in vivo *21*: 829-834 (2007)

Biological Activity of 3-Formylchromones and Related Compounds

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Abstract. *Several 3-formylchromone derivatives were examined for their tumor cell-cytotoxic, anti-Helicobacter pylori, urease inhibitory and anti-HIV activity. Comparing their relative cytotoxicity against four human tumor cell lines and three normal human cells, tumor cell-specific cytotoxicity was detected in some 3-formylchromone derivatives. There was no clear-cut relationship between the cytotoxicity and the chemical structures of the compounds. 6,8-Dichloro-3-formylchromone (**FC10**) showed comparable anti-H. pylori activity with metronidazole and potent urease inhibition against jack bean urease. On the other hand, 6,8-dibromo-3-formylchromone (**FC11**) exhibited potent inhibitory activity against the urease, but had no anti-H. pylori activity. No chromones (**FC1-16**) exhibited anti-HIV activity.*

The chromones (4*H*-1-benzopyran-4-ones) have attracted attention from the point of view of both biological activity (1, 2) and organic synthesis (2, 3). Chromone derivatives have been found to exhibit a broad range of biological activities, including antifungal, antiviral, antimicrobial, antiallergenic, antitubulin and antitumor activity (1, 4). In addition, many flavonoids are based on the chromone structure and have been found to possess several therapeutically interesting biological activities (5).

In our previous studies on the structure-cytotoxic activity relationship with a series of β-diketones in human tumor cells, 3-formylchromone (**FC1**) was shown to exhibit potent cytotoxic activities against some tumor cells and had tumor cell-specific cytotoxicity (6). The presence of a formyl group at the C-3 position of the chromone seems to be responsible for such biological activity, because compound **FC1** possesses an α,β-unsaturated reactive aldehyde which can react as Michael acceptors. It is known that 3-formylchromone derivatives show induction of the chloroplast-free mutants in *Euglena gracilis* (7), have antiproliferative activity (8), and act as $p56^{lck}$ tyrosine kinase inhibitors (9) and modifiers of multidrug resistance in mouse lymphoma cells and human Colo320 colon cancer cells (10). It is also reported that some 3-formylchromone derivatives showed a relatively strong anti-anaphylactic reaction but generally had a low LD_{50} (11).

In the present study, we describe the effects produced by 3-formylchromones substituted at the C-6 position. We evaluated their cytotoxicity against human tumor cell lines and normal cells, and their anti-*Helicobacter pylori* (*H. pylori*), urease inhibitory and anti-HIV activity; the structurally related coumarine (2*H*-1-benzopyran-2-one) derivatives were also investigated.

Materials and Methods

Chemicals. 3-Formylchromone (**FC1**) (MW=174), 3-formyl-6-methylchromone (**FC2**) (MW=188), 3-formyl-6-isopropylchromone (**FC3**) (MW=216), 3-formyl-6-nitrochromone (**FC5**) (MW=219), 6-chloro-3-formylchromone (**FC7**) (MW=208.5), 6-bromo-3-formylchromone (**FC8**) (MW=253), 6-chloro-3-formyl-7-methylchromone (**FC9**) (MW=222.5), 6,8-dichloro-3-formylchro-mone (**FC10**) (MW=243), 6,8-dibromo-3-formylchromone (**FC11**) (MW=332), 3-cyanochromone (**FC12**) (MW=171), chromone (**FC13**) (MW=146), 3-acetylcoumarin (**FC14**) (MW=188), coumarin-3-carboxylic acid (**FC15**) (MW=190) and ethyl coumarin-3-carboxylate (**FC16**) (MW=218) were all obtained from Aldrich Chemical Co. Inc. Milwaukee, USA. 6-Methoxy-(**FC4**) (MW=204) and 6-fluoro-3-formylchromone (**FC6**) (MW=192) were synthesized as described in the literature (12). The structures of these compounds are given in Figure 1. Their log *p*-values have been reported elsewhere (10).

