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Quantitative Structure-cytotoxicity Relationship of 3-Benzylidenechromanones

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Abstract. *Aim: Sixteen 3-benzylidenechromanones were subjected to quantitative structure–activity relationship (QSAR) analysis based on their cytotoxicity and tumor-specificity, in order to examine their new biological activities. Materials and Methods: Cytotoxicity against two human oral squamous cell carcinoma cell lines, two mesenchymal and two epithelial normal oral cells, was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Tumor-specificity (TS) was evaluated by the ratio of the mean CC_{50} (50% cytotoxic concentration) against normal cells to that against tumor cell lines. Physicochemical, structural and quantum-chemical parameters were calculated based on the conformations optimized by the LowModeMD method. Results: 3-Benzylidenechromanone derivatives that have a methoxy group at 7-position of the chromanone ring and hydroxyl or methoxy group at 4'-position of benzene ring showed relatively higher TS values, exceeding those of doxorubicin (DXR) and 5-fluorouracil (5-FU). Since these anticancer drugs were highly cytotoxic to normal keratinocytes, QSAR analysis was performed with oral carcinoma and mesenchymal normal cells. Tumor-specificity was well correlated with 3D-MoRSE descriptors (that relate to three dimensional shapes) and Edge adjacency indices (that relate to two dimensional shapes and polarization). Introduction of hydroxyl group at 3'-position of benzene ring significantly elevated the tumor-specificity. Conclusion: Molecular shape, size and polarization are useful markers for the evaluation of tumor-specificity of 3-benzylidenechromanone derivatives.*

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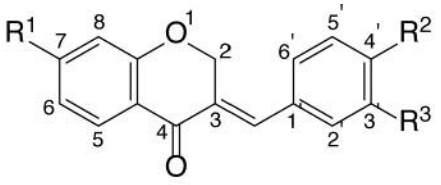
Key Words: 3-benzylidenechromanones, QSAR analysis, cytotoxicity, tumor selectivity, oral carcinoma, mesenchymal and epithelial normal oral cells.

(3*E*)-3-Benzylidene-2,3-dihydro-4*H*-1-benzopyran-4-ones (3-benzylidene-4-chromanones) constitute a small class of natural products primarily isolated from *Hyacinthaceae* and *Caesalpinioideae* (1-5). Several natural and synthetic 3-benzylidene-4-chromanones are related structurally to flavonoids and found to possess various biological properties, such as antioxidant, antifungal, antiviral, anti-mutagenic, anti-proliferative, anti-allergic, antihistaminic, anti-inflammatory and monoamine oxidase inhibitory activity (6-17). However, no systematically evaluated data are available on the cytotoxic activity of 3-benzylidene-4-chromanone derivatives against both malignant and non-malignant cells.

In order to further explore novel biological activities of 3-benzylidenechromanones, we recently synthesized a series of seventeen derivatives (Figure 1). In the present study, we first investigated their cytotoxicity against human oral squamous cell carcinoma (OSCC) cell lines (Ca9-22, HSC-2), human normal oral cells derived from mesenchymal tissue (gingival fibroblast, HGF; pulp cells, HPC) and epithelial tissue (oral keratinocyte, HOK; primary gingival epithelial cells, HGEP). Tumor-specificity index (TS) was calculated by dividing the mean 50% cytotoxic concentration (CC_{50}) against normal oral cells by that against OSCC cell lines. We have previously confirmed that the TS value thus determined reflects the anti-tumor potential of test samples, although normal and tumor cells are derived from different tissues (mesenchymal or epithelial) (18). We next performed the quantitative structure–activity relationship (QSAR) analysis, using the cytotoxicity data obtained with OSCC cells (Ca9-22, HSC-2) and normal oral mesenchymal cells (HGF, HPC).

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) from GIBCO BRL, Grand Island, NY, USA; fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and doxorubicin (DXR) from Sigma-Aldrich Inc., St. Louis, MO, USA; dimethyl sulfoxide (DMSO) from Wako Pure



Compound	R^1	R^2	R^3
1	H	H	H
2	H	OH	H
3	H	OH	OH
4	H	OMe	H
5	H	OMe	OMe
6	H	NMe_2	H
7	H	F	H
8	H	Cl	H
9	OH	OH	H
10	OH	OH	OH
11	OH	OMe	H
12	OH	NMe_2	H
13	OMe	H	H
14	OMe	OH	H
15	OMe	OH	OH
16	OMe	OMe	H
17	OMe	NMe_2	H

Figure 1. *Structure of seventeen 3-benzylidenechromanones.*

Chem. Ind., Osaka, Japan; 5-fluorouracil (5-FU) from Kyowa, Tokyo, Japan. Culture plastic dishes and plates (96-well) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

