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Synthesis and Biological Activities of Fluorinated Chalcone Derivatives

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Abstract—We have designed and synthesized new 5-lipoxygenase inhibitors, fluorinated 3,4-dihydroxychalcones, and evaluated their biological activities with respect to antiperoxidation activity and in vitro antitumor activities. All fluorinated chalcones tested showed 5-lipoxygenase inhibition on rat basophilic leukemia-1 (RBL-1) cells and inhibitory action on Fe^{3+}-ADP induced NADPH-dependent lipid peroxidation in rat liver microsomes. The potencies were comparable or better to that of the lead 3,4-dihydroxychalcone. 6-Fluoro-3,4-dihydroxy-2′,4′-dimethoxy chalcone (**7**) was the most effective compound in the in vitro assay using a human cancer cell line panel (HCC panel) consisting of 39 systems.

Introduction

The leukotrienes are mediators of such important functions as smooth muscle contraction, increased vascular permeability, leukocyte chemotaxis, hypersensitivity reactions and inflammation.[1,2] These eicosanoides are formed from the unstable epoxide, leukotriene A_4 (LTA_4), which is transformed either by LTB_4 synthase to give LTB_4 or by LTC_4 synthase to produce the cysteinyl leukotrienes. The synthesis of LTA_4 from arachidonic acid is mediated by the enzyme, 5-lipoxygenase (5-LO). Because of the potent activities of the leukotrienes, development of 5-LO inhibitors has become the focus of much research.[3–5] Chemotherapeutic goals of this research include treatment of diseases associated with inflammatory responses, including asthma and inflammatory skin diseases such as psoriasis and contact dermatitis.[1] The ability of a 5-LO inhibitor to trigger massive apoptosis in human prostate cancer cells is further indication of the potential utility of these agents.[6] Inhibition of 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) biosynthesis from arachidonic acid has been implicated in this response.

It was previously shown that a series of 3,4-dihydroxychalcones had potent inhibitory effects on 5-LO with antioxidative effects, and some inhibited cyclooxygenase.[7]

The C–F bond frequently is used as a replacement of a C–H or C–OH bond in biologically active compounds because these substitutions result in altered physicochemical properties of the molecule, but introduce no major steric changes.[8] Because of this, useful alterations in biological activities often result from these substitutions.[9] This report deals with the synthesis of fluorinated chalcone derivatives and their 5-LO inhibiting action on rat basophilic leukemia-1 (RBL-1) cells, preventive action on Fe^{3+}-ADP induced NADPH-dependent lipid peroxidation in rat liver microsomes, and action against a human cancer cell line panel (HCC panel) consisting of 39 systems.

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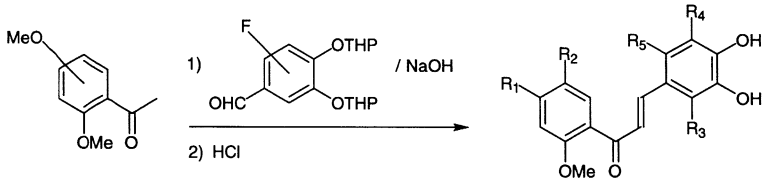

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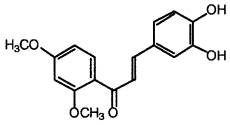
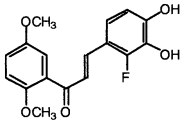
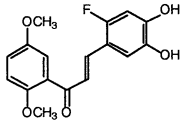
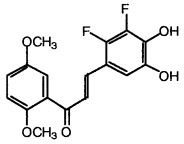
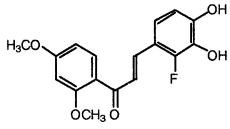
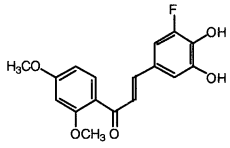
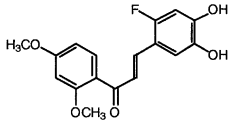
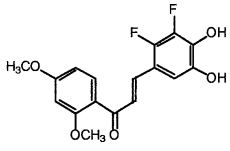
3: R_2=OMe; R_5=F; R_1, R_3, R_4=H
4: R_2=OMe; R_4, R_5=F; R_1, R_3=H
5: R_1=OMe; R_3=F; R_2, R_4, R_5=H
6: R_1=OMe; R_4=F; R_2, R_3, R_5=H
7: R_1=OMe; R_5=F; R_2, R_3, R_4=H
8: R_1=OMe; R_4, R_5=F; R_2, R_3=H

Scheme 1.

