Quantitative Structure–Cytotoxicity Relationship of Phenylpropanoid Amides

CHIYAKO SHIMADA[1], YOSHIHIRO UESAWA[2], MARIKO ISHIHARA[3], HAJIME KAGAYA[2], TAISEI KANAMOTO[4], SHIGEMI TERAKUBO[4], HIDEKI NAKASHIMA[4], KOICHI TAKAO[5], TAKAYUKI SAITO[5], YOSHIAKI SUGITA[5] and HIROSHI SAKAGAMI[1]

[1]*Division of Pharmacology and* [3]*Basic Chemistry, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama, Japan;*
[2]*Department of Clinical Pharmaceutics, Meiji Pharmaceutical University, Noshio, Kiyose, Tokyo, Japan;*
[4]*St. Marianna University School of Medicine, Kanagawa, Japan;*
[5]*Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama, Japan*

Abstract. *Background: A total of 12 phenylpropanoid amides were subjected to quantitative structure–activity relationship (QSAR) analysis, based on their cytotoxicity, tumor selectivity and anti-HIV activity, in order to investigate on their 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 CC_{50} (50% cytotoxic concentration) against normal oral cells to that against OSCC cell lines. Anti-HIV activity was evaluated by the ratio of CC_{50} to EC_{50} (50% cytoprotective concentration from HIV infection). Physicochemical, structural, and quantum-chemical parameters were calculated based on the conformations optimized by the LowModeMD method followed by density functional theory (DFT) method. Results: Twelve phenylpropanoid amides showed moderate cytotoxicity against both normal and OSCC cell lines. N-Caffeoyl derivatives coupled with vanillylamine and tyramine exhibited relatively higher tumor selectivity. Cytotoxicity against normal cells was correlated with descriptors related to electrostatic interaction such as polar surface area and chemical hardness, whereas cytotoxicity against tumor cells correlated with free energy, surface area and ellipticity. The tumor-selective cytotoxicity correlated with molecular size (surface area) and electrostatic interaction (the maximum electrostatic potential). Conclusion: The molecular size, shape and ability for electrostatic interaction are useful parameters for estimating the tumor selectivity of phenylpropanoid amides.*

Correspondence to: Hiroshi Sakagami, Division of Pharmacology, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: +81 492792758, Fax: +81 492855171, e-mail: sakagami@dent.meikai.ac.jp

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Phenylpropanoid amides, synthesized by coupling reaction with cinnamic acid derivatives and serotonin or phenylalkylamines showed antioxidant (1, 2), tyrosinase-inhibitory activity (2-4), cyclo-oxygenase 2-inhibitory activity (5), and anti-microbial (6) and anti-fungal activities (7). Due to their inhibitory action on melanin synthesis and skin pigmentation, these groups of compounds are useful materials for cosmetic ingredients, and functional foods (8). However, the studies of biological activities of phenylpropanoid amides have been limited to these research areas.

In order to further explore their biological activities, a total of 12 synthetic phenylpropanoid amides [cinnamic acid derivatives (*p*-coumaric acid, ferulic acid and caffeic acid) coupled with vanillylamine (compounds **1-3**), tyramine (compounds **4-6**), dopamine (compounds **7-9**) or serotonin (compounds **10-12**)] (Figure 1) were investigated for their cytotoxicity and anti-HIV activity, and then subjected to quantitative structure–activity relationship (QSAR) analysis.

For cytotoxicity assay, both human normal oral cells (gingival fibroblast, HGF; pulp cells, periodontal ligament fibroblast, HPLF; pulp cell, HPC) and human oral squamous cell carcinoma (OSCC) cell lines (Ca9-22, HSC-2, HSC-3, HSC-4) were used as target cells. The tumor-selectivity index (TS) was calculated by dividing the mean 50% cytotoxic concentration (CC_{50}) against normal oral cells by that against OSCC cell lines.

