

Quantitative Structure–Cytotoxicity Relationship of Newly Synthesized Piperic Acid Esters

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Abstract. *Background/Aim:* Eleven piperic acid esters were subjected to quantitative structure–activity relationship (QSAR) analysis based on their cytotoxicity and tumor-specificity, in order to find their new biological activities. *Materials and Methods:* Cytotoxicity against four human oral squamous cell carcinoma cell lines and three oral normal mesenchymal cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Tumor specificity (TS) was evaluated by the ratio of the mean 50% cytotoxic concentration (CC₅₀) against normal cells to that against tumor cell lines. Potency-selectivity expression (PSE) value was calculated by dividing the TS value by CC₅₀ against tumor cells. Apoptosis markers were detected by western blot analysis. Physicochemical, structural and quantum-chemical parameters were calculated based on the conformations optimized by force-field minimization. *Results:* One phenylmethyl ester and five phenylethyl esters showed relatively higher cytotoxicity and tumor specificity, that were significantly modified by introduction of hydroxyl and methoxy groups. On the other hand, phenylpropyl ester, phenylbutyl ester and decyl ester were essentially inactive.

(2E,4E)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid 2-(3,4-dihydroxyphenyl)ethyl ester [4] had the highest TS and PSE values. This compound also stimulated the cleavage of caspase-3, suggesting the induction of apoptosis. TS values were correlated with molecular size, ionization potential, molecular shape, ionization potential and electronegativity. None of the compounds had any anti-HIV activity. *Conclusion:* Chemical modification of the lead compound may be a potential choice for designing a new type of anticancer drugs.

Piperine {1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl] piperidine}, along with its isomer chavicine, is a pungent alkaloid contained in black pepper (1). Piperine has been reported to enhance the oral absorption and hence the bioavailability of accompanying compounds (2, 3) and some antibacterial activity (4, 5). In animal studies, piperine showed anti-depression/cognitive-enhancing (6), anti-inflammatory (7) and anti-angiogenic (8) activities.

Piperine induced apoptotic cell death in human colon cells (9), melanoma cells (10), and lung (11) and rectal carcinoma cell lines (12), whereas it induced autophagy in human prostate cancer cells (13). On the other hand, piperine protected normal cells from apoptosis induced by cadmium (14), cisplatin (15) and glutamate (16). However, as far as we know only one article has performed the rigorous antitumor investigation with appropriate normal cells from the same tissues or same type of cells (epithelial or mesenchymal) (11). Furthermore, there is no report about the antiviral activity of piperine. We have recently reported that among a total of 12 synthetic piperic acid amides, *N*-piperoyldopamine showed the highest tumor selectivity (17).

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In continuation of discovering new biological activities of piperine derivatives, a total of 11 synthetic piperic acid esters were investigated for their cytotoxicity and anti-HIV activity, and then subjected to quantitative structure–activity relationship (QSAR) analysis.

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), doxorubicin, azidothymidine, 2',3'-dideoxycytidine from Sigma-Aldrich Inc., St. Louis, MO, USA; dimethyl sulfoxide (DMSO), dextran sulfate (molecular mass, 5 kDa) from Wako Pure Chem. Ind., Osaka, Japan; methotrexate from Nacalai Tesque, Inc., Kyoto, Japan; curdlan sulfate (molecular mass, 79 kDa) from Ajinomoto Co. Ltd., Tokyo, Japan. Culture plastic dishes and plates (96-well) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

Synthesis of test compounds. (2*E*,4*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid (4-methoxyphenyl)methyl ester [1], (2*E*,4*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid (4-hydroxy-3-methoxyphenyl)methyl ester [2], (2*E*,4*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid 2-(4-hydroxyphenyl)ethyl ester [3], (2*E*,4*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid 2-(3,4-dihydroxyphenyl)ethyl ester [4], (2*E*,4*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid 2-(4-methoxyphenyl)ethyl ester [5], (2*E*,4*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid 2-(3,4-dimethoxyphenyl)ethyl ester [6], (2*E*,4*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid 2-phenylethyl ester [7], (2*E*,4*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid 3-phenylpropyl ester [8], (2*E*,4*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid 4-phenyl-2-butyl ester [9], (2*E*,4*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid 4-phenylbutyl ester [10] and (2*E*,4*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid decyl ester [11] (structures shown in Figure 1) were synthesized by coupling of piperic acid with the appropriate alcohols by means of a modified procedure described elsewhere (18). To a mixture of piperic acid (1.1 mmol) and the appropriate alcohol (1.0 mmol) in dry tetrahydrofuran (4 ml) were added triphenylphosphine (1.5 mmol) and diisopropyl azodicarboxylate (1.5 mmol). The reaction mixture was stirred for 2–24 h at room temperature and the whole was extracted with ethyl acetate and saturated NaHCO₃ solution. The organic layer was dried over Na₂SO₄ and the solvent was evaporated under reduced pressure. The residue was then purified by silica gel column chromatography to give the corresponding piperic acid ester. All compounds were dissolved in DMSO at 40 mM and stored at –20°C before use.