Table 1. Effect of chalcone derivatives on Fe^{3+}-ADP induced NADPH dependent lipid peroxidation in rat liver microsomes and on RBL-1 cell 5-lipoxygenase activity

	Compd	IC_{50} (M)		log*P* (C)[c]
		5-Lipoxygenase[a]	Lipid peroxidation[b]	
1		4.0×10^{-8}	4.5×10^{-6}	3.07
2		8.3×10^{-8}	2.3×10^{-6}	3.12
3		1.2×10^{-7}	3.7×10^{-6}	3.33
4		1.2×10^{-7}	7.4×10^{-6}	3.39
5		7.0×10^{-8}	1.0×10^{-6}	3.17
6		1.2×10^{-7}	1.8×10^{-6}	3.38
7		8.7×10^{-8}	3.5×10^{-6}	3.38
8		4.3×10^{-8}	3.5×10^{-6}	3.43

[a]Test compound concentration: 1×10^{-6} – 1×10^{-8} M.
[b]Test compound concentration: 1×10^{-5} M – 3×10^{-7} M.
[c]log*P*(C) was calculated using PrologP 5.1 (PALLAS for Windows, CompuDrug International Inc., South San Francisco, CA, USA).

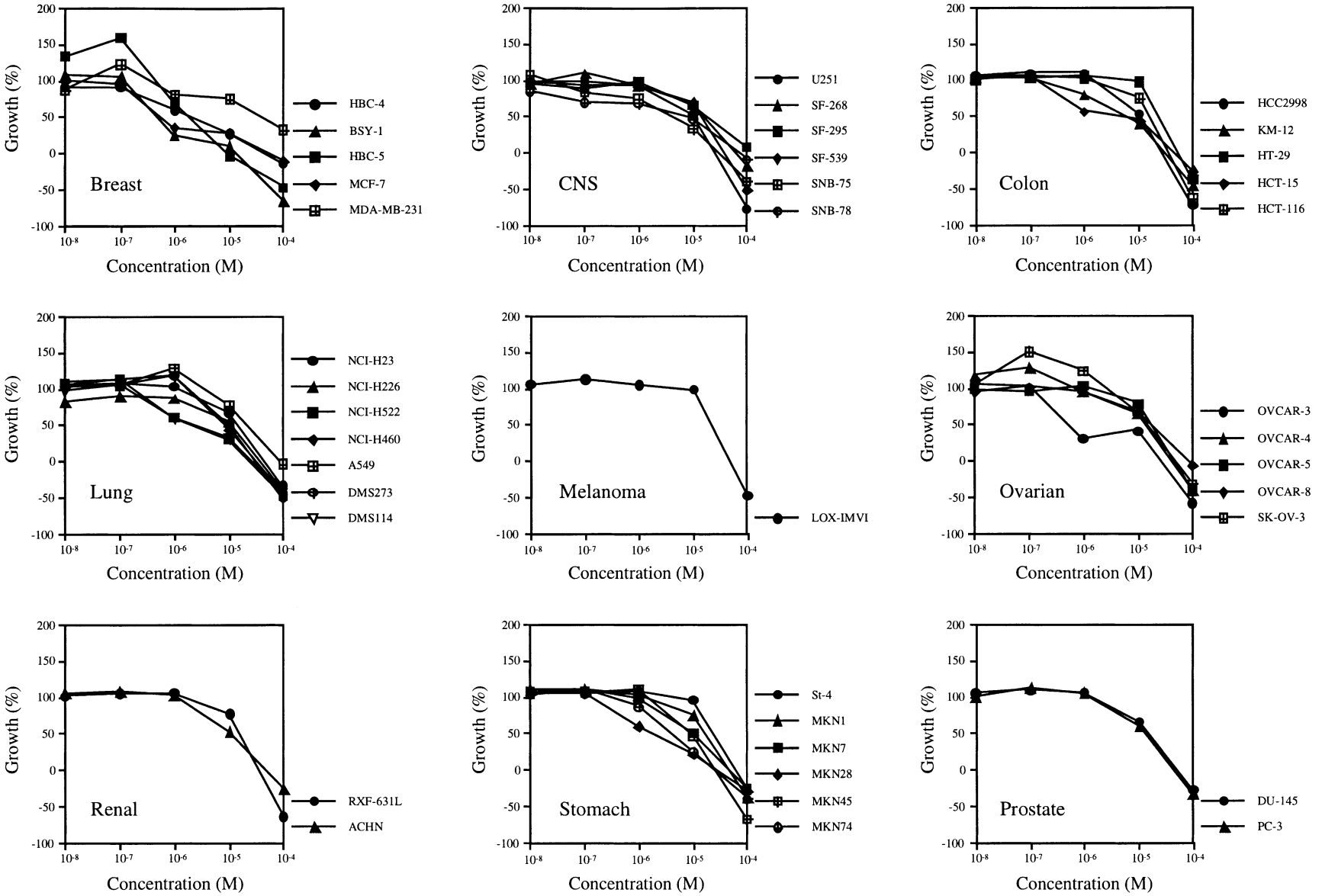


Figure 1. Dose–response curves of compound **7** on HCC panel. Test compound concentration; 10^{-4}–10^{-8} M.

Chemistry

Claisen–Schmidt condensations of acetophenones with bis-THP-protected fluorinated dihydroxybenzaldehydes were carried out as previously reported (Scheme 1).[7] Without purification, the protecting groups were removed by acid treatment, and the catecholic chalcones (**2**–**8**) were purified by chromatography and/or recrystallization.

Results and Discussion

Preventive action on Fe^{3+}-ADP induced NADPH-dependent lipid peroxydation in rat liver microsomes and 5-LO inhibitory action on RBL-1 cells were investigated for the fluorinated chalcone derivatives synthesized (Table 1). The lipid peroxidation preventing action was induced with Fe^{3+}-ADP in rat liver microsomes and the amount formed of peroxidized lipids was determined as the amount of malondialdehyde produced in their reaction with 2-thiobarbituric acid (TBA). The percentage of prevention was calculated against the control on the basis of the experimental data obtained. All of the chalcone derivatives exhibited a concentration-dependent lipid peroxidation preventing action at concentrations from 1×10^{-5} to 3×10^{-7} M and the value of IC_{50} was in a range of 1.0×10^{-6} to 7.4×10^{-6}M. The IC_{50} value for nonfluorinated 3,4-dihydroxy-2′,4′-dimethoxychalcone (**1**) was 4.5×10^{-6} M.

