazine **[8a, 8d, 8e, 8f]** was not affected by the fluorine functional group. These data suggest that the addition of the

fluorine functional group differently modified the cytotoxic activity of the compounds, depending upon their three-dimensional structure. The most active compounds [**7a, 8a, 8f**] induced internucleosomal DNA fragmentation in human promyelocytic leukemia HL-60 cells, but not in HSG cells (Figure 2). We have previously reported that various natural and synthetic compounds failed to induce internucleosomal DNA fragmentation in oral tumor cell lines, in contrast to human leukemic cells (1-5). This suggests that chromatin DNA of oral tumor cells might be resistant to DNase attack (12, 13), or DNase can not be fully activated by treatment with these agents.

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