Cell culture. Human normal oral mesenchymal cells (human gingival fibroblast, HGF; human periodontal ligament fibroblast, HPLF; human pulp cells, HPC) were established from the first premolar tooth extracted from the lower jaw of a 12-year-old girl (19), and cells at 10–18 population doubling levels were used in this study. Human oral squamous cell carcinoma (OSCC) cell lines [Ca9-22 (derived from gingival tissue); HSC-2, HSC-3, HSC-4 (derived from tongue)] were purchased from Riken Cell Bank (Tsukuba, Japan). All of these cells were cultured at 37°C in DMEM

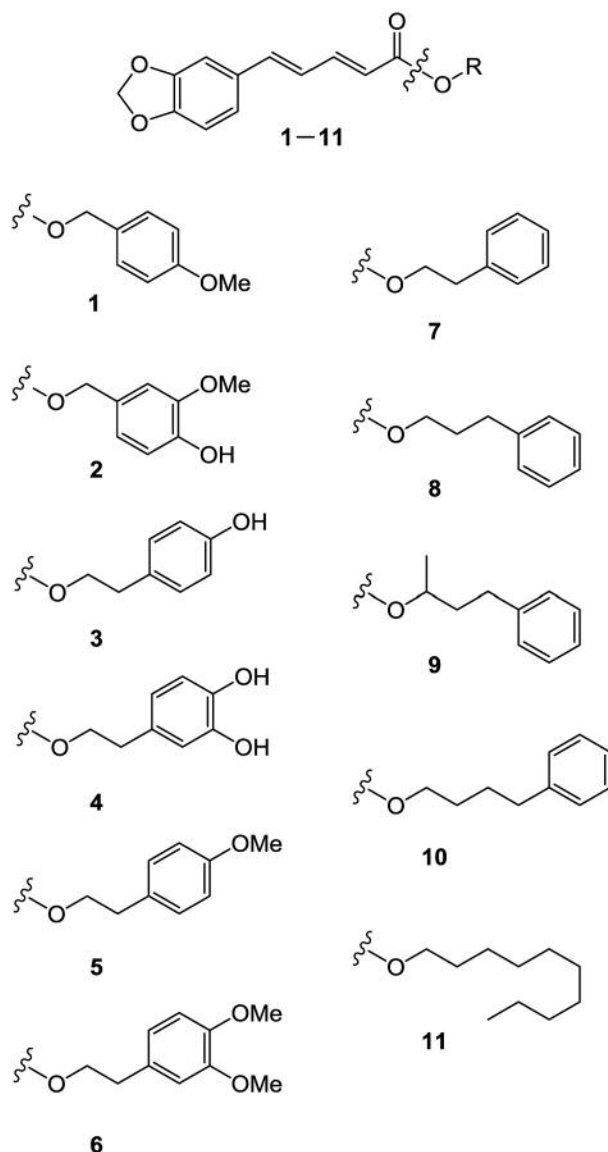


Figure 1. Structure of eleven piperic acid esters.

supplemented with 10% heat-inactivated FBS, 100 units/ml, penicillin G and 100 µg/ml streptomycin sulfate under a humidified 5% CO₂ atmosphere.

Assay for cytotoxic activity. Cells were inoculated at 2.5×10³ cells/0.1 ml in a 96-microwell plate. After 48 h, the medium was replaced with 0.1 ml of fresh medium containing different concentrations of single test compounds. Cells were incubated further for 48 h and the relative viable cell number was then determined by the MTT method (20). The relative viable cell number was determined by the absorbance of the cell lysate at 560 nm, using a microplate reader (Infinite F 50 R, TECAN, Kawasaki, Japan). Control cells were treated with the same amounts of DMSO

Table I. Cytotoxic activity of 11 piperic acid esters against oral malignant and non-malignant cells. Each value represents the mean of triplicate determinations.