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% cytoprotective concentration from HIV infection (EC_{50}).

Table I. *Cytotoxic activity of twelve phenylpropanoid amide derivatives. Each value represents the mean±S.D. of triplicate assays.*

	CC_{50} (μM)										
	Human oral squamous cell carcinoma cell					Human normal oral cell					
Phenylpropanoid amides	Ca9-22 (A)	HSC-2	HSC-3	HSC-4	mean±S.D. (B)	HGF (C)	HPLF	HPC	mean±S.D. (D)	TS (D/B)	TS (C/A)
1	68±39	155±9.4	262±11	218±37	176±8.4	180±25	243±3.8	255±5.5	226±40	1.3	2.6
2	66±38	229±8.6	238±40	269±60	201±91	207±13	295±11	257±2.9	253±44	1.3	3.1
3	79±44	94±28	89±10	227±20	122±70	>400	>400	334±42	>378	>3.1	>5.1
4	21±7.5	25±7.3	88±22	53±14	47±31	62±9.9	65±18	51±6.4	59±7.4	1.3	3.0
5	222±49	274±18	277±7.5	299±11	268±33	211±7.0	220±13	275±29	235±35	0.9	1.0
6	153±11	45±9.6	74±7.5	126±4.0	100±49	325±73	333±115	>363	>340	>3.4	2.1
7	261±74	54±6.5	174±29	83±1.5	143±84	269±16	212±12	271±3.6	251±34	1.8	1.0
8	>400	87±8.7	316±40	272±7.8	268±132	352±54	278±62	320±8.7	317±37	1.2	0.9
9	361±46	72±25	172±49	221±2.1	207±120	318±12	306±13	322±6.7	315±8.3	1.5	0.9
10	236±92	74±5.5	122±24	184±5.0	154±71	111±11	146±12	212±24	156±51	1.0	0.5
11	134±7.8	142±1.0	139±17	181±2.0	149±22	164±5.5	175±2.0	242±7.5	194±42	1.3	1.2
12	157±16	102±11	139±20	130±4.6	132±23	117±26	117±15	158±3.5	131±24	1.0	0.7
Positive controls											
Docetaxel	<0.0078	<0.0078	<0.0078	<0.0078	<0.0078	>1	>1	>1	>1	>128	>128
5-FU	59.4±17.7	12.1±3.1	41.5±10.5	<7.8	30.2±24.6	>2000	>2000	>2000	>2000	>66	>34
Doxorubicin	0.74±0.23	0.22±0.03	0.40±0.11	0.20±0.05	0.39±0.25	1.70±0.47	1.30±0.20	>5.0	2.67±2.0	6.8	2.3

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

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 and 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; 5-fluorouracil (5-FU) from Kyowa, Tokyo, Japan; docetaxel from Toronto Research Chemicals, NY, USA; 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. *N-p*-Coumaroylvanillylamine (**1**), *N*-feruloylvanillylamine (**2**), *N*-caffeoylvanillylamine (**3**), *N-p*-coumaroyltyramine (**4**), *N*-feruloyltyramine (**5**), *N*-caffeoyltyramine (**6**), *N-p*-coumaroyldopamine (**7**), *N*-feruloyldopamine (**8**), *N*-caffeoyldopamine (**9**), *N-p*-coumaroylserotonin (**10**), *N*-feruloylserotonin (**11**) and *N*-caffeoylserotonin (**12**) (Figure 1) were synthesized by coupling of cinnamic acid derivatives with vanillylamine, tyramine dopamine, or serotonin in *N,N*-dimethylformamide and dichloromethane in the presence of triethylamine and 1-hydroxy-1*H*-benzotriazole, and 1-ethyl-3-(3-dimethyaminopropyl) carbodiimide as a coupling reagent, according to previous methods (9). 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 (10), 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 5% CO_2 atmosphere. Cells were then harvested by treatment with 0.25% trypsin-0.025% EDTA-2Na in PBS(−) 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, NJ, USA). 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 (0, 3.1, 6.3, 12.5, 25, 50, 100, 200 or 400 μM). Control cells were treated with the same amounts of DMSO present in each diluent solution (0.0078, 0.0156. 0.03125, 0.0625, 0.125, 0.25, 0.5 or 1%). 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 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 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