5-LO inhibition was evaluated from the amount formed of 5-HETE determined by HPLC after adding arachidonic acid as the substrate to cell homogenate. The percentage of inhibition was calculated against the control using the analytical data obtained. All fluorinated chalcone derivatives showed a concentration-dependent 5-LO inhibiting action in their concentration range from 1×10^{-6} to 1×10^{-8} M. The IC_{50} value for the fluorinated compounds ranged from 1.2×10^{-7} to 4.3×10^{-8} M while that for the nonfluorinated compound (**1**) was 4.0×10^{-8} M.

Among the fluorinated chalcone derivatives synthesized, 2′,4′-dimethoxy type compounds (**5**–**8**) showed stronger antioxydative and 5-LO inhibitory actions than 2′,5′-dimethoxy type compounds (**2**–**4**). Fluorination was found to cause no significant change in these activities.

HCC-panel[10,11] was employed to examine the anticarcinogenic activity of the fluorinated compounds. Each of the test compounds was added to a microtiter plate 24 h after cancer cells were enclosed into it. The percentage of cell proliferation was evaluated against the control by determining colorimetrically[12] the amount of the cells after 48 h incubation. First, prescreening was performed using three cell lines, MCF-7 (breast), NCI-H460 (lung), and SF-268 (CNS), at a compound concentration of 10^{-4} M (Table 2). The results on the nonfluorinated chalcone (**1**) and the fluorinated chalcone derivatives (**3**, **5**, **7**, **8**) showed that all of these compounds have a cell proliferation suppressing action on the three cell lines used. Among these

Table 2. Effect of chalcone derivatives on human cancer cell lines

Compd	Growth (%)		
	NCH-H460	MCF-7	SF-268
1	−73	−65	−30
3	−59	−50	−65
5	−51	−79	−26
7	−84	−100	−100
8	−30	10	19

Test compound concentration: 10^{-4} M.