Synthesis of test compounds. (3*E*)-2,3-Dihydro-3-(phenylmethylene)-4*H*-1-benzopyran-4-one [1], (3*E*)-2,3-dihydro-3-[(4-hydroxyphenyl) methylene]-4*H*-1-benzopyran-4-one [**2**], (3*E*)-2,3-dihydro-3-[(3,4-dihydroxyphenyl)methylene]-4*H*-1-benzopyran-4-one [**3**], (3*E*)-2,3-dihydro-3-[(4-methoxyphenyl)methylene]-4*H*-1-benzopyran-4-one [**4**], (3*E*)-2,3-dihydro-3-[(3,4-dimethoxyphenyl)methylene]-4*H*-1-benzopyran-4-one [**5**], (3*E*)-2,3-dihydro-3-[(4-dimethylaminophenyl)methylene]-4*H*-1-benzopyran-4-one [**6**], (3*E*)-2,3-dihydro-3-[(4-fluorophenyl)methylene]-4*H*-1-benzopyran-4-one [**7**], (3*E*)-3-[(4-chlorophenyl)methylene]-2,3-dihydro-4*H*-1-benzopyran-4-one [**8**], (3*E*)-2,3-dihydro-7-hydroxy-3-[(4-hydroxyphenyl)methylene]-4*H*-1-benzopyran-4-one [**9**], (3*E*)-2,3-dihydro-3-[(3,4-dihydroxyphenyl)methylene]-7-hydroxy-4*H*-1-benzopyran-4-one [**10**], (3*E*)-2,3-dihydro-7-hydroxy-3-[(4-methoxyphenyl)methylene]-4*H*-1-benzopyran-4-one [**11**], (3*E*)-2,3-dihydro-3-[(4-dimethylaminophenyl)methylene]-7-hydroxy-4*H*-1-benzopyran-4-one [**12**], (3*E*)-2,3-dihydro-7-methoxy-3-(phenylmethylene)-4*H*-1-benzopyran-4-one [**13**], (3*E*)-2,3-dihydro-3-[(4-hydroxyphenyl)methylene]-7-methoxy-4*H*-1-benzopyran-4-one [**14**], (3*E*)-2,3-dihydro-3-[(3,4-dihydroxyphenyl)methylene]-7-methoxy-4*H*-1-benzopyran-4-one [**15**], (3*E*)-2,3-dihydro-7-methoxy-3-[(4-methoxyphenyl)methylene]-4H-1-benzopyran-4-one [**16**] and (3*E*)-2,3-dihydro-3-[(4-dimethylaminophenyl)methylene]-7-methoxy-4*H*-1-benzopyran-4-one [**17**] (structures shown in Figure 1) were synthesized by base-catalyzed condensation of appropriate 4-chromanone with substituted benzaldehyde derivatives according to previous methods (19, 20). All compounds were dissolved in DMSO at 40 mM and stored at –20˚C before use.

Cell culture. HGF, HPLF and HPC cells, established from the first premolar tooth extracted from the lower jaw of a 12-year-old girl (21), as well as OSCC cell lines (Ca9-22, HSC-2, HSC-3, HSC-4), purchased from Riken Cell Bank, Tsukuba, Japan, were cultured at 37˚C in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml, penicillin G and 100 μg/ml streptomycin sulfate under a humidified 5% CO_2 atmosphere. HOK cells (purchased from COSMO BIO Co/ Ltd., Tokyo, Japan) were cultured in keratinocyte growth supplement (OKGS, Cat, No. 2652). HGEP cells (purchased from CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) were grown in CnT-PR medium. Cells were then harvested by treatment with 0.25% trypsin-0.025% EDTA-2Na in phosphate buffer without magnesium and calcium ions and either sub-cultured or used for experiments.

Assay for cytotoxic activity. Cells were inoculated at 2.5×10^3 cells/0.1 ml in a 96-microwell plate (Becton Dickinson Labware). After 48 h, the medium was removed by suction with an aspirator and replaced with 0.1 ml of fresh medium containing different concentrations of single test compounds. Control cells were treated with the same amounts of DMSO present in each diluent solution (0.0078, 0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1%) and the cell damage induced by DMSO was subtracted. Cells were incubated for 48 h and the relative viable cell number was then determined by the MTT method. In brief, the treated cells were incubated for another three hours in fresh culture medium containing 0.2 mg/ml MTT. Cells were then lysed with 0.1 ml of DMSO and the absorbance at 562 nm of the cell lysate was determined using a microplate reader (Sunrise Rainbow RC-R; TECAN, Männedorf, Switzerland). CC_{50} was determined from the dose–response curve and the mean value of CC_{50} for each cell type was calculated from triplicate assays.

Calculation of TS. The tumor-selectivity index (TS) was calculated by the following equation: TS=mean CC_{50} against normal cells/mean CC_{50} against tumor cells, that is, D/B [HGF+HPC (normal mesenchymal) (D) *vs*. Ca9-22+HSC-2 (tumor epithelial) (B)], C/A [HGF (C) *vs*. Ca9-22 cells (A), derived from gingival tissue (22)] and E/B [HOK+HGEP (normal epithelial)(E) *vs*. Ca9-22+HSC-2 (tumor epithelial)(B)] (Table II). A, B, C, D and E are indicated in Table I.

