Quantitative Structure–Cytotoxicity Relationship of Piperic Acid Amides

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Abstract. *Background: A total of 12 piperic acid amides, including piperine, were subjected to quantitative structure–activity relationship (QSAR) analysis, based on their cytotoxicity, tumor selectivity and anti-HIV activity, in order to find new biological activities. Materials and Methods: Cytotoxicity against four human oral squamous cell carcinoma (OSCC) cell lines and three human oral normal cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Tumor selectivity was evaluated by the ratio of the mean 50% cytotoxic concentration (CC_{50}) against normal oral cells to that against OSCC cell lines. Anti-HIV activity was evaluated by the ratio of the CC_{50} to 50% HIV infection-cytoprotective concentration (EC_{50}). Physicochemical, structural, and quantum-chemical parameters were calculated based on the conformations optimized by LowModeMD method followed by density functional theory method. Results: All compounds showed low-to-moderate tumor selectivity, but no anti-HIV activity. N-Piperoyldopamine (**8**) which has a catechol moiety, showed the highest tumor selectivity, possibly due to its unique molecular shape and electrostatic interaction, especially its largest partial equalization of orbital electronegativities and vsurf descriptors. Conclusion: The present study suggests that molecular shape and ability for electrostatic interaction are useful parameters for estimating the tumor selectivity of piperic acid amides.*

Piperine {1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl] piperidine)} (Compound **1** in Figure 1), along with its isomer chavicine, is an alkaloid of black pepper (1). The pungency of piperine is generated by activating the heat and acidity-sensing human transient receptor potential vanilloid receptor (TRPV1) in pain-sensing nerve cells (2). Piperine has been reported to enhance the oral absorption and hence the bioavailability of accompanying compounds, possibly by inhibiting P-glycoprotein-mediated cellular efflux and cytochrome *P450* (CYP) *3A4* (3, 4). Piperine also enhanced the antimicrobial activity of rifampicin by inhibiting the multi-drug efflux pump (5). In animal studies, piperine showed anti-depression/cognitive-enhancing (6), anti-inflammatory (7) and anti-angiogenic (8) activities. Piperine also showed mild-to-moderate antibacterial activity against selected Gram-positive and Gram-negative bacteria (9).

Piperine induced apoptotic cell death in human colon (10), melanoma cells (11), and lung (12) and rectal carcinoma cell lines (13), whereas it induced autophagy in human prostate cancer cells (14), arresting the cells at G_0/G_1 phase regardless of the type of cell death (10, 11, 14). Piperine induced erythroleukemia cells to mature into monocytes/macrophages (15). On the other hand, piperine protected normal cells against apoptosis induced by cadmium (16), cisplatin (17) and glutamate (18). However, rigorous antitumor investigation with appropriate normal cells from the same tissues or same type of cells (epithelial or mesenchymal) have not yet been performed. As far as we know, only one study has reported that piperine showed no cytotoxicity against WI38 lung fibroblast as normal counterpart *vs.* human adenocarcinoma cell line A549 derived from lung cancer (12). There is no report on the antiviral activity of piperine.

In order to discover new biological activities of piperine, a total of 12 synthetic piperic acid amides including piperine were investigated for their cytotoxicity and anti-HIV activity, and then subjected to quantitative structure–activity relationship (QSAR) analysis.

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Key Words: Piperic acid amides, QSAR analysis, cytotoxicity, tumor selectivity, anti-HIV activity.