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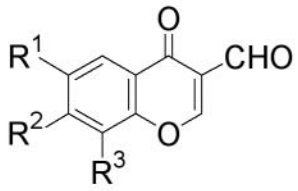
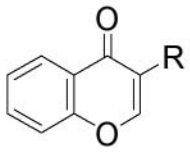
Key Words: 3-Formylchromone, urease, anti-*Helicobacter pylori*, cytotoxic activity, anti-HIV.



FC1-11

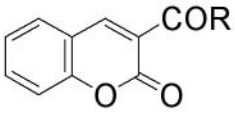
Compd	R^1	R^2	R^3	Log P[a]
FC1	H	H	H	0.35
FC2	CH_3	H	H	0.86
FC3	i-Pr	H	H	1.76
FC4	CH_3O	H	H	0.56
FC5	NO_2	H	H	-
FC6	F	H	H	0.60
FC7	Cl	H	H	1.17
FC8	Br	H	H	1.32
FC9	Cl	CH_3	H	1.67
FC10	Cl	H	Cl	1.91
FC11	Br	H	Br	2.21

[a] Log P reported in (10).



FC12, 13

Compd	R	Log P[a]
FC12	CN	0.45
FC13	H	1.01



FC14-16

Compd	R	Log P[a]
FC14	CH_3	1.15
FC15	OH	1.54
FC16	OC_2H_5	1.91

Figure 1. *Chemical structures and calculated log p values of chromone (**FC1-13**) and coumarin (**FC14-16**) derivatives.*

Cell culture. Normal human gingival fibroblast (HGF), human pulp cell (HPC) and human periodontal ligament fibroblast (HPLF) were obtained from human periodontal tissue after informed consent, according to the guideline of Meikai University Ethic Committee (No. 0206). Since normal cells have a limited life-span, cells at 3-7 population doubling levels (PDL) were used for the present study. Human oral squamous cell carcinoma cell lines (HSC-2, HSC-3) were supplied by Prof. Nagumo, Showa University, and Dr. Fukuda, Meikai University, respectively. Human submandibular gland carcinoma cell line (HSG) was supplied by Drs. Atsumi and Kurihara, Meikai University. Human promyelocytic leukemia cell line (HL-60) was supplied by Prof. Nakaya, Showa University.

HL-60 cells were maintained at 37˚C in RPMI-1640 medium supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Other cells were cultured as a monolayer culture at 37˚C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere, and subcultured by trypsinization.

Cytotoxic activity. The relative viable cell number of adherent cells was determined by MTT methods, while that of non-adherent cells (HL-60 cells) was determined by trypan blue dye exclusion.

For the MTT assay, the cells were treated for 24 h without (control) or with different concentrations (1-1000 μM) of test samples. The samples were dissolved in dimethyl sulfoxide (DMSO) at 100 mM, and added to the medium after dilution with the cuture medium. The final DMSO concentration was below 1%, that did not significantly affect the cell growth. The cells were washed once with phosphate-buffered saline without Mg^{2+} or Ca^{2+} and further incubated for 4 h with 0.2 mg/mL MTT in DMEM + 10% FBS. After removal of the medium, the cells were lysed with 0.1 mL of DMSO. The absorbance at 540 nm of the solubilized formazan pellet (which reflects the relative viable cell number) was then determined by microplate reader (Biochromatic Labsystem, Helsinki, Finland).

For the trypan blue dye exclusion assay, the number of cells which did not incorporate the trypan blue dye was calculated as the viable cell number by counting using a hemocytometer. From the dose-response curve, the 50% cytotoxic concentration (CC_{50}) was determined (13). Tumor-specific cytotoxicity (SI value) was determined by the following equation: SI=[CC_{50} (HGF + HPC + HPLF)/CC_{50} (HSC-2 + HSC-3 + HSG + HL-60)] x 4/3.