Table I. *Cytotoxic activity of seventeen 3-benzylidenechromanones against human oral malignant and non-malignant cells. Each value represents the mean±S.D. of triplicate determinations.*

	CC_{50} (μM)														
	Human oral squamous cell carcioma cell line							Human oral normal cells							
								Epithelial			Mesenchymal				
	(A) Ca9-22	SD	HSC-2	SD	(B) mean	(C) HGF	SD	HPC	SD	(D) mean	HOK	SD	HGEP	SD	(E) mean
1	7.6	3.2	11.2	0.3	9.4	75.7	14.0	77.0	11.5	76.3					
2	24.2	1.2	32.3	2.1	28.3	344.7	48.3	>400	0.0	372.3					
3	9.9	1.5	17.3	4.0	13.6	210.7	164.3	>400	0.0	>305.3					
4	8.8	0.4	21.3	1.5	15.1	70.7	6.4	190.3	181.6	130.5					
5	7.7	1.7	8.0	1.1	7.8	70.7	7.6	69.0	35.5	69.8					
6	18.4	3.2	21.0	3.6	19.7	>400	0.0	>400	0.0	>400	41.7	3.5	44.7	25.9	43.2
7	7.6	0.3	9.0	1.3	8.3	140.0	25.5	107.3	6.8	123.7					
8	10.2	0.1	17.7	3.5	14.0	>400	0.0	383.7	15.2	>391.8	80.8	1.0	18.0	2.0	49.4
9	24.7	0.6	29.7	2.5	27.2	205.3	75.8	192.7	14.5	199.0					
10	16.5	5.3	16.6	6.0	16.5	>400	0.0	>400	0.0	>400	16.3	2.5	20.3	7.5	18.3
11	23.7	2.3	27.3	5.8	25.5	138.7	49.7	207.3	32.8	173.0					
12	5.1	3.5	4.5	3.5	4.8	33.0	6.6	306.7	161.7	169.8					
13	7.6	3.4	13.7	3.1	10.7	85.3	6.4	92.0	28.8	88.7					
14	24.2	0.4	27.0	3.6	25.6	143.3	78.5	172.7	38.5	158.0					
15	3.2	0.5	11.3	0.6	7.3	>400	0.0	>400	0.0	>400	3.8	1.3	3.3	0.3	3.6
16	7.2	2.1	16.0	2.6	11.6	>400	0.0	116.0	23.4	>258.0	23.9	1.1	25.0	13.1	24.5
17	254.7	186.0	>400	0.0	>327	>400	0.0	>400	0.0	>400					
DXR	0.40	0.08	0.21	0.03	0.31	>10	0.00	>10	0.00	>10	0.27	0.15	0.51	0.08	0.39
5-FU	76.3	31.9	>1000	0.0	>538	>1000	0.0	>1000	0.0	>1000	44.7	25.1	14.5	2.2	29.6

HGF, human gingival fibroblast; HPC, human pulp cells; HPLF, human periodontal ligament fibroblast; Ca9-22 (derived from gingival tissue), HSC-2, HSC-3 and HSC-4 (derived from tongue), oral squamous cell carcinoma cell lines; CC_{50}, 50% cytotoxic concentration; DXR, doxorubicin; 5-FU, 5-fluorouracil.

Calculation of PSE. Potency-selectivity expression (PSE) was calculated by the following equation: PSE=TS /meanCC_{50} against tumor cells (B) ×100 (23), that is, (D/B^2) ×100 (HGF+HPC *vs*. Ca9-22+HSC-2) and (E/B^2) ×100 (HOK+HGEP *vs*. Ca9-22+HSC-2) (Table II).

Estimation of CC_{50} values. Original data contain the sign of inequality, such as ">". For the convenience of analysis, these values were changed into forms suitable for arithmetic calculation. Since ">400" is equal to "from 400 to ∞", we calculated the harmonic mean as follows: 1/[average(1/400,1/∞)]=800. Since the CC_{50} values had a distribution pattern close to a logarithmic normal distribution, we used the pCC_{50} (*i.e*. the −log CC_{50}) for the comparison of the cytotoxicity between the compounds. The mean pCC_{50} values for normal cells and tumor cell lines were defined as N and T, respectively (24).

Calculation of chemical descriptors. Each chemical structure was optimized by the LowModeMD method (25), a suitable search method for minimum energy conformers of flexible molecules, with Merck Molecular Force Field (MMFF94x) in Molecular Operating Environment (MOE) version 2014.09 (Chemical Computing Group Inc., Quebec, Canada). We used the software MOE (341 descriptors), Dragon 7.0 (Kode srl., Pisa, Italy) (2783 descriptors) and 10 descriptors (that relate the location of substituent groups and types) to calculate descriptors. The significant descriptors used were:

(a) RDF095i (Radial Distribution Function - 095/weighted by ionization potential), RDF095u (Radial Distribution Function - 095/unweighted) and RDF095e (Radial Distribution Function - 095/weighted by Sanderson electronegativity) (26);