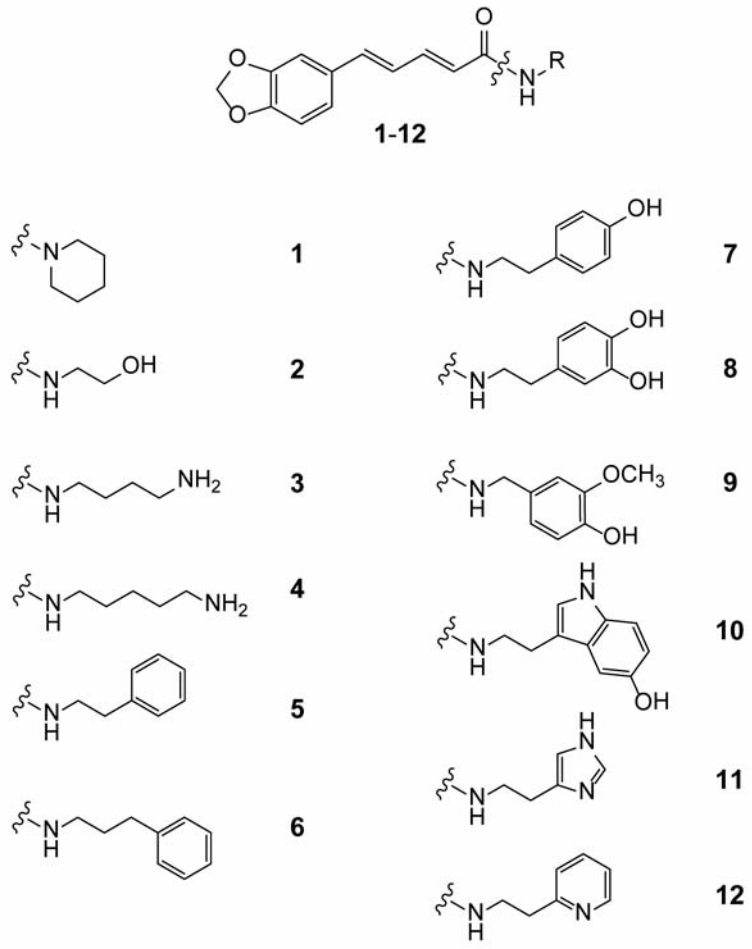

Figure 1. *Structure of piperic acid amides: piperine {1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine)} (**1**) N-Piperoylethanolamine (**2**), N-piperoylputrescine (**3**), N-piperoylcadaverine (**4**), N-piperoylphenethylamine (**5**), N-piperoyl-3-phenylpropylamine (**6**), N-piperoyltyramine (**7**), N-piperoyldopamine (**8**), N-piperoylvanillylamine (**9**), N-piperoylserotonin (**10**), N-piperoylhistamine (**11**) and N-piperoyl-2-(2-pyridinyl)ethylamine (**12**).*

For the cytotoxicity assay, both human normal oral cells (gingival fibroblast, HGF; periodontal ligament fibroblast, HPLF; pulp cells, HPC) and human oral squamous cell carcinoma (OSCC) cell lines (Ca9-22, HSC-2, HSC-3, HSC-4) were used as target cells. The antitumor potential was evaluated by the tumor-selectivity index (TS), calculated by dividing the mean 50% cytotoxic concentration (CC_{50}) against normal oral cells by that against OSCC cell lines. We have recently reported that among 24 plant extracts, leaves of *Camptotheca acuminate*, a well-known source of camptothecin, had the highest TS value (88.3) among 24 plant extracts, suggesting that the TS value determined by this method seems to reflect the antitumor potential of each plant extract, although these oral normal and OSCC cell lines of oral origin are classified as different types of cells (mesenchymal or epithelial) (19).

For anti-HIV assay, mock- and HIV-infected human T-cell lymphotropic virus-I (HTLV-I)-carrying human T-cell line MT4 was used. The selectivity index (SI) was calculated by diving the CC_{50} by the 50% HIV infection-cytoprotective concentration (EC_{50}).

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), azidothymidine and 2',3'-dideoxycytidine from Sigma-Aldrich Inc., St. Louis, MO, USA; piperine, dimethyl sulfoxide (DMSO), dextran sulfate (molecular mass, 5 kDa) from Wako Pure Chem. Ind., Osaka, Japan; 5-fluorouracil (5-FU) from Kyowa, Tokyo, 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 Labware (Franklin Lakes, NJ, USA).