Measurement of anti-H. pylori activity. The micro-dilution broth method was used to determine the minimum inhibitory concentration (MIC). Brain heart infusion (BHI) broth containing 10% FBS (Biofluid, Inc. Rockville, MD, USA) and 0.1% glucose was used as the medium and was cultured in a jar conditioned with AnaeroPack (Campylo)(Mitsubishi Gas Chemical Co., Inc.). Briefly, *H. pylori* was inoculated in the medium and cultured at 37˚C for 2 days. The collected bacterial suspension was diluted to approximately 10^7 colony forming units (CFU)/mL with the

Table I. *Cytotoxic activity of 3-formylchromones and related compounds against normal and tumor cells.*

Compound	50% Cytotoxic concentration (CC_{50}, μM)							SI[a]= CC_{50} (normal)/ CC_{50} (tumor)
	Human tumor cell lines				Normal cells			
	HSC-2	HSC-3	HSG	HL-60	HGF	HPC	HPLF	
FC1	89	225	332	59	552	322	678	2.9
FC2	47	184	128	13	307	188	404	3.2
FC3	42	172	95	20	171	102	235	2.1
FC4	80	99	166	30	490	175	635	4.6
CF5	84	262	546	78	750	521	679	2.7
FC6	22	165	91	8	327	293	651	5.9
FC7	46	73	92	17	166	74	208	2.6
FC8	64	81	169	41	260	97	419	2.9
FC9	42	102	115	30	173	86	159	1.9
CF10	56	117	215	52	390	215	388	3.0
FC11	75	131	216	40	246	193	276	2.1
FC12	250	692	687	445	754	866	876	1.6
FC13	784	679	>1000	853	>1000	>1000	>1000	>><1.2
FC14	314	469	>1000	843	945	>1000	861	>><1.4
CF15	786	763	966	951	801	887	824	1.0
FC16	638	875	932	461	813	1000	844	1.2
Doxorubicin	9	8	37	20	>1000	>1000	>1000	>54.1
EGCG[b]	215	375	496	83	>1000	>1000	>1000	>3.4

Near confluent cells were incubated with or without different concentrations of each compound for 24 h and the relative viable cell number (absorbance at 540 nm of the MTT-stained cell lysate) was determined by the MTT method. The viable cell number of HL-60 cells was determined by trypan blue exclusion. The CC_{50} was determined from the dose-response curve. Each value represents the mean from duplicate determinations. [a]Determined by the equation: SI=[CC_{50}(HGF + HPC + HPLF) / CC_{50}(HSC-2 + HSC-3 + HSG + HL-60)] x 4/3. [b]EGCG: (–)-Epigallocatechin-3-gallate.

medium. The compounds were dissolved in DMSO and diluted stepwise with the medium, and put them in a microplate reader. To the solution of the compounds, a suspension of bacteria was added to make 10^6 CFU/150 μL/well. The mixture was incubated at 37˚C for 5 days. The MIC values of the compounds were determined from the dose-response curves. These values in Table III represent the mean from duplicate determinations (13).

Measurement of urease. Jack bean urease was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The assay mixture, containing 25 μL (4U) of jack bean urease and 25 μL (100 μg) of the test compound, was preincubated for 0.5 or 3 h at 25˚C in a 96-well assay plate. After preincubation, 0.2 mL of 100 mM phosphate buffer pH 6.8 containing 500 mM urea and 0.002% phenol red were added and incubated at room temperature. The reaction time, required for enough ammonium carbonate to form to raise the pH of the phosphate buffer from 6.8 to 7.7, was measured by micro plate reader (570 nm) (14, 15). The IC_{50} values in Table III represents the mean from duplicate determinations.

Assay for anti-HIV activity. Human T-cell leukemia virus-I (HTLV-I)-bearing CD4-positive human T-cell lines (MT-4 cells) were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. HIV- or mock-infected MT-4 cells ($1.5x10^5$/μL, 200 μL) were placed into 96-well microtiter plates in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and incubated in the presence of different concentrations (5-fold serial dilutions) of the compounds tested. The compounds **FC1-16**, DS and CRDS were tested between 1000 to 0.0026 μg/mL concentrations. Those of AZT and ddC were between 500 to 0.0013 μM and 5000 to 0.0128 μM, respectively. After incubation for 5 days at 37˚C in a CO_2 incubator, the cell viability was quantified by a colorimetric assay (at 540 nm and 690 nm), monitoring the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan product (16). All data represent the mean values of triplicate measurements. The values are expresed as percentage cytotoxicity and percentage antiviral protection, from which 50% cytotoxic concentration (CC_{50}) and 50% effective concentration (EC_{50}) are calculated. Thus, the selectivity index (SI) in Table II was defined as follows: SI=CC_{50}/EC_{50}.