(b) Vsurf_IW6 (Hydrophilic interaction-energy moment 6), Vsurf_ID7 (Hydrophobic interaction-energy moment 7), Vsurf_ID1 (Hydrophobic interaction-energy moment 1) and Vsurf_HB7 (H-bond donor capacity 7) that are similar to the VolSurf descriptors (27) and depend on the structure connectivity and conformation;

(c) Mor03v (signal 03/weighted by van der Waals volume), Mor03m (signal 03/weighted by mass), Mor09m (signal 09/weighted by mass), Mor03p (signal 03/weighted by polarizability) and Mor25v (signal 25/weighted by van der Waals volume) that are 3D-MoRSE (3D-Molecule Representation of Structures based on Electron diffraction) descriptors (28);

(d) G1u (1st component symmetry directional WHIM index/unweighted encoding molecular symmetry that extracts the global symmetry information) (29);

(e) R3m+ (R maximal autocorrelation of lag 3/weighted by mass) that reflects three-dimensional structure and molecular volume (30); and

(f) SpMAD_AEA(dm) (Spectral mean absolute deviation from augmented edge adjacency matrix weighted by dipole moment edge adjacency indices) (31).

Table II. *Tumor-specificity and PSE values of seventeen 3-benzylidenechromanones and anticancer drugs, determined with the data of Table I. Each value represents the mean of triplicate determinations.*

	TS			PSE	
Normal cells:	Epi	Epi	Epi	Epi	
	vs.	*vs.*	*vs.*	*vs.*	
Tumor cells:	Mes	Epi	Mes	Epi	
	(D/B)	(C/A)	(E/B)	$(D/B^2)\times100$	$(E/B^2)\times100$
1	8.1	9.9		86	
2	13.2	14.3		47	
3	>22.4	21.2		>164	
4	8.7	8.1		58	
5	8.9	9.2		114	
6	>20.3	>21.7	2.2	>103	11
7	14.9	18.4		180	
8	>28.1	>39.1	3.5	>201	25
9	7.3	8.3		27	
10	>24.2	>24.2	1.1	>146	6.7
11	6.8	5.9		27	
12	35.4	6.4		737	
13	8.3	11.2		78	
14	6.2	5.9		24	
15	>55.2	>126.3	0.5	>761	6.8
16	>22.2	>55.6	2.1	>191	18
17	1.2	1.6		<0.37	
DXR	>32.8	>24.8	1.3	>10754	421
5-FU	><1.9	>13.1	<0.06	<0.35	<0.011

Epi, epithelial; Mes, mesenchymal; TS, Tumor selectivity index; PSE, potency-selectivity expression; CC_{50}, 50% cytotoxic concentration; DXR, doxorubicin; 5-FU, 5-fluorouracil; A, B, C, D, E are explained in Table I.

Statistical treatment. The relation among cytotoxicity, tumor specificity index, anti-UV activity and chemical descriptors was investigated using simple regression analyses by JMP Pro version 12.2.0 (SAS Institute Inc., Cary, NC, USA). The significance level was set at $p<0.05$.

Results

Cytotoxicity. A total of seventeen 3-benzylidenechromanones derivatives (Figure 1) generally showed higher cytotoxicity against human oral squamous cell lines (Ca9-22 and HSC-2) (CC_{50}=4.8~327 μM, mean=33.7 μM) than against human mesenchymal normal oral cells (HGF and HPC) (CC_{50}=69.8~400 μM, mean=242 μM) with an averaged tumor-specificity index (TS) of 7.2 (=242/34) (Table I). Among them, compounds [**3, 6, 8, 10, 12, 15, 16**] showed much higher TS (20.3~55.2), comparable with that of the anticancer drug DXR (32.8). When tumor-selectivity was calculated using cells both derived from the gingival tissue (Ca9-22 *vs*. HGF), compounds [**6, 8, 10, 15, 16**] showed much higher TS (21.7~126.3), exceeding that of DXR (24.8) and 5-FU (13.1). When tumor-selectivity was calculated using cells both derived from the epithelial tissues, compounds [**6, 8, 16**] showed much reduced tumor-selectivity (2.1~3.5), while DXR and 5-FU showed little or no tumor-specificity (0.06~1.3) due to their cytotoxicity against human keratinocytes (Table II).

In order to identify compounds, which have both good potencies and are selectively toxic to neoplasms, the potency-selectivity expression (PSE) values of the compounds were calculated by the following equation: PSE= $(TS/CC_{50})\times100$ (23). When mesenchymal (HGF and HPC) *vs*. Ca9-22+HSC-2 were used, DXR showed the highest PSE value (10754), followed by 3-benzylidenechromanones (0.37~761, mean=173, two orders lower than DXR) and then 5-FU (0.35) (Table II). When epithelial (HOK and HGEP) *vs*. Ca9-22+HSC-2 were used, DXR again showed the highest PSE value (421), followed by compounds [**6, 8, 10, 15, 16**] (6.7~25, mean=14) and then 5-FU (0.011) (Table II).