	CC ₅₀ (μM)												TS		PSE	
	Human oral squamous cell carcinoma cell lines						Human normal oral cells									
	(A)		HSC-2	HSC-3	HSC-4	(B)		SD	(C)		(D)		(D/B)	(C/A)	(D/B ²) ×100	(C/A ²) ×100
Ca9-22		mean				HGF	HPLF		HPC	mean	SD					
1	26	44	40	41	38	8	>400	>400	>400	>400		>10.5	>15.1	>27.8	>57.2	
2	270	>400	>400	>400	>368	65	>400	>375	>393	>390	13	><1.1	>1.5	><0.3	>0.5	
3	45	67	42	30	46	16	105	139	121	121	17	2.6	2.3	5.7	5.2	
4	5	28	22	13	17	10	118	151	138	136	17	8.0	21.6	47.0	396.0	
5	29	48	49	43	42	9	59	81	68	70	11	1.6	2.0	3.9	6.8	
6	92	87	102	127	102	18	130	340	103	191	130	1.9	1.4	1.8	1.5	
7	60	75	41	68	61	15	>400	>400	>400	>400		>6.6	>6.6	>10.8	>11.0	
8	>400	>400	>400	>400	>400		>400	>400	>400	>400		><1.0	><1.0	><0.3	><0.3	
9	>400	>400	>400	>400	>400		>400	>400	>400	>400		><1.0	><1.0	><0.3	><0.3	
10	>400	>400	>400	>400	>400		>400	>400	>400	>400		><1.0	><1.0	><0.3	><0.3	
11	>400	>400	>400	>400	>400		>400	>400	>400	>400		><1.0	><1.0	><0.3	><0.3	
DXR	0.27	0.12	0.14	<0.078	<0.15	0.08	>10	1.1	8.8	6.6	4.8	>43.3	>37.3	>28322.4	>13922.9	
MTX	>1000	78	8	8	>273	486	>1000	>1000	>1000	>1000		><3.7	><1.0	><1.3	><0.1	

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 (OSCC) cell lines; CC₅₀: 50% cytotoxic concentration; DXR: doxorubicin; MTX: methotrexate. TS: tumor-selectivity index; PSE: potency-selectivity expression; CC₅₀: 50% cytotoxic concentration.

and the cell damage induced by DMSO was subtracted from that induced by test agents. The concentration of compound that reduced the viable cell number by 50% (CC₅₀) was determined from the dose–response curve and the mean value of CC₅₀ for each cell type was calculated from triplicate assays. Changes in cell morphology were observed under the light microscope (EVOSfl, ThermoFisher Scientific, Waltham, Mass, USA).

Calculation of tumor-selectivity index (TS). TS was calculated using the following equation: TS=mean CC₅₀ against three normal cells/mean CC₅₀ against for OSCC cell lines [(D/B) in Table I]. Since both Ca9-22 and HGF cells were derived from the gingival tissue (21), the relative sensitivity of these cells was also compared [(C/A) in Table I]. We did not use human normal oral keratinocytes as controls, since many anticancer drugs showed potent cytotoxicity against normal keratinocytes by inducing apoptosis (20).

Calculation of potency-selectivity expression (PSE). PSE was calculated using the following equation: PSE=TS/CC₅₀ against tumor cells ×100 (22) [that is, (D/B²) ×100 (HGF, HPLF, HPC vs. Ca9-22, HSC-2, HSC-3, HSC-4) and (C/A²) ×100 (HGF vs Ca9-22) in Table I].

Estimation of CC₅₀ values. Since the CC₅₀ values had a distribution pattern close to a logarithmic normal distribution, we used the pCC₅₀ (*i.e.*, the –log CC₅₀) for the comparison of the cytotoxicity between the compounds. The mean pCC₅₀ values for normal cells and tumor cell lines were defined as N and T, respectively (22).

Calculation of chemical descriptors. The 3D-structure of each chemical structure (drawn by Marvin Sketch ver 16, ChemAxon, Budapest, Hungary, <http://www.chemaxon.com>) was optimized by

Table II. Anti-HIV activity of piperic acid esters and chemotherapeutic agents. Each value represents the mean of triplicate determinations.

Piperic acid esters	CC ₅₀ (μM)	EC ₅₀ (μM)	Anti-HIV activity SI (=CC ₅₀ /EC ₅₀)
1	266	>400	<1
2	322	>400	<1
3	119	>400	<1
4	>400	>400	><1
5	>400	>400	><1
6	137	>400	<1
7	>400	>400	><1
8	390	>400	<1
9	>400	>400	><1
10	>400	>400	><1
11	>400	>400	><1
Positive controls			
Dextran sulfate (μg/ml)	233	0.777	300
Curdan sulfate (μg/ml)	>1000	0.172	>5805
Azidothymidine	53	0.026	2017
2',3'-Dideoxycytidine	1859	1.113	1670

CC₅₀: 50% Cytotoxic concentration; EC₅₀: 50% effective concentration; SI: selectivity index (CC₅₀/EC₅₀).