Synthesis of test compounds. *N*-Piperoylethanolamine (**2**), *N*-piperoylputrescine (**3**), *N*-piperoylcadaverine (**4**), *N*-piperoylphenethylamine (**5**), *N*-piperoyl-3-phenylpropylamine (**6**), *N*-piperoyltyramine (**7**), *N*-piperoyldopamine (**8**), *N*-piperoylvanillylamine (**9**), *N*-piperoylserotonin (**10**), *N*-piperoylhistamine (**11**) and *N*-piperoyl-2-(2-pyridinyl)ethylamine (**12**) (Figure 1) were synthesized by coupling of piperic acid with the appropriate amines by means of a modified procedure described elsewhere (20). To a mixture of piperic acid (1.0 mmol) in CH_2Cl_2 (5 ml) was added oxalyl chloride (10 mmol), and the mixture was stirred at room temperature for 3 h. The solvent and excess oxalyl chloride were then evaporated under reduced pressure. The crude acid chloride generated was dissolved in CH_2Cl_2 or dimethylformamide (DMF) (2 ml), and was added dropwise to a mixture of the appropriated amine or its hydrochloride salt (1.2 mmol) and Et3N (8 mmol) in CH_2Cl_2 or DMF (5 ml) under ice-cooling. The reaction mixture was stirred for 5 h at room temperature. Ice-water was added to the mixture and the whole was extracted with $CHCl_3$. The organic layer was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was then purified by silica gel column chromatography to give the corresponding piperic acid amide. All the conjugates were characterized by ^1H nuclear magnetic resonance (NMR) and mass spectrometry (MS) data. 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), and 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 atmosphere with 5% CO_2. Cells were then harvested by treatment with 0.25% trypsin-0.025% EDTA-2Na in phosphate-buffered saline without calcium and magnesium [PSB(–)] and either subcultured 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 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. Cells were incubated for 48 h, and the relative viable cell number was then determined by MTT method. In brief, the treated cells were incubated for another 3 h in fresh culture medium containing 0.2 mg/ml MTT. Cells were then lysed with 0.1 ml of DMSO, and the absorbance at 540 nm of the cell lysate was determined using a microplate reader (Biochromatic Labsystem, Helsinki, Finland). The CC_{50} was determined from the dose–response curve and the mean value of CC_{50} for each cell type was calculated from three independent experiments.

Calculation of TS. The TS was calculated by the following equation: TS=mean CC_{50} against normal cells/mean CC_{50} against tumor cells. Since Ca9-22 cells were derived from gingival tissue (22), the relative sensitivity of Ca9-22 and HGF was also compared.

Assay for HIV activity. HTLV-I-carrying human T-cell line MT4 cells (supplied by Dr. Naoki Yamamoto), highly sensitive to Human Immunodeficiency Virus-1 (HIV-1), were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. HIV- and mock-infected (control) MT-4 cells were incubated for five days with different concentrations of test compounds and the relative viable cell number was determined by MTT assay. The CC_{50} and EC_{50} were determined from the dose–response curve for mock-infected and HIV-infected cells, respectively (23). All data represent the mean values of triplicate measurements. The anti-HIV activity was evaluated by SI (CC_{50}/EC_{50}).

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 the representative value for tumor selectivity. Tumor selectivity is defined by the balance between pCC_{50} values for normal (N) and tumor (T) cells. The difference (T−N) was used as a tumor-selectivity index only for the following QASR analyses.

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) 2013.08 (Chemical Computing Group Inc., Quebec, Canada). Each structure was refined with density functional theory (DFT-B3LYP/6-31G**) by using Spartan10 for Windows (Wavefunction, Inc., Irvine, CA, USA) (26). During each step of the calculation, quantum chemical, molecular shape, and molecular property parameters including the partial equalization of orbital electronegativities (PEOE) and vsurf descriptors, were obtained. The parameters used were: a_hyd (number of hydrophobic atoms), a_nO (number of oxygen atoms), logP(o/w) (log of the octanol/water partition coefficient), logS (log of the aqueous solubility), PEOE_VSA_FNEG (fractional negative van der Waals surface area), PEOE_VSA_FPOS (fractional positive van der Waals surface area), PEOE_VSA_NEG (total negative van der Waals surface area), PEOE_VSA_PNEG (total negative polar van der Waals surface area), PEOE_VSA_POL (total polar van der Waals surface area), PEOE_VSA_PPOS (total positive polar van der Waals surface area), PEOE_VSA+4 (sum of vi where qi is in the range 0.20-0.25; vi and qi denote the van der Waals surface area and the partial charge of atom i, respectively), vsurf_EWmin1 (lowest hydrophilic energy 1), vsurf_HB6 (H-bond donor capacity 6), vsurf_HB7 (H-bond donor capacity 7), vsurf_IW7 (hydrophilic interaction-energy moment 7), vsurf_IW8 (hydrophilic interaction-energy moment 8), vsurf_W7 (hydrophilic volume 7).

Statistical analysis. The relation among cytotoxicity, tumor-specificity and chemical descriptors was investigated using simple regression analyses by JMP Pro version 10.0.2 (SAS Institute Inc., Cary, NC, USA). The significance level was set at $p<0.05$.