Computational analysis. Since compound [**17**] showed very week tumor-specificity (1.2) and PSE (0.37) (Table II), we performed QSAR analysis with compounds [**1~16**]. More than 3,000 kinds of feature values with or without each functional group were calculated, based on chemical structures.

Cytotoxicity of 3-benzylidenechromanones against human OSCC cell lines (Ca9-22, HSC-2) was correlated with RDF095 (Radial Distribution Function – 095)i (ionization potential) (r^2=0.497, p=0.0023), RDF095u (unweighted) (r^2=0.442, p=0.0049), RDF095e (Sanderson electronegativity) (r^2=0.442, p=0.0049), Hydrophilic integy moment, such as Vsurf_IW6 (r^2=0.401, p=0.0085), Vsurf_ID7 (r^2=0.381, p=0.0108) and Vsurf_ID1 (r2=0.352, p=0.0154) (Figure 2).

Cytotoxicity of 3-benzylidenechromanones against mesenchymal normal cells (HGF, HPC) was correlated with Mor03v (van der Waals volume) (r^2=0.505, p=0.002), Mor03m (mass) (r^2=0.477, p=0.003), Mor09m (mass) (r^2=0.465, p=0.0036), G1u (1st component symmetry directional WHIM index/unweighted) (r^2=0.410, p=0.0075), Mor03p (polarizability) (r^2=0.408, p=0.0077) and R3m+ (mass) (r^2=0.398, p=0.0088) (Figure 3).

Tumor-specificity of 3-benzylidenechromanones was correlated with Mor03m (mass) (r^2=0.607, p=0.0004), Mor03v (van der Waals volume) (r^2=0.560, p=0.0009), SpMAD_AEA (dm) (dipole moment edge adjacency indices) (r^2=0.537, p=0.0012), Vsurf_HB7 (H-bond donor capacity) (r^2=0.502, p=0.0021), R3m+ (mass) (r^2=0.497, p=0.0023) and Mor25v (van der Waals volume) (r^2=0.488, p=0.0026) (Figure 4).

Introduction of hydroxyl group at 3'-position of benzene ring was correlated more significantly with tumor-specificity (p=0.0051), as compared to cytotoxicity against normal cells (p=0.0217) or tumor cells (p=0.547) (Figure 5).