CORINA Classic (Molecular Networks GmbH, Germany) and force-field calculations (amber-10: EHT) in Molecular Operating Environment (MOE) version 2015.1001 (Chemical Computing Group Inc., Quebec, Canada). The number of structural descriptors

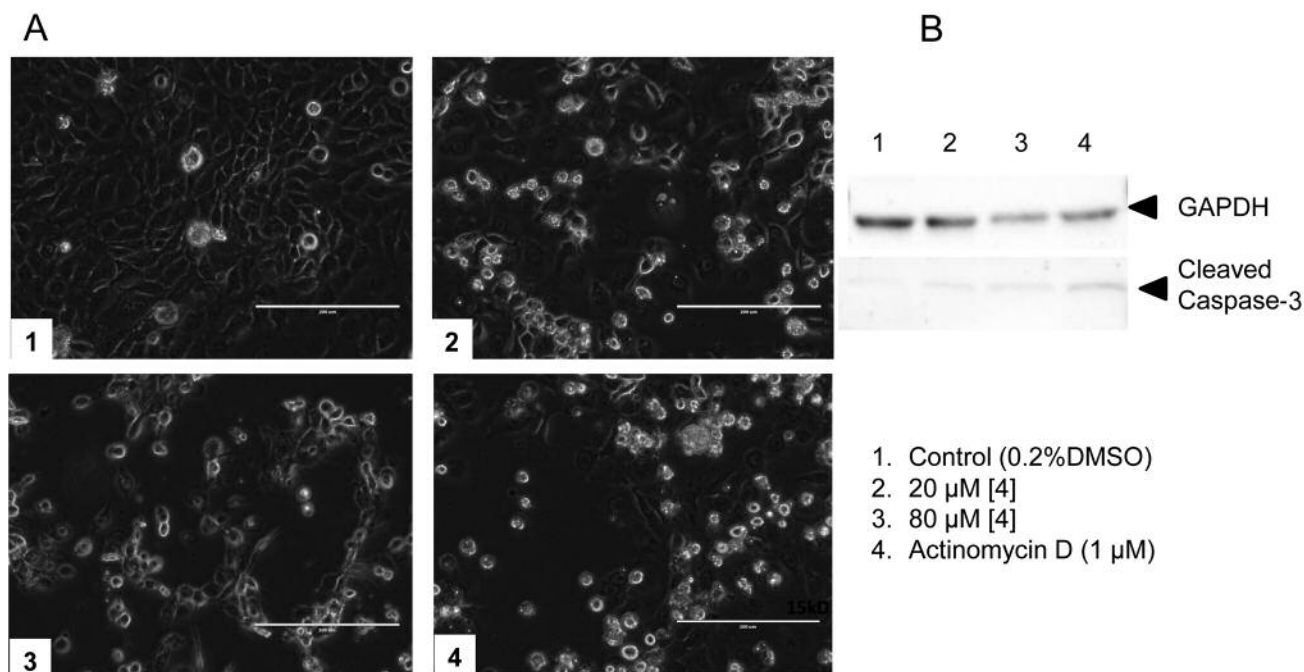


Figure 2. Apoptosis induction by [4] in HSC-2 human oral squamous cell carcinoma cell line. Bars in A, 200 μ m.

calculated from MOE and Dragon 7.0 (Kode srl., Pisa, Italy) after the elimination of overlapped descriptors were 302 and 2747, respectively.

The following 15 Dragon descriptors and 2 MOE descriptors were significantly correlated with T, N and T-N.

Dragon descriptors (23): CATS2D_04_AL (CATS2D Acceptor-Lipophilic at lag 04), CATS3D_13_AL (CATS3D Acceptor-Lipophilic BIN 13), CATS3D_16_AA (CATS3D Acceptor-Acceptor BIN 16), CATS3D_17_AA (CATS3D Acceptor-Acceptor BIN 17), Dp (D total accessibility index/weighted by polarizability), HATS5i (leverage-weighted autocorrelation of lag 5/weighted by ionization potential), JGI10 (mean topological charge index of order 10), MATS5v (Moran autocorrelation of lag 5 weighted by van der Waals volume), R2e+ (R maximal autocorrelation of lag 2/weighted by Sanderson electronegativity), R5i+ (R maximal autocorrelation of lag 5/weighted by ionization potential), R5u+ (R maximal autocorrelation of lag 5/unweighted), SPH (sphericity), TDB08e (3D Topological distance based descriptors - lag 8 weighted by Sanderson electronegativity), TDB08i (3D Topological distance based descriptors - lag 8 weighted by ionization potential), and TDB08u (3D Topological distance based descriptors - lag 8 unweighted).