Table 3. Effect of compound **7** on HCC panel

Cell	GI_{50} (μM)	TGI (μM)	LC_{50} (μM)
Breast			
HBC-4	1.9	46	>100
BSY-1	0.48	13	64
HBC-5	1.9	9.5	>100
MCF7	0.55	54	>100
MDA-MB-231	38	>100	>100
CNS			
U251	10	26	63
SF-268	16	63	>100
SF-295	19	>100	>100
SF-539	14	38	99
SNB-75	4.0	29	>100
SNB-78	7.4	72	>100
Colon			
HCC2998	10	27	67
KM-12	5.4	29	>100
HT-29	22	53	>100
HCT-15	3.9	44	>100
HCT-116	14	35	80
Lung			
NCI-H23	14	47	>100
NCH-H226	11	39	>100
NCI-H522	2.3	24	>100
NCI-H460	8.2	32	>100
A549	22	93	>100
DMS273	10	33	>100
DMS114	2.2	27	>100
Melanoma			
LOX-IMVI	21	47	>100
Ovarian			
OVCAR-3	0.53	26	83
OVCAR-4	13	41	>100
OVCAR-5	18	46	>100
OVCAR-8	17	84	>100
SK-OV-3	15	49	>100
Renal			
RXF-631L	16	36	81
ACHN	11	47	>100
Stomach			
St-4	24	61	>100
MKN1	17	45	>100
MKN7	9.8	45	>100
MKN128	1.8	28	>100
MKN45	9.0	26	71
MKN74	3.9	24	>100
Prostate			
DU-145	14	51	>100
PC-3	12	44	>100

Test compound concentration: 10^{-4}–10^{-8} M.