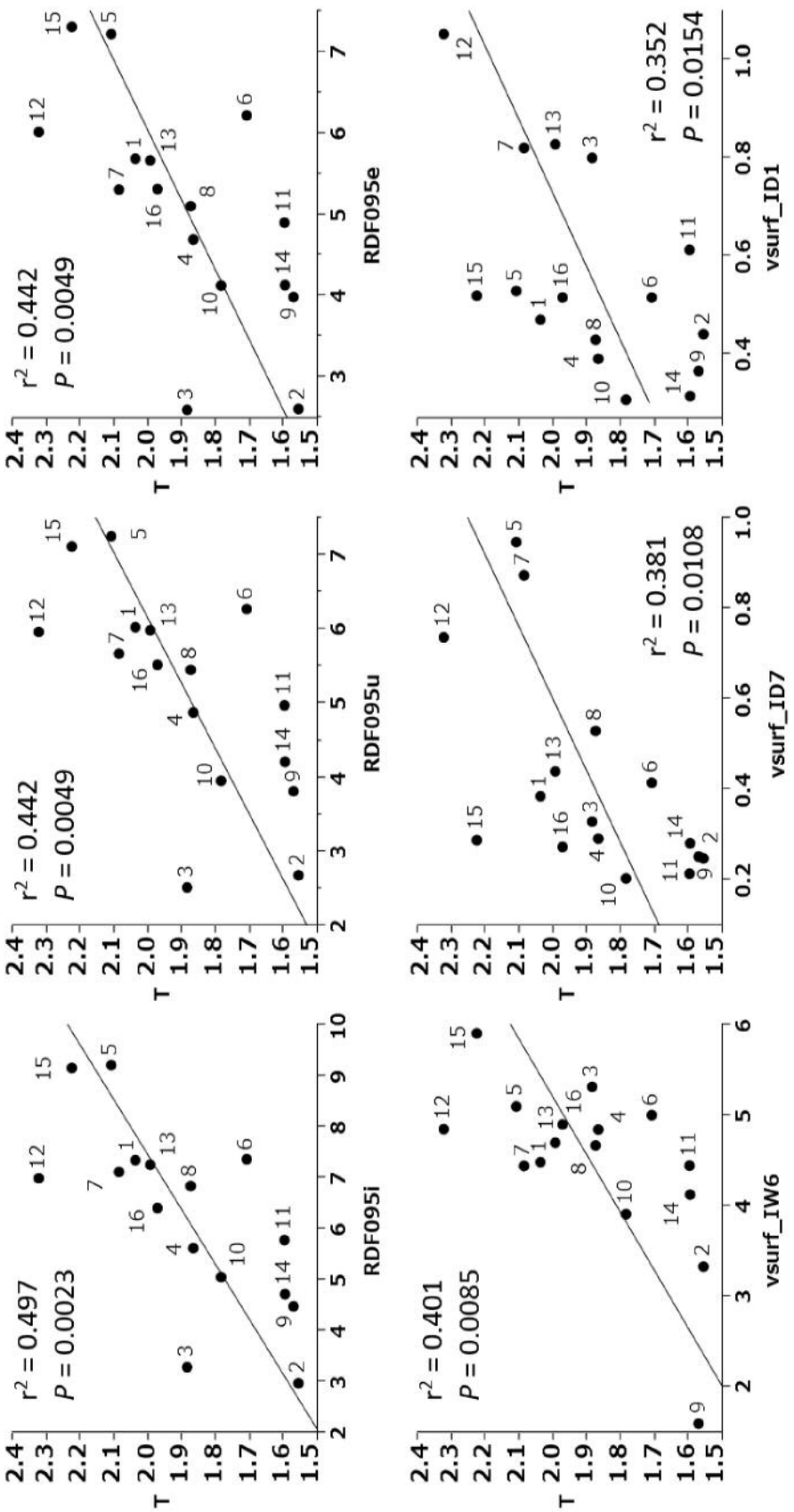


Figure 2. *Determination of coefficient between chemical descriptors and cytotoxicity of 3-benzylidenechromanones against tumor cells (defined as T). The mean (pCC_{50}, i.e. the $-log\ CC_{50}$) values for tumor cell lines were defined as T.*

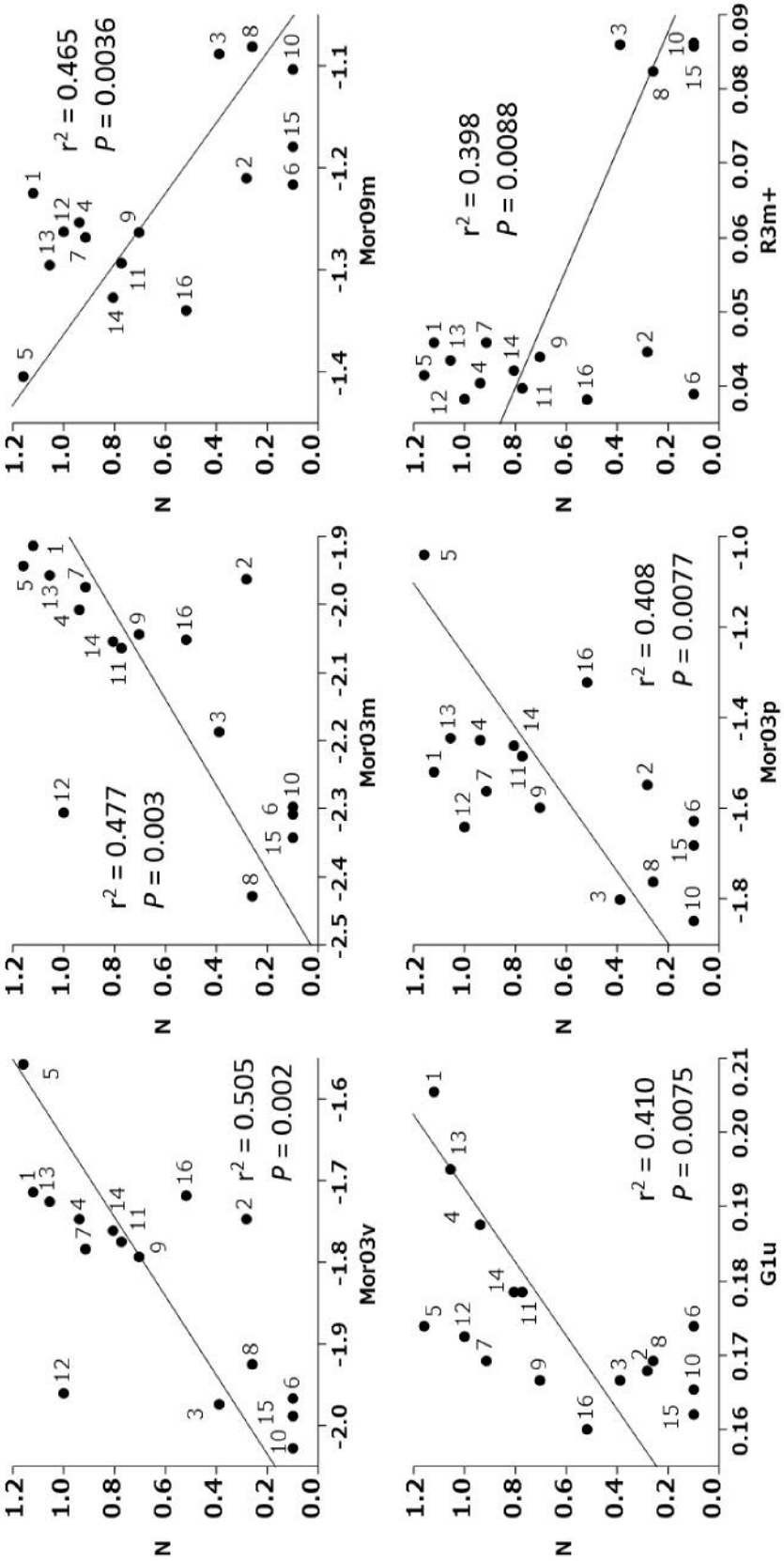


Figure 3. *Determination of coefficient between chemical descriptors and cytotoxicity of 3-benzylidenechromanones against normal cells (defined as N). The mean (pCC_{50}, i.e. the $-log\ CC_{50}$) values for normal cells were defined as N.*