MOE descriptors: rsynth (the synthetic reasonableness, or feasibility, of the chemical structure), and vsurf_DW13 (Contact distances of vsurf_Ewmin 13; Surface Area, Volume and Shape Descriptor. The vsurf_ descriptors are similar to the VolSurf descriptors (24).

Western blot analysis. The cells were washed with PBS and processed for western blot analysis, as described previously (22).

Antibodies against cleaved caspase-3 (Cell Signaling Technology Inc., Beverly, MP, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Trevigen, Gaithersburg, MD, USA) were used as primary antibodies. As secondary antibodies, we used α -rabbit IgG (DAKO, Tokyo, Japan) antibodies which were conjugated with horseradish peroxidase.

Assay for anti-HIV activity. HTLV-I-carrying human T-cell line MT-4 cells, highly sensitive to human immunodeficiency virus-1 (HIV-1), were infected with HIV-1_{IIIB} at a multiplicity of infection of 0.01. HIV- and mock-infected (control) MT-4 cells were incubated for 5 days with different concentrations of samples and the relative viable cell number was determined by the MTT assay. The CC₅₀ and 50% effective concentration (EC₅₀) were determined from the dose-response curve for mock-infected and HIV-infected cells, respectively (25). All data represent the mean values of triplicate measurements. The anti-HIV activity was evaluated by selectivity index (SI) (=CC₅₀/EC₅₀).

Statistical treatment. The relation among cytotoxicity, tumor specificity index 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. Among eleven piperic acid ester derivatives, 2-(3,4-dihydroxyphenyl)ethyl ester [4] showed the highest cytotoxicity against OSCC cell lines (mean CC₅₀=17 μ M),

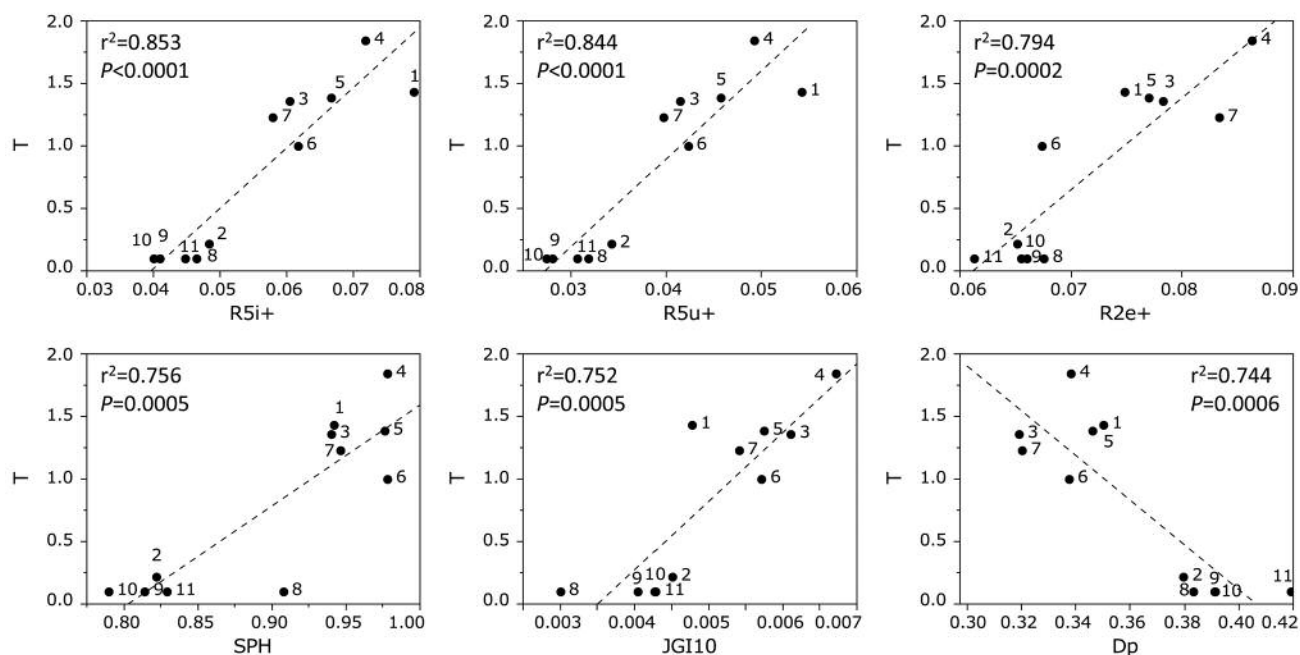


Figure 3. Determination of coefficient between chemical descriptors and cytotoxicity of piperic acid esters against tumor cells (defined as T). The mean (pCC_{50} i.e., the $-\log CC_{50}$) values for tumor cell lines were defined as T .