Results

Cytotoxic activity. The 6-substituted 3-formylchromone derivatives (**FC1-8**) screened for their tumor-specific cytotoxic potentials are shown in Figure 1. Their CC_{50} values for the four tumor cell lines and three normal cells are summarized in Table I. Cytotoxic data for the related chromone derivatives (**FC9-13**), coumarin derivatives (**FC14-16**), doxorubicin and (–)-epigallocatechin-3-gallate (EGCG) are also included in the Table.

Table II. *anti-HIV activity of 3-formylchromones and related compounds.*

Compound	CC_{50} (μg/mL)	EC_{50} (μg/mL)	SI [CC_{50}/EC_{50}]
FC1	30.8	>40	<1
FC2	39.8	>40	<1
FC3	10.6	>40	<1
FC4	96.7	>200	<1
FC5	35.4	>40	<1
FC6	17.0	>40	<1
FC7	17.9	>40	<1
FC8	28.1	>40	<1
FC9	19.2	>40	<1
FC10	87.8	>200	<1
FC11	92.3	>200	<1
FC12	121.9	>200	<1
FC13	146.2	>200	<1
FC14	395.5	>1000	<1
FC15	612.9	>1000	<1
FC16	439.9	>1000	<1
DS	125.8	2.26	56
CRDS	402.2	0.12	3392
AZT(μM)	152.8	0.02	8726
ddC(μM)	3459.7	2.41	1433

DS: dextran-sulfate; CRDS: curdlan-sulfate; AZT: 3'-azido-3'- dideoxythymidine; ddC: dideoxycytidine.

Table III. *Urease inhibitory and anti-H. pylori activity of 3-formylchromones and related compounds.*

Compound	Anti-*H. pylori* MIC (μg/mL)	IC_{50} (μg/mL)[a]	
		0.5 h	3 h
FC1	>100	>200	>200
FC2	>100	>200	>200
FC3	41	>200	>200
FC4	>100	>200	>200
FC5	100	>200[b]	>200[b]
FC6	>100	>200	>200
FC7	81	>200	>200
FC8	75	>200	>200
FC9	>100	>200	>200
FC10	23	0.195	0.169
FC11	>100	0.220	0.136
FC12	>100	>200	>200
FC13	>100	>200	>200
FC14	>100	>200	>200
FC15	>100	>200	>200
FC16	>100	>200	>200
Hydroxyurea (HU)	-	0.016[c]	0.0074[c]
Clarithromycin	0.022	-	-
Amoxicillin	0.16	-	-
Metronidazole	45[d]	-	-

[a]IC_{50}: 50% inhibitory concentration; [b]the deep yellow color of the solution of the sample interfered with the assay; [c]our previous data (15); [d]our previous data (13).

The average CC_{50} value of **FC1** to HSC-2, HSC-3, HSG and HL-60 cells was 176 μM towards these four cell lines. 6-Substituted 3-formylchromones (**FC2-4** and **6-11**), but 6-nitro derivative (**FC5**), showed higher cytotoxicity than the compound **FC1**, and 6-fluoro (**FC6**), 6-chloro (**FC7**) and 6-chloro-7-methyl (**FC9**) derivatives were the most cytotoxic.

The cytotoxicity of **FC12** and **13** was much less than that of **FC1-11**, suggesting that the presence of 3-formyl group was beneficial. In order to assess the preferential toxicity for tumor cells, a selectivity index (SI) was calculated for each compound. All 3-formylchromones (**FC1-13**) displayed preferential toxicity for malignant cells and their SI values were shown in Table I. Normal cells exhibited a higher resistance to all of these compounds as compared with the tumor cell lines, which resulted in an elevation of the SI values of some derivatives. In particular, **FC4** and **6** showed excellent selectivity (SI=4.6 and 5.9, respectively).