Table I. *Cytotoxic activity of twelve piperic acid amides. Each value represents the mean ±S.D. of triplicate assays.*

	CC_{50} (μM)										
	Human oral squamous cell carcinoma cell line					Human normal oral cells					
	Ca9-22	HSC-2	HSC-3	HSC-4	mean±S.D.	HGF	HPLF	HPC	mean±S.D.	TS	
Piperic acid amide	(A)				(B)	(C)			(D)	(D/B)	(C/A)
1	128±14	512±38	583±202	600±24	456±222	473±22	513±13	501±39	496±21	1.1	3.6
2	239±50	335±54	487±57	450±72	378±113	539±19	510±7.0	518±19	522±15	1.4	2.3
3	103±15	114±14	134±14	122±11	118±13	81±22	127±1.5	137±13	115±30	1.0	0.8
4	107±7.6	118±51	170±31	152±18	137±29	122±8.0	137±7.0	131±47	130±7.5	1.0	1.1
5	7.4±0.8	13±2.1	73±23	199±174	73±89	21±4.0	68±45	82±31	57±32	0.8	2.8
6	13±4.6	18±7.0	81±2.1	208±88	80±91	16±3.8	41±2.6	19±1.5	25±14	0.3	1.3
7	11±0.1	16±6.4	18±4.2	14±2.5	15±3.0	13±0.58	23±3.2	20±1.0	19±5.1	1.3	1.2
8	38±8.5	51±13	131±60	80±17	75±41	>800	>800	>800	>800	>10.7	>21.1
9	79±11	447±331	97±3.2	>800	>356	535±8.7	535±13	573±26	548±22	<1.5	6.8
10	33±1.2	51±13	38±7.2	58±15	45±12	41±1.7	46±2.3	75±1.2	54±18	1.2	1.2
11	455±123	696±105	>800	500±53	>613	658±36	617±33	680±41	652±32	<1.1	1.4
12	183±9.0	262±14	268±6.5	250±3.5	241±39	343±104	497±5.5	467±50	436±82	1.8	1.9
5-FU	88±11	24±7.8	38±7.6	28±4.9	45±30	>1000	>1000	>1000	>1000	>22.2	>11.4

HGF: Human gingival fibroblast; HPC, human pulp cells; HPLF, human periodontal ligament fibroblast; Ca9-22, HSC-2, HSC-3, HSC-4: human oral squamous cell carcinoma cell lines; TS: tumor-selectivity index; CC_{50}: 50% cytotoxic concentration; 5-FU: 5-fluorouracil.

Results

Cytotoxicity. Compared to the positive control, 5-FU, piperine exhibited minor tumor specificity (Table I). Among 11 other analogs, compound [**8**] exhibited the highest tumor specificity, whereas other compounds [**2-7**, **9-12**] exhibited much lower tumor specificity (Table I).

Anti-HIV activity. In contrast to the higher anti-HIV activity of positive controls (dextran sulfate, curdlan sulfate, azidothymidine, 2',3'-dideoxycytidine) (SI=1789-15882), none of the piperic acid amides **1-12** were able to protect cells from 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 amides.

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

Piperic acid amides	CC_{50} (μM)	EC_{50} (μM)	SI
1	324	>800	<1
2	253	>800	<1
3	112	>800	<1
4	89	>800	<1
5	36	>800	<1
6	279	>800	<1
7	2	>800	<1
8	46	>800	<1
9	688	>800	<1
10	279	>800	<1
11	32	>800	<1
12	175	>800	<1
Positive controls			
Dextran sulfate (μg/ml)	621	0.05	12363
Curdlan sulfate (μg/ml)	>1000	0.18	5523
Azidothymidine	233	0.015	15882
2',3'-Dideoxycytidine	2145	1.2	1789

CC_{50}: 50% Cytotoxic concentration; EC_{50}: 50% effective concentration; SI: selectivity index (CC_{50}/EC_{50}).

Computational analysis. Cytotoxicity of piperic acid amides against tumor cells (defined by T) correlated with the partial equalization of orbital electronegativity in total negative van der Waals surface area (r^2=0.751, p<0.0005), fractional positive van der Waals surface area (r^2=0.701. p<0.001), fractional negative van der Waals surface area (r^2=0.701, p<0.001), log of the octanol/water partition coefficient (r^2=0.492, p<0.05), number of hydrophobic atoms (r^2=0.473, p<0.05) and log of the aqueous solubility (r^2=0.432, p<0.05) (Figure 2A).