followed by (4-methoxyphenyl)methyl ester (38 μM) [1], 2-(4-methoxyphenyl)ethyl ester [5] (42 μM), 2-(4-hydroxyphenyl)ethyl ester (46 μM) [3], 2-phenylethyl ester [7] (61 μM) and 2-(3,4-dimethoxyphenyl)ethyl ester [6] (102 μM). On the other hand, (4-hydroxy-3-methoxyphenyl)methyl ester [2] (>368 μM), 3-phenylpropyl ester [8], 4-phenyl-2-butyl ester [9], 4-phenylbutyl ester [10] and decyl ester [11] (>400 μM) were essentially inactive (Table I).

Tumor-specificity. When four OSCC cell lines and three normal oral cells were used, [1] showed the highest TS value (>10.5), followed by [4] (8.0), [7] (>6.6), [3] (2.6), [6] (1.9), [5] (1.6), [2] (><1.1) and [8, 9, 10, 11] (><1.0) (D/B in Table I). [4] showed the highest PSE value (47.0), followed by [1] (>27.8), [7] (>10.8), [3] (5.7), [5] (3.9), [6] (1.8), and [2, 8, 9, 10, 11] (><0.3) (D/B² $\times 100$ in Table I).

When Ca9-22 and HGF cells, both derived from gingival tissue, were used, [4] showed the highest TS value (21.6), followed by [1] (>15.1), [7] (>6.6), [3] (2.3), [5] (2.0), [2] (>1.5), [6] (1.4) and [8, 9, 10, 11] (><1.0) (C/A in Table I). [4] showed the highest PSE value (396.0), followed by [1] (>57.2), [7] (>11.0), [5] (6.8), [3] (5.2), [6] (1.5), [2] (>0.5) and [8, 9, 10, 11] (><0.3) (C/A² $\times 100$ in Table I).

Treatment with HSC-2 cells with [4] induced cell shrinkage (A) and stimulated the cleavage of caspase-3 (B), suggesting the induction of apoptosis (Figure 2).

Anti-HIV activity of chalcones. In contrast to popular anti-HIV agents (dextran sulfate, curdlan sulfate, azidothymidine, 2',3'-dideoxycytidine) (SI=300-5805), none of the piperic esters protected cells from the cytopathic effect of HIV infection (SI<1) (Table II). Based on these data, the subsequent QSAR analysis was focused on the cytotoxicity of piperic acid esters.

Computational analysis. We next performed the QSAR analysis of piperic acid esters in regards to their cytotoxicity against tumor cells and normal cells. Among a total of 3049 descriptors, 17 descriptors described below correlated well with cytotoxicity and tumor specificity.

Cytotoxicity of piperic acid esters against human OSCC cell lines was correlated with $R5i+$ (molecular shape and ionization potential) ($r^2=0.853$, $p<0.0001$), $R5u+$ (molecular shape) ($r^2=0.844$, $p<0.0001$), $R2e+$ (molecular shape and electronegativity) ($r^2=0.794$, $p=0.0002$), SPH (molecular sphericity) ($r^2=0.756$, $p=0.0005$), $JGI10$ (topological charge) ($r^2=0.752$, $p=0.0005$), Dp (molecular shape and polarizability) ($r^2=0.744$, $p=0.0006$) (Figure 3).