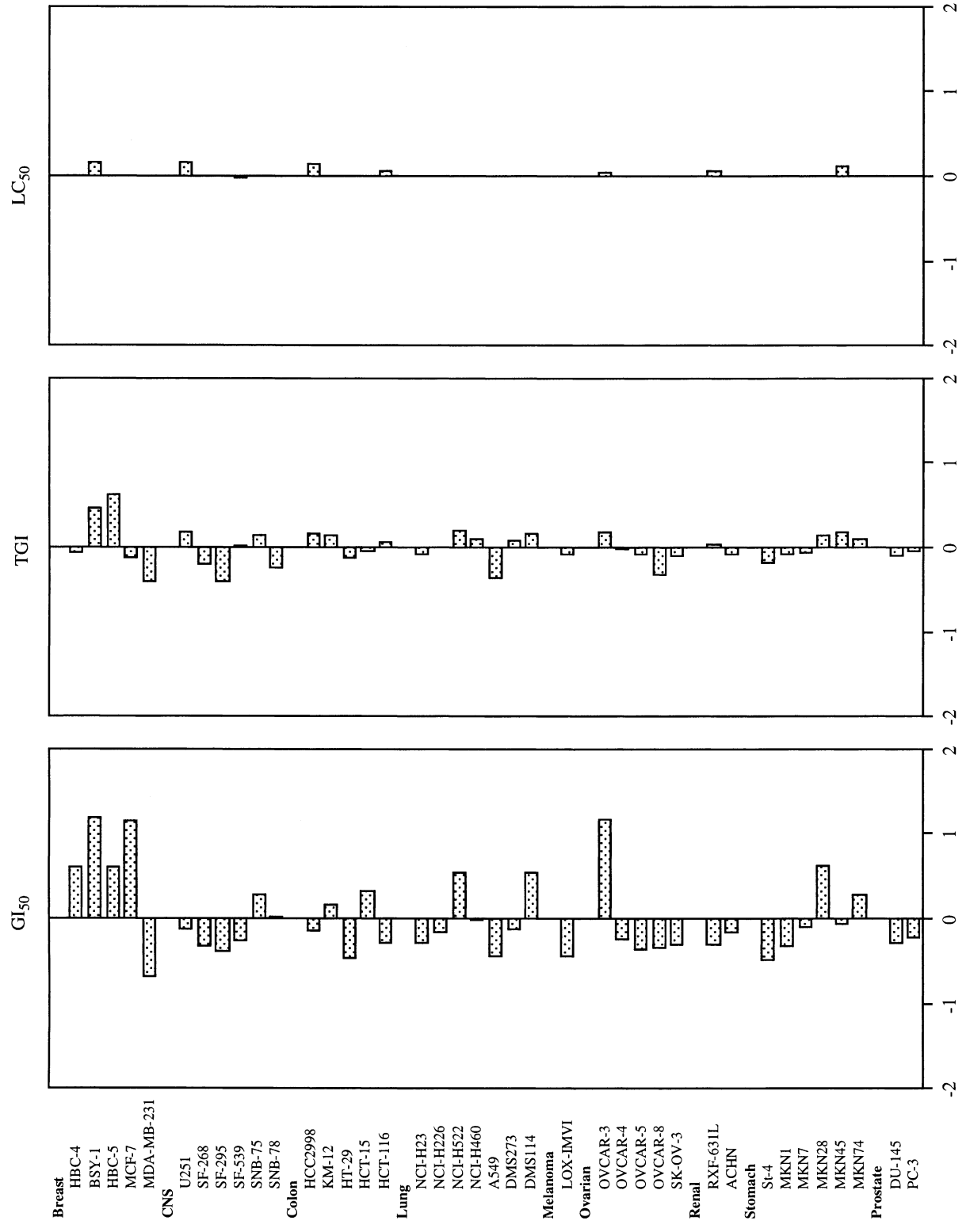


Figure 2. Mean graph of compound **7** on HCC panel.

compounds, **7** was most anticarcinogenic and killed the cells of MCF-7 and SF-268 almost completely.

Screening was then conducted for **7** making use of an HCC panel consisting of 39 cell lines. The compound exhibited a concentration-dependent inhibitory action on cell proliferation for all cell lines (Fig. 1). Evaluations were made on the values of GI_{50} (compound concentration necessary to suppress cell proliferation to 50% of the control), TGI (compound concentration necessary to suppress cell proliferation to the cell number at time zero), and LC_{50} (compound concentration necessary to reduce cell number to 50% of that at time zero) for these cell lines (Table 3). The values obtained for each of the three indexes were averaged respectively over the 39 cell lines and the mean values thus calculated were used to denote the relative sensitivity of each cell line to the test compound (Fig. 2). This sort of figure is called mean graph and allows to grasp the specificity of cancer cells to drug. Many **7**-sensitive cell strains were found in breast cancer cell lines. Based on these mean values, an analysis was carried out using the compare program[13,14] to make a comparison between the compound and the conventional anticancer agents. As a result, the most similar agent was found to be vincristine[15,16] with a tubulin inhibiting action but the correlation coefficient was small (0.57). Examination of the tubulin inhibiting action of **7** revealed that the compound has no such action (data not shown). This suggests an action mechanism of **7** differing from that of the conventional anticancer agents to make the compound a unique anticarcinogenic agent.

In summary, we have synthesized fluorinated derivatives of 3,4-dihydroxy-dimethoxychalcone and shown that they have antioxidative and 5-LO preventing actions which are almost equivalent to those of the non-fluorinated compound. We have also shown that the compounds synthesized exhibit an anticarcinogenic action and suggested that **7** may act on cancer cells in a way quite different from that of the conventional anticancer agents. The compound may thus be an extremely useful anticancer agent.

Experimental

Chemistry

Melting points are uncorrected. ^1H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, with CD_3OD and DMSO-d_6 as solvents. Commercial reagents and solvents were used without further purification. All reactions were performed under a dry N_2 atmosphere. Analytical TLC was performed with Kisegel 60 GF_{254}(Merck) and flash column chromatography was performed with silica gel 60 (230–400 mesh, Merck).

General procedure for formation of THP-protected fluorinated catechualdehydes

A solution of 3,4-dihydro-2*H*-pyran in 120 mL of methylene chloride was added dropwise to a stirred mixture of fluorinated 3,4-dihydroxybenzaldehyde (20 mmol) and pyridinium *p*-toluenesulfonate (0.12 g, 0.48 mmol) in 80 mL of methylene chloride. The reaction mixture was stirred at room temperature until all the components dissolved. The reaction mixture was washed twice with water, dried, and evaporated in vacuo. The residual 3,4-bis-(tetrahydro-pyran-2-yloxy)-fluorobenzaldehydes were used in the condensation step without further purification.

General condensation procedure

A solution of the above crude 3,4-bis-(tetrahydro-pyran-2-yloxy)-fluorobenzaldehyde and 2′,4′- or 2′,5′-dimethoxyacetophenone (20 mmol) and barium hydroxide octahydrate (6.52 g, 20 mmol) in 100 mL of MeOH was stirred at 40 °C for 12 h. The mixture was concentrated in vacuo to give the crude protected chalcone. This was dissolved in 80 mL of MeOH and *p*-toluenesulfonic acid monohydrate (0.091 g, 0.48 mmol) was added to it. The mixture was stirred at room temperature for 3 h (monitored by TLC for completion of the reaction) and then concentrated in vacuo. Water (100 mL) was added to the mixture, the aqueous solution was neutralized with 5% $NaHCO_3$ and extracted with EtOAc. The organic layer was separated, washed with water and brine, dried over $MgSO_4$, filtered and evaporated. The residue was chromatographed on silica gel to afford the fluorinated chalcones, data for which are given below.