Anti-HIV activity. The inhibition of HIV-induced cytopathic effects by **FC** compounds was studied. However, there was no significant inhibition by **FC1-16** of the cytopathic effects of HIV infection in MT-4 cells using effective concentrations of >200 μg/mL (selectivity index (SI) <1), compared with four positive controls-dextran sulfate (DS)(SI=56), curdlan sulfate (CRDS)(SI=3392), AZT (SI=8726) and dideoxycytidine (ddC)(SI=1433). The cytotoxic concentration (CC_{50}) against mock-infected MT-4 cells varied greatly (Table II); values of **FC3**, **6**, **7** or **9** were well fitted to their cytotoxic activity against tumor cell lines (Table I).

Anti-*H. pylori* activity. *H. pylori* is considered to be the major causative agent of several gastric pathologies, such as chronic gastritis, peptic ulcer and stomach cancer (17). Today, control of this organism is an important goal of medicinal science. As a result, there is a need to develop new therapeutic agents with selective antibacterial activity against *H. pylori* (18). As a part of a screening program of a number of compounds, a series of chromone derivatives (**FC1-16**) were screened against *H. pylori* and the MIC figures are given in Table III. **FC3**, **7**, **8** and **10** showed potent anti-*H. pylori* activity. The anti-*H. pylori* activity of **FC10** was the most potent, equipotent to that of metronidazole. Thus, the addition of Cl at the C8-position (**FC10**) increased anti-*H. pylori* activity, suggesting that this position is important as the electrophilic and lipophilic features. On the other hand, interestingly, the addition of an extra bromine substituent at position 8 (**FC11**) reduced the activity.

Urease inhibition activity. Ureases are sulfhydryl enzymes which have a cysteine residue in the active site and catalyse

the hydrolysis of urea (19). The enzymatic hydrolysis of urea causes an abrupt increase in overall pH, which negatively influences human health and agriculture. In particular, urease activity constitutes a virulence factor in human infections of the urinary and gastrointestinal tracts. *H. pylori* produces a large amount of urease which is believed to play an essential role in facilitating its survival. Urease inhibitors have been regarded as targets for new anti-ulcer drugs (20). A number of α,β-unsaturated ketones have been demonstrated to show urease-inhibitory activities (21). **FC10** and **FC11** showed potent urease inhibitory activity. However, **FC1-4**, **6-9** and **12-16** exhibited little or no ability to inhibit urease (>200 μg/mL). The deep yellow color of the solution of **FC5** interfered with the assay.

The anti-urease inhibitory potency of 6-chloro-(**FC7**) and 6-bromo-(**FC8**) compounds was much less in comparison with 6,8-dichloro-(**FC10**) and 6,8-dibromo-(**FC11**) compounds. Therefore, including a weak electron-withdrawing group, such as Cl or Br, at both C-6 and C-8 positions increased the lipophilicity and the activity.

Discussion

We found that 3-formylchromones generally possess several interesting biological activities which strongly depend on the nature and position of substituents. The hydrophobicity of an individual chromone is certainly not the only determinant of its activity (10). The results showed that the all 6-substituted 3-formylchromones, but not the 6-nitro-substituted one (**FC5**), exhibit more potent cytotoxicity against tumor cell lines as compared to the parent 3-formylchromone (**FC1**). However, there was no clear-cut relationship between the chemical structure of the compounds tested and their cytotoxic activity. On the other hand, only the compounds with two halogen groups at C-6 and C-8 positions exhibited urease inhibitory activity. Anti-*H. pylori* activity is only shown by derivatives with Br group at C-8 position.

The concept of privileged structures or scaffolds was emerged as one of the guiding principles of modern drug discovery (2). Molecules with the chromone scaffold are privileged substructures exhibiting a range of biological activities (1, 4, 7, 10) and are present in large quantities in human diets. In addition, chromones represent an attractive source of medicinally interesting compounds due to their low toxicity. 3-Formylchromones bearing an electron-withdrawing formyl group at C-3 are highly functional molecules, capable of reacting as Michael acceptors. It seems reasonable, therefore, that the exploration of more potent or better bioavailable agent and cancer cell line-specific cytotoxic agent can be achieved through further modifications of the 3-formylchromone.

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Received March 28, 2007
Revised June 15, 2007
Accepted July 2, 2007