Cytotoxicity of piperic acid esters against human normal oral mesenchymal cells was correlated with CATS3D_17_AA (molecular shape and H-bond acceptor) ($r^2=0.957$, $p<0.0001$), rsynth (synthetic feasibility) ($r^2=0.942$, $p<0.0001$),

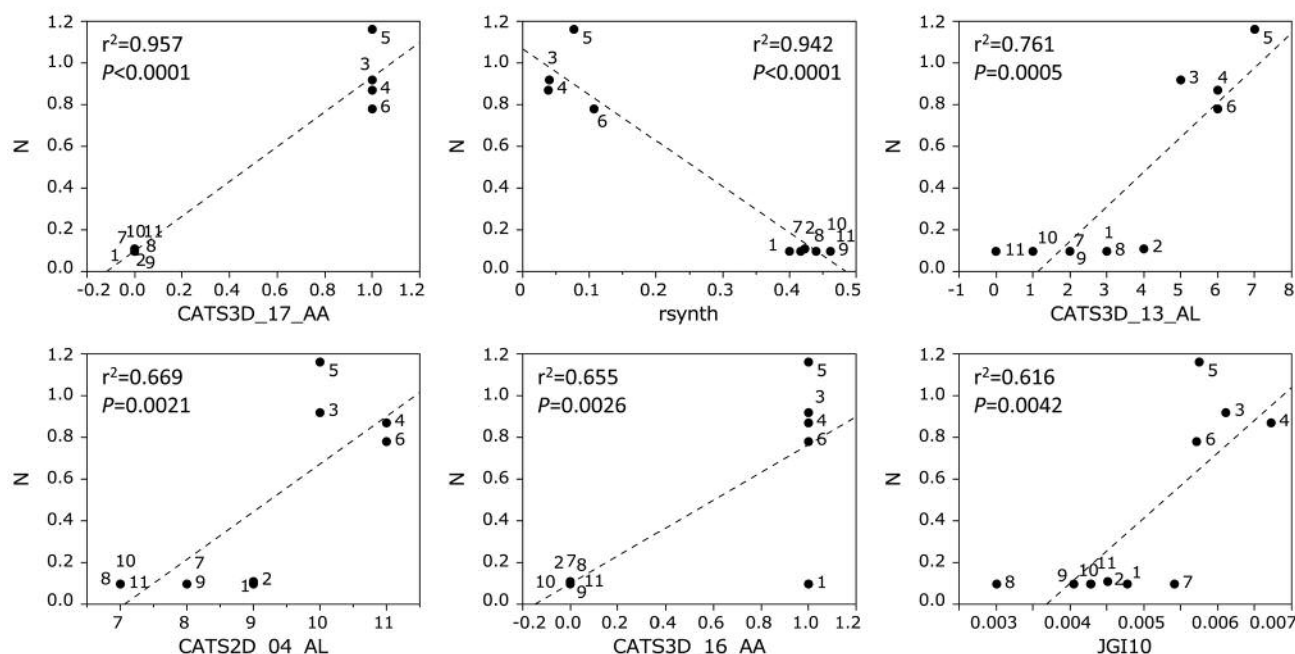


Figure 4. Determination of coefficient between chemical descriptors and cytotoxicity of piperic acid esters against normal cells (defined as N). The mean (pCC_{50} i.e., the $-\log CC_{50}$) values for normal cells were defined as N.

CATS3D_13_AL (molecular shape, H-bond acceptor and lipophilicity) ($r^2=0.761$, $p=0.0005$), CATS2D_04_AL (topological shape, H-bond acceptor and lipophilicity) ($r^2=0.669$, $p=0.0021$), CATS3D_16_AA (molecular shape and H-bond acceptor) ($r^2=0.655$, $p=0.0026$), JGI10 (topological charge) ($r^2=0.616$, $p=0.0042$) (Figure 4).

Tumor specificity of piperic acid esters was correlated with vsurf_DW13 (molecular size and shape) ($r^2=0.668$, $p=0.0021$), HATS5i (topological shape and ionization potential) ($r^2=0.659$, $p=0.0024$), TDB08u (molecular shape) ($r^2=0.658$, $p=0.0024$), TDB08i (molecular shape and ionization potential) ($r^2=0.653$, $p=0.0026$), TDB08e (molecular shape and electronegativity) ($r^2=0.653$, $p=0.0026$), MATS5v (topological shape and molecular size) ($r^2=0.646$, $p=0.0029$) (Figure 5).

Discussion

We have synthesized eleven piperic acid esters, of which ten compounds except [11] are new compounds. The present study demonstrated that five phenylethyl esters [3, 4, 5, 6, 7] showed relatively higher cytotoxicity and tumor specificity, without or with the introduction of hydroxyl group or methoxy group in the benzene ring. Phenylmethyl ester [1] also showed higher tumor-specificity, but removal of the methoxy group [2] resulted in significant reduction in both cytotoxicity and tumor-specificity. On the other hand,

compounds with longer side chains such as phenylpropyl ester [8], phenylbutyl ester [9, 10] and decyl ester [11] were essentially inactive (Table I).