2′,5′-Dimethoxy-2-fluoro-3,4-dihydroxychalcone (2). Yield 35%; dark yellow solid, mp 185–186 °C; ^1H NMR (300 MHz, CD_3OD) δ 7.62 (d, $J=15.9$ Hz, 1H), 7.34 (d, $J=15.9$ Hz, 1H), 7.09 (brs, 3H), 7.04 (d, $J=8.1$ Hz, 1H), 6.65 (dd, $J=8.7$ and 1.8 Hz, 1H), 3.86 (s, 3H), 3.79 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 191.5, 153.1, 151.8, 151.4 (d, $J=244.7$ Hz), 150.5 (d, $J=6.2$ Hz), 136.2, 133.5 (d, $J=13.7$ Hz), 129.4, 125.5 (d, $J=6.3$ Hz), 119.4 (d, $J=4.5$ Hz), 118.4, 114.2, 114.1, 113.9 (d, $J=1.1$Hz), 111.8, 56.3, 55.6; HRMS (FAB^+) m/z calcd for $C_{17}H_{16}O_5F$ (M + 1) 319.0982, obsd 319.0972.

2′,5′-Dimethoxy-6-fluoro-3,4-dihydroxychalcone (3). Yield 30%; yellow solid, mp 180–183 °C; ^1H NMR (300 MHz, DMSO-d_6) δ 10.19 (brs, 1H), 9.24 (brs, 1H), 7.48 (d, $J=15.9$ Hz, 1H), 7.18 (d, $J=15.9$ Hz, 1H), 7.12–7.09 (m, 3H), 7.03 (brd, $J=1.8$ Hz, 1H), 6.63 (d, $J=11.7$ Hz, 1H), 3.82 (s, 3H), 3.74 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 191.3, 155.4 (d, $J=242.5$ Hz), 153.1, 151.9, 149.9 (d, $J=11.9$ Hz), 142.5, 135.3 (d, $J=2.9$ Hz), 129.4, 125.0 (d, $J=5.1$ Hz), 118.5, 114.02, 113.96, 113.3 (d, $J=4.0$ Hz), 112.2 (d, $J=12.5$ Hz), 103.3 (d, $J=25.6$ Hz), 56.4, 55.6; HRMS (FAB^+) m/z calcd for $C_{17}H_{16}O_5F$ (M + 1) 319.0982, obsd 319.0975.

2′,5′-Dimethoxy-5,6-difluoro-3, 4-dihydroxy chalcone (4). Yield 35%, dark yellow solid, mp 129–130 °C; ^1H NMR (300 MHz, CD_3OD) δ 7.60 (d, $J=15.9$ Hz, 1H), 7.33 (d, $J=15.9$ Hz, 1H), 7.11 (brs, 3H), 6.88 (dd, $J=6.6$ and 2.1 Hz, 1H), 3.88 (s, 3H), 3.79 (s, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 194.3, 155.2, 154.2, 146.4 (dd, $J=243.9$ and 11.4 Hz), 144.6 (dd, $J=4.6$ and 2.3 Hz),

142.3 (dd, $J=238.4$ and 15.4 Hz), 139.4 (dd, $J=11.4$ and 2.9 Hz), 136.7 (brt, $J=3.4$ Hz), 130.5, 127.9 (d, $J=5.7$ Hz), 120.3, 115.7, 114.8, 114.7, 108.6, 56.9, 56.3; HRMS (FAB^+) m/z calcd for $C_{17}H_{15}O_5F_2$ (M + 1) 337.0888, obsd 337.0877.

2′,4′-Dimethoxy-2-fluoro-3,4-dihydroxy chalcone (5). Yield 32%; yellow solid, mp 185–186 °C; ^1H NMR (300 MHz, DMSO-d_6) δ 10.12 (brs, 1H), 9.29 (brs, 1H), 7.60 (d, $J=8.4$ Hz, 1H), 7.53 (d, $J=16.2$ Hz, 1H), 7.43 (d, $J=15.9$ Hz, 1H), 7.11 (t, $J=8.4$ Hz, 1H), 6.68–6.62 (m, 3H), 3.89 (s, 3H), 3.85 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 189.2, 163.9, 160.1, 151.4 (d, $J=244.1$ Hz), 150.2 (d, $J=6.2$ Hz), 134.7, 133.5 (d, $J=13.7$ Hz), 132.0, 125.8 (d, $J=6.3$ Hz), 121.5, 119.2 (d, $J=4.0$ Hz), 114.5 (d, $J=9.7$ Hz), 111.7, 106.0, 98.6, 55.9, 55.6; HRMS (FAB^+) m/z calcd for $C_{17}H_{16}O_5F$ (M + 1) 319.0982, obsd 319.0974.