On the other hand, cytotoxicity of piperic acid amides against normal cells (defined by N) was correlated with hydrophilic interaction-energy moment 7 (r^2=0.530, p<0.01), lowest hydrophilic energy 1 (r^2=0.491, p<0.05), H-bond donor capacity 7 (r^2=0.484, p<0.05), hydrophilic volume 7 (r^2=0.484, p<0.05), H-bond donor capacity 6 (r^2=0.476,

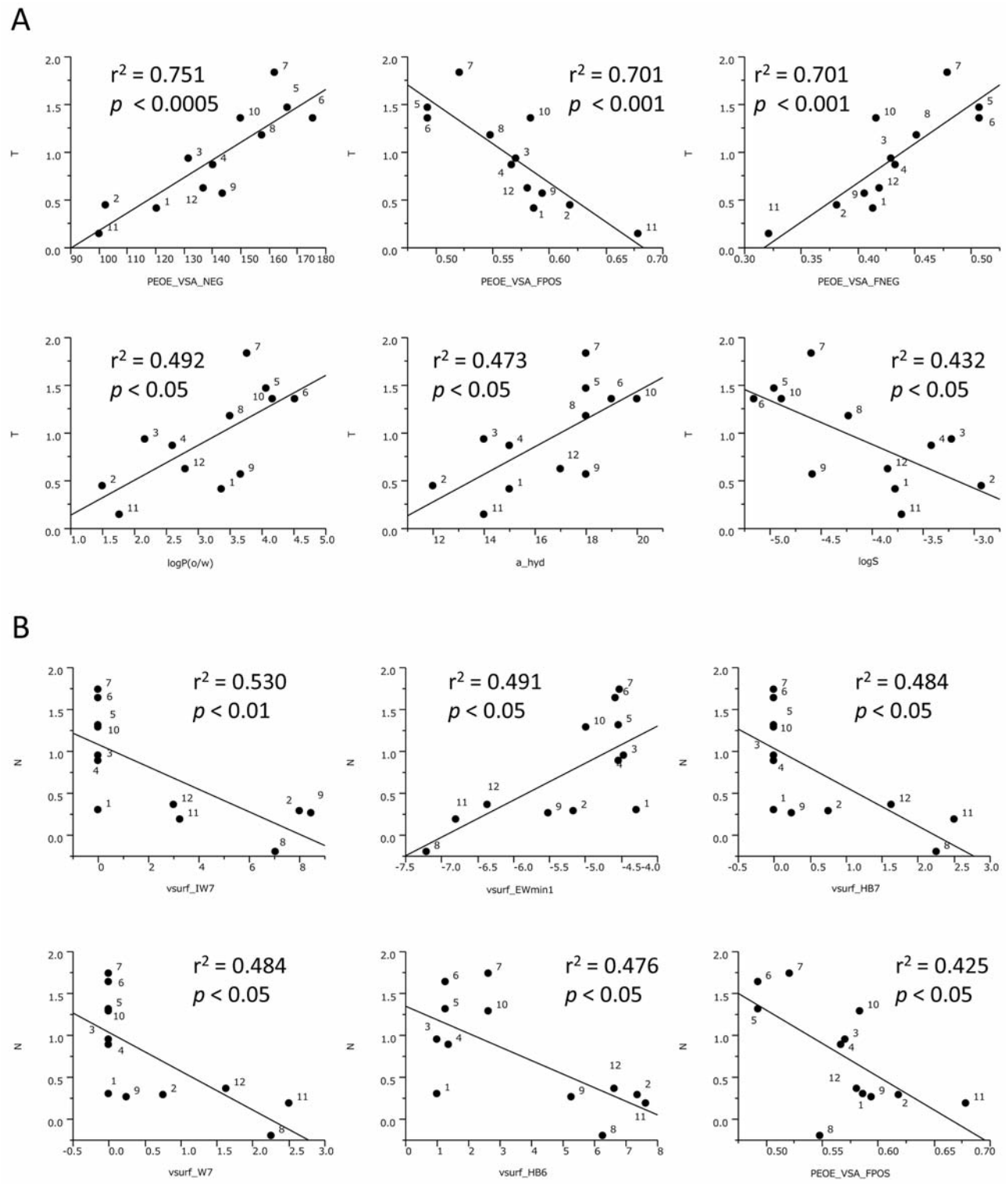

Figure 2. *Correlation coefficient of chemical descriptors and cytotoxicity of piperic acid amides against tumor cells (defined as T) (A) and normal cells (defined as N) (B). The mean (pCC_{50} i.e, the $-\log CC_{50}$) values for normal cells and tumor cell lines were defined as N and T, respectively. The descriptors used were: a_hyd (Number of hydrophobic atoms), logP(o/w) (Log of the octanol/water partition coefficient), logS (Log of the aqueous solubility), PEOE_VSA_FNEG (Fractional negative van der Waals surface area), PEOE_VSA_FPOS (Fractional positive van der Waals surface area), PEOE_VSA_NEG (Total negative van der Waals surface area), vsurf_EWmin1 (Lowest hydrophilic energy 1), vsurf_HB6 (H-bond donor capacity 6), vsurf_HB7 (H-bond donor capacity 7), vsurf_IW7 (Hydrophilic interaction-energy moment 7), vsurf_W7 (Hydrophilic volume 7).*