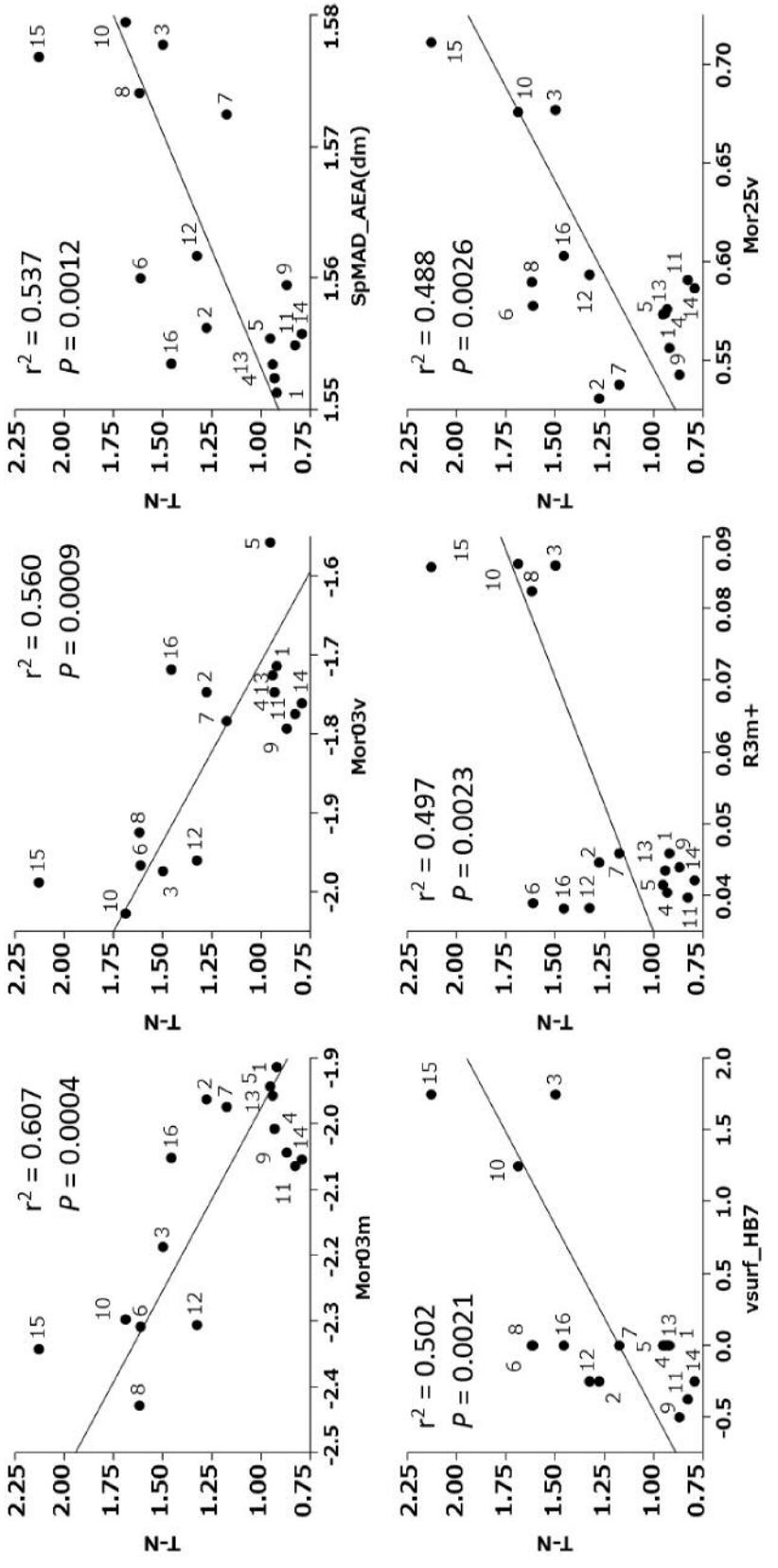


Figure 4. *Determination of coefficient between chemical descriptors and tumor specificity of 3-benzylidenechromanones (defined as T–N).*