2′,4′-Dimethoxy-5-fluoro-3,4-dihydroxy chalcone (6). Yield 29%; yellow solid, mp 194–195 °C; ^1H NMR (300 MHz, CD_3OD) δ 7.65 (d, $J=8.7$ Hz, 1H), 7.44 (d, $J=15.9$ Hz, 1H), 7.36 (d, $J=15.9$ Hz, 1H), 6.93–6.89 (m, 2H), 6.62 (d, $J=9.0$ Hz, 1H), 6.62 (d, $J=11.1$ Hz, 1H), 3.93 (s, 3H), 3.88 (s, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 193.0, 166.4, 162.3, 153.6 d, $J=236.8$ Hz), 149.0 (d, $J=6.3$ Hz), 143.7 (d, $J=3.1$ Hz), 137.5 (d, $J=15.4$ Hz), 133.7, 127.7 (d, $J=8.6$ Hz), 126.5, 123.0, 112.1 (d, $J=1.7$ Hz), 108.7 (d, $J=19.9$ Hz), 107.0, 99.7, 56.5, 56.3; HRMS (FAB^+) m/z calcd for $C_{17}H_{16}O_5F$ (M + 1) 319.0982, obsd 319.0983.

2′,4′-Dimethoxy-6-fluoro-3,4-dihydroxy chalcone (7). Yield 41%; yellow crystals, mp 179–180 °C; ^1H NMR (300 MHz, DMSO-d_6) δ 10.11 (brs, 1H), 9.23 (brs, 1H), 7.60 (d, $J=8.4$ Hz, 1H), 7.52 (d, $J=15.6$ Hz, 1H), 7.34 (d, $J=15.6$ Hz, 1H), 7.09 (d, $J=7.5$ Hz, 1H), 6.69–6.62 (m, 3H), 3.90 (s, 3H), 3.85 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 188.9, 163.9, 160.2, 155.3 (d, $J=241.9$ Hz), 149.5 (d, $J=12.0$ Hz), 142.4, 133.8, 132.1, 125.4 (d, $J=5.2$ Hz), 121.5, 113.3 (d, $J=4.0$ Hz), 112.6 (d, $J=12.5$ Hz), 106.1, 103.3 (d, $J=26.2$ Hz), 98.7, 56.0, 55.6; HRMS (FAB^+) m/z calcd for $C_{17}H_{16}O_5F$ (M + 1) 319.0982, obsd 319.0974.

2′,4′-Dimethoxy-5, 6-difluoro-3,4-dihydroxychalcone (8). Yield 30%; yellow solid, mp 218–219 °C; ^1H NMR (300 MHz, DMSO-d_6) δ 10.31 (brs, 1H), 9.80 (brs, 1H), 7.63 (d, $J=8.7$ Hz, 1H), 7.50 (d, $J=16.2$ Hz, 1H), 7.42 (d, $J=15.9$ Hz, 1H), 6.94 (dd, $J=6.9$ and 1.8 Hz, 1H), 6.69 (d, $J=2.1$ Hz, 1H), 6.65 (dd, $J=8.7$ and 2.4 Hz, 1H), 3.91 (s, 3H), 3.86 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 188.6, 164.2, 160.3, 144.0 (dd, $J=242.5$ and 11.4 Hz), 143.4 (brd, $J=3.4$ Hz), 140.6 (dd, $J=237.9$ and 15.4 Hz), 137.6 (dd, $J=10.9$ and 2.3 Hz), 132.8, 132.2, 127.0 (d, $J=5.7$ Hz), 121.2, 112.7 (d, $J=9.1$ Hz), 107.7, 106.2, 98.7, 56.0, 55.6; HRMS (FAB^+) m/z calcd for $C_{17}H_{15}O_5F_2$ (M + 1) 337.0888, obsd 337.0884.