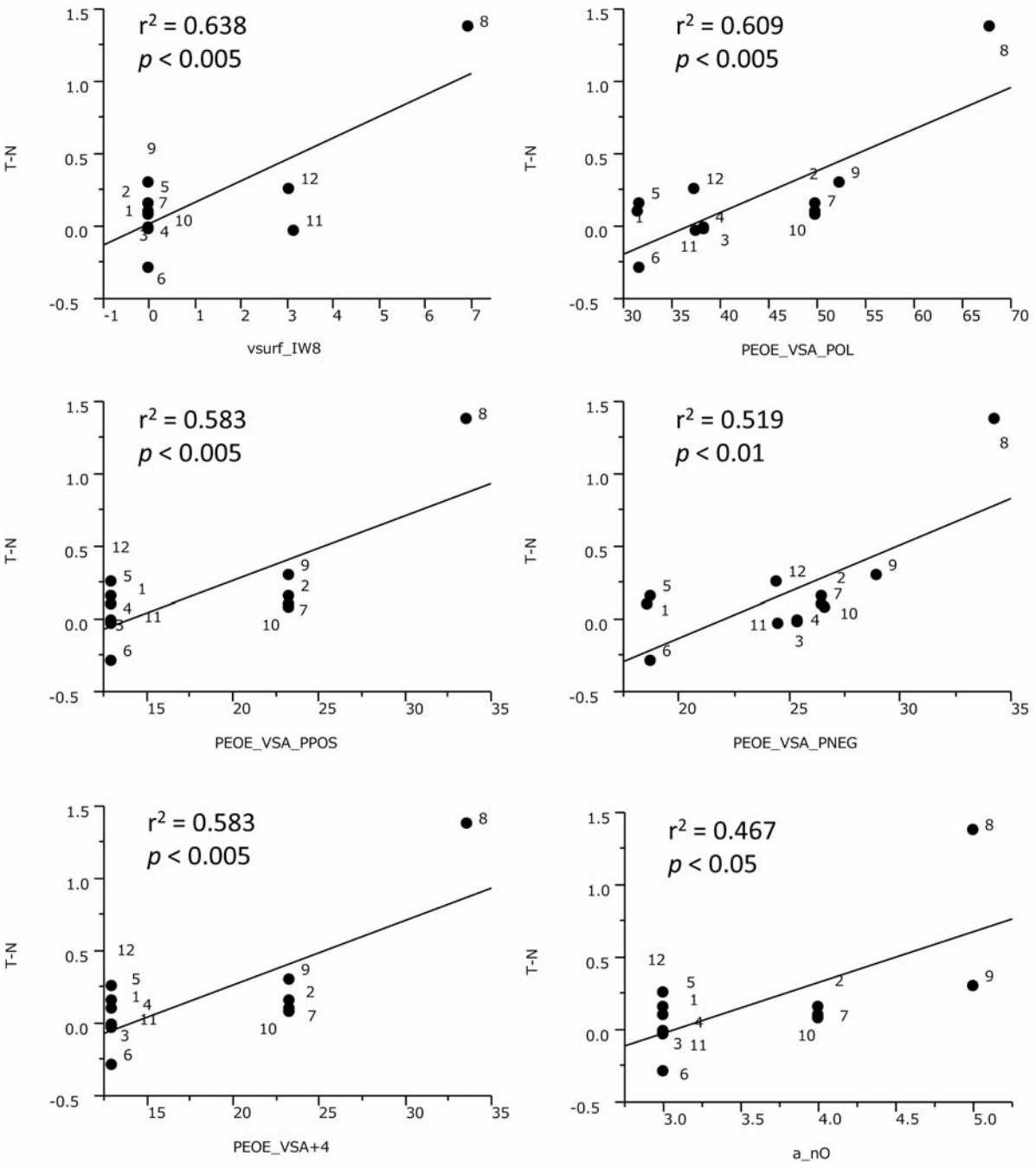

Figure 3. *Correlation coefficient of chemical descriptors and tumor specificity of piperic acid amides, defined as T−N. The descriptors used were: a_nO (Number of oxygen atoms), PEOE_VSA_PNEG (Total negative polar van der Waals surface area), PEOE_VSA_POL (Total polar van der Waals surface area), PEOE_VSA_PPOS (Total positive polar van der Waals surface area), PEOE_VSA+4 (Sum of vi where qi is in the range [0.20,0.25]; vi and qi denote the van der Waals surface area and the partial charge of atom i, respectively), vsurf_IW8 (Hydrophilic interaction-energy moment 8).*

$p<0.05$) and total positive polar van der Waals surface area ($r^2=0.425$, $p<0.05$) (Figure 2B).

Tumor selectivity of piperic acid amides (defined by T–N) correlated with the vsurf descriptor in standard hydrophilic interaction-energy moment 8 (vsurf_IW) ($r^2=0.638$, $p<0.005$), the partial equalization of orbital electronegativity in total polar van der Waals surface area ($r^2=0.609$, $p<0.005$), sum of van der Waals surface area in atoms with the partial charge ($r^2=0.583$, $p<0.005$), fractional positive van der Waals surface area ($r^2=0.583$, $p<0.005$),

total negative polar van der Waals surface area (r^2=0.519, p<0.01) and number of oxygen atoms (r^2=0.467, p<0.05) (Figure 3).

Discussion

The present study demonstrated for the first time that piperine has minor antitumor potential but no anti-HIV activity, and introduction of a catechol moiety [**8**] significantly enhanced the tumor specificity. We found that TS values determined by two different equations (either D/B or C/A, see Table I) were considerably variable, suggesting the considerable difference in sensitivity of seven cell lines used to the 12 piperic acid amide derivatives. It is, thus, necessary that we should use more than three cell lines for both normal and tumor cell groups. Based on these experimental data, we performed the QASR analysis using the D/B value.