Discussion

The present study demonstrated that compounds [**3, 10, 15**] with hydroxyl group in the 3'-position of the benzene ring significantly contribute to the tumor-specificity (p=0.0051). Compound [**10**], also known as sappanone A, has been recently reported to exert its anti-inflammatory activity by activating nuclear factor erythroid 2-related factor (Nrf2)/ heme oxygenase-1 (HO-1) pathway and suppressing the nuclear factor-κB (NF-κB) activation (32). Compound [**16**] showed antibacterial and antifungal activities (33). Compounds [**9, 11**] inhibited the efflux of antimicrobials, such as ethidium bromide from the *Mycobacterium smegmatis* mc^2 155, suggesting their possible candidacy as anti- tuberculosis drugs by reducing drug-resistance (10). On the other hand, the anti-cancer study of homoisoflavonoids has been limited, possibly due to the absence of appropriate assay method for tumor-selectivity. We have recently explored a new method by which tumor-selectivity can be estimated (34). Although this system uses two different types of cells, human oral normal mesenchymal cells with human OSCC cell lines, the TS value obtained yielded the clinically expected values, judging from the evidence that most of the anticancer drugs and herbs produced extremely higher TS values (18, 34). The use of human oral keratinocytes led to our unexpected finding that OSCC cell lines were very much sensitive to DXR and 5-FU, confirming our recent papers (35, 36). This may be a new type of side-effect induced by anticancer drugs. We found that compounds [**6, 8, 16**] showed much lower cytotoxicity against human normal keratinocytes as compared with DXR and 5-FU (Table II). It is urgent to explore the method by which this type of side-effect can be alleviated.

We also found that the introduction of dimethylamino groups [**6, 12**] and chloride [**8**] in the 4'-position of benzene ring produced comparable TS values with [**3, 10, 15**]. This indicates that molecular shape, size and polarization are useful for the evaluation of tumor-specificity of 3-benzylidenechromanone derivatives.

Conflicts of Interest

The Authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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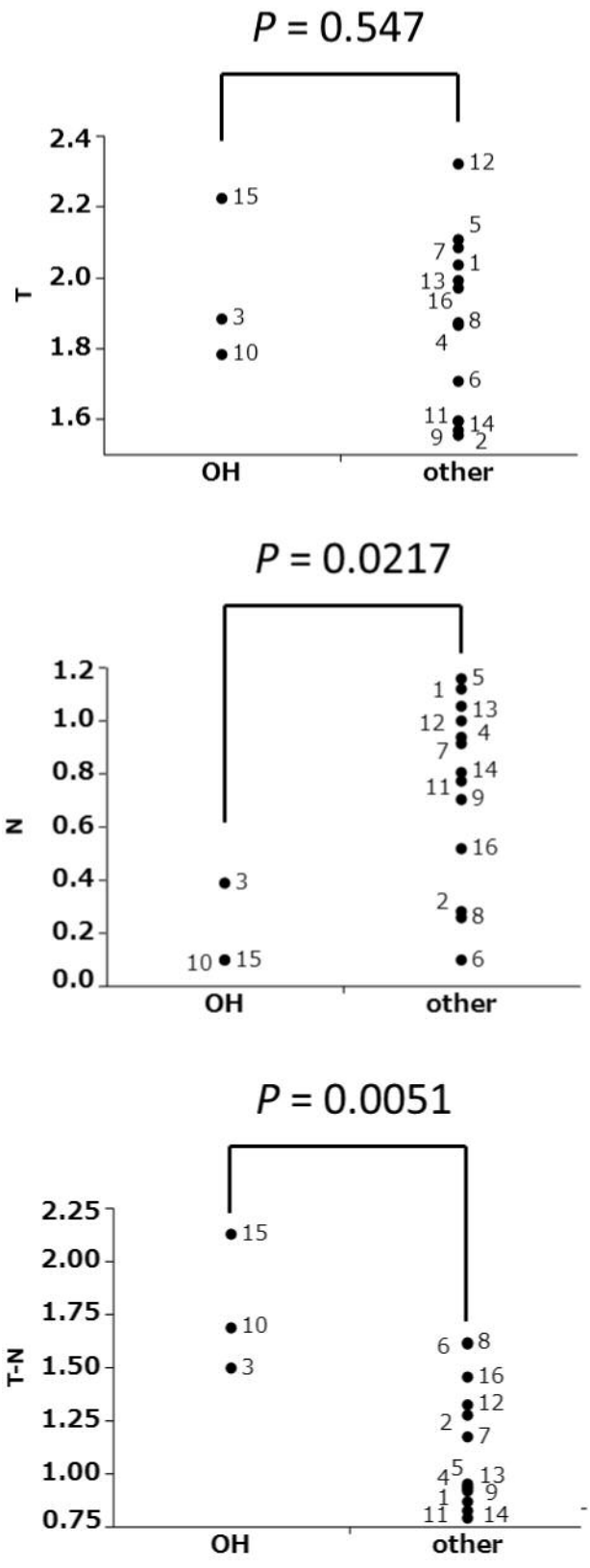

Figure 5. *Effect of introduction of hydroxyl group on 3'-position of benzene ring on T, N and N-T.*

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