Pharmacology

Materials. Male Wistar rats were obtained from Japan SLC Inc. RBL-1 cell was obtained from Dainippon Pharmaceutical Co., Ltd. 5-HETE was obtained from Sigma Chemical Co., Ltd. ADP and β-NADPH were obtained from Wako Pure Chemical Industries, Ltd. Arachidonic acid was obtained from Nacalai Tesque, Inc. HCC panel screening were performed by National Cancer Institute, National Institutes of Health, USA and Cancer Chemotherapy Center, Screening Committee of New Anticancer Agents supported by Grant-in-Aid for Scientific Research on Priority Area 'Cancer', from The Ministry of Education, Science, Sports and Culture, Japan.

Measurement of Fe^{3+}–ADP induced NADPH dependent lipid peroxidation in rat liver microsome.[17] The modified method of Kiso et al.[18] was used. Microsomes were prepared from male Wistar rats weighing about 200 g. The rat liver was homogenized in cold 0.25 M sucrose. The homogenate was centrifuged at 4 °C (8000×g, 10 min). The supernatant fraction was then collected and ultracentrifuged at 4 °C (105,000×g, 30 min). The pellet obtained was resuspended in 83.5 mM KCl–37.2 mM Tris–HCl buffer (pH 7.4) and stocked at −20 °C until use. Protein concentration was determined by the method of Lowry et al. [19] The assay system (1 mL) consisted of 83.5 mM KCl–37.2 mM Tris–HCl buffer (pH 7.4), the test compound in 1% DMSO, 0.2 mM NADPH, 1 mM ADP, 1 mg protein/mL rat liver microsomes and 10 μM $FeCl_3$. The reaction mixture was incubated at 37 °C for 20 min, and then cooled on ice to terminate the reaction. Lipid peroxide was measured by the method of Ohkawa et al. [20] Thus, 8.1% sodium dodecyl sulfate (0.2 mL), 20% AcOH containing 0.27 M HCl adjusted to pH 3.5 with NaOH (1.5 mL) and 0.8% TBA (1.5 mL) were added to the reaction mixture. The mixture was then boiled at 100 °C for 20 min and the reaction was stopped by cooling on ice. Thereafter, n-BuOH–pyridine (15:1, 4 mL) was added, and vigorous mixing was performed. After centrifugation (780×g, 10 min), the organic layer was separated, and the absorbance was measured at 532 nm. The amount of TBA-positive material was expressed as a corresponding amount of malondialdehyde.

Measurement of RBL-1 cell 5-lipoxygenase activity.[7] The modified method of Blackham et al. [21] was used. RBL-1 cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% heat-inactivated newborn calf serum, 100 units/mL penicillin and 0.1 mg/mL streptomycin. Cells were cultured at 37 °C in 5% CO_2/air. Cells in the growth phase (5×10^5 to 10^6 cells/mL) were collected by centrifugation (100×g, 5 min) and suspended at 3×10^7 cells/mL in 50 mM phosphate buffer (0.25 M sucrose, 1 mM EDTA, 2 mM glutathione, pH 7.4). Cells were stored at −80 °C until use. The assay system (0.5 mL) consisted of 50 mM phosphate buffer (0.25 M sucrose, 1mM EDTA, 2 mM glutathione, pH 7.4), the test compound in 1% DMSO, 2 mM $CaCl_2$, 0.2 mg/mL arachidonic acid (10 mg/mL MeOH soln; 10 μL) and 10^7 cells/mL RBL-1 cells homogenate. Reaction mixture was incubated at 37 °C for 3 min, and then 0.5 mL of MeOH was added to terminate the reaction. The mixture was centrifuged (2000×g, 15 min), 5-HETE in the supernatant was

analyzed by HPLC (column; COSMOSIL 5C18-MS Waters 4.6×150 mm (Nacalai Tesque, Inc.), mobile phase; CH_3CN–0.1% AcOH aq (6:4), flow rate; 1 mL/min, temperature; rt, absorbance; 235 nm).

Evaluation of anticarcinogenic action using HCC.[10,11] Each of the test compounds was added to a microtiter plate 24 h after cancer cells were enclosed into it. After 48 h incubation, the amount of the cells was colorimetrically determined using sulforhodamine B as the reagent[12] and the percentage of cell proliferation was calculated against the control. An HCC panel consisting of 39 cell lines was employed for screening tests, in which GI_{50}, TGI, and LC_{50} were evaluated at 10^{-4}–10^{-8} M compound concentrations for each cell line, the mean value for each index over the cell lines was calculated, the relative sensitivity of each cell line to each test compound was estimated using the mean index values obtained, and comparisons were made between each test compound and the conventional anticancer agents through analyses making use of the compare program.[13, 14]

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