We could not obtain significant descriptors for T from the quantum chemical approaches. Therefore, with the assistance of descriptors calculated by MOE, a total 330 parameters were searched. We found that many PEOE descriptors, which provide information on electric charge, and vsurf descriptors, which reflect the molecular shape, explain well the cytotoxicity and tumor-selectivity of piperic acid amides. The PEOE method of calculating atomic partial charges (27) is a method in which charge is transferred between bonded atoms until equilibrium. The vsurf descriptors are similar to the VolSurf descriptors (28), and depend on the structure connectivity and conformation. We found good correlation of T with van der Waals surface area (total negative, fractional positive and negative) and hydrophobic property (Figure 2A). N correlated well with hydrophilic interaction-energy moment and energy, H-bond donor capacity and volume, and total positive polar van der Waals surface area (Figure 2B). The tumor selectivity (T–N) correlated well with van der Waals surface area (total polar, positive polar and negative polar), and number of oxygen atoms (Figure 3). Compound [**8**] had the highest tumor specificity, possibly due to its unique molecular shape and electrostatic interaction, especially its largest PEOE and vsurf descriptors.

Curcumin (diferuloylmethane), a natural compound extracted from *Curcuma longa* L, has been reported by many investigators to inhibit the proliferation of various tumor cells in culture, prevent carcinogenesis and inhibit the growth of implanted tumors (29). However, the evaluation system used herein for TS demonstrated that curcumin had a very narrow therapeutic window (TS=1.7) (30). Previous attempts to enhance the antitumor potential of curcumin by introducing piperic acid and glycine (31) or demethoxy, bisdemethoxy or piperoyl (32) groups were unsuccessful.

In conclusion, the present study demonstrates there are many chemical descriptors specific to cytotoxicity against normal and tumor cells, and TS. Tumor selectivity was well-correlated with molecular shape and electrostatic interaction. Multivariate statistics with these chemical descriptors may be useful for designing the most favorable compound with higher tumor selectivity.

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