Among the 11 compounds, [4] had the highest TS ($D/B=8.0$, $C/A=21.6$) and PSE values ($D/B^2 \times 100=47.0$, $C/A^2 \times 100=396.0$) (Table I). It should be noted that TS/PSE values of [4] were higher than those of methotrexate ($TS=>1.0\sim3.7$, $PSE=>0.1\sim1.3$), albeit much lower than those of doxorubicin ($TS=>3.7\sim43.3$, $PSE=>12922.9\sim28322.4$) (Table I). Apoptosis-inducing activity of [4] further suggests its antitumor potential.

QSAR analysis demonstrated that tumor specificity of piperic acid esters was rather correlated with vsurf_DW13, HATS5i, TDB08u, TDB08i, TDB08e and MATS5v that reflects molecular size, ionization potential, molecular shape, ionization potential, electronegativity, and molecular size, respectively (Figure 5). However, chemical descriptors that were correlated with cytotoxicity against normal cells (CATS3D_17_AA, rsynth, CATS3D_13_AL, CATS2D_04_AL, CATS3D_16_AA and JGI10) (Figure 3) and tumor cells (R5i+, R5u+, R2e+, SPH, JGI10, DP) were quite different with each other. This suggests that modification of backbone structure of piperic acid esters can produce more selective compounds.

The present study demonstrated that eleven piperic acid esters had no ant-HIV activity. We reported previously that

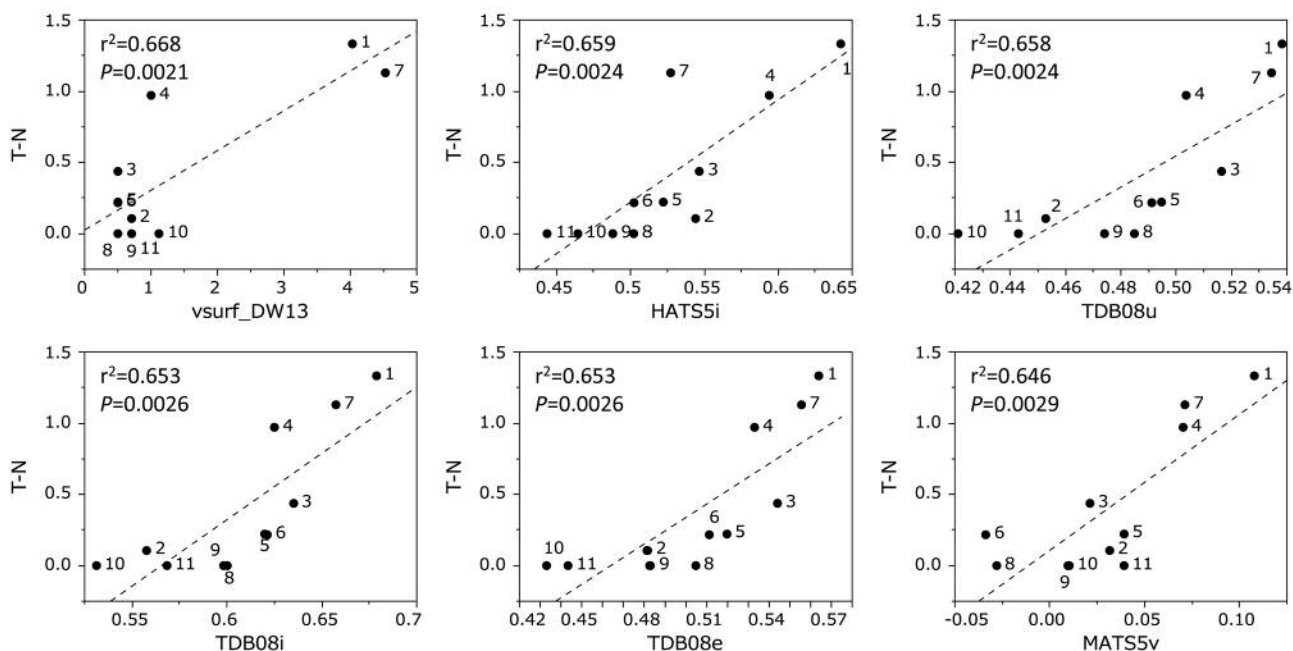


Figure 5. Determination of coefficient between chemical descriptors and tumor specificity of piperic acid esters (defined as T-N).

twelve piperic acid amides had no anti-HIV activity, in agreement with the absence of publication regarding the antiviral activity of piperic acid esters on pubmed search.

In conclusion, compound **4** is a potential lead compound for synthesizing more potent compounds targeted against OSCC cells.

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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