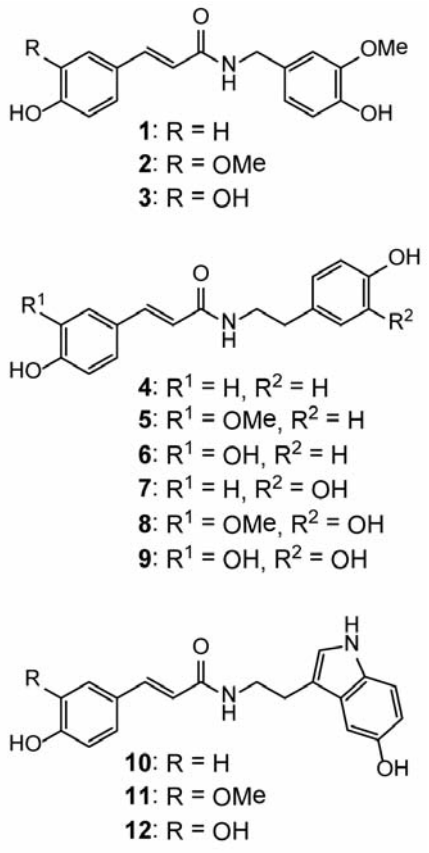

Figure 1. *Structure of phenylpropanoid amides.*

[(D/B) in Table I]. Since Ca9-22 cells were derived from gingival tissue (11), the relative sensitivity of Ca9-22 and HGF was also compared [(C/A) in Table I].

Assay for anti-HIV activity. HTLV-I-carrying human T-cell line MT4 cells, 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 samples 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 (12). 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 (13).

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 for the following analyses as a tumor-selectivity index.

Calculation of chemical descriptors. Each chemical structure was optimized by the LowModeMD method (14), a suitable search method for minimum energy conformers of flexible molecules, with Merck Molecular Force Field (MMFF94) 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) (12). During each step of the calculation, quantum chemical, molecular shape, and molecular property parameters were obtained. The parameters used were: Gibbs free energy (G˚), entropy (S˚), enthalpy (H˚), surface area (molecular surface area), water-accessible polar surface area (acc. visible polar area), highest occupied molecular orbital (HOMO) energy, lowest unoccupied molecular orbital (LUMO) energy, hardness (chemical hardness), ovality (oval ellipticity), lipophilicity ($logP_{o/w}$), molecular hydrogen bond acceptor count (HBACount) (*i.e.* the number of acceptor atoms), maximum electrostatic potential (MaxElPot).

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 10.0.2 (SAS Institute Inc., Cary, NC, USA). The significance level was set at $p<0.05$.

Results

Cytotoxicity. Twelve phenylpropanoid amides showed moderate cytotoxicity against both normal human oral cells and human OSCC cell lines. In most cases, OSCC cells showed slightly higher sensitivities than normal cells, yielding weak tumor selectivity (TS=0.9 to >3.4), as compared with popular chemotherapeutic agents (docetaxel, fluorouracil, doxorubicin: TS=6.8 to >128) (expressed as D/B in Table I).

Phenypropanoid/vanillylamine derivatives (**1-3**) showed some tumor selectivity [TS=1.3 to >3.1 (D/B), 2.6 to >5.1 (C/A)]. Among them, **3** having a caffeoyl group showed the highest cytotoxicity (CC_{50}=122 μM) and tumor selectivity (TS>3.1).

Phenylpropanoid/tyramine derivatives (**4-6**) showed comparable tumor selectivity [TS=0.9 to >3.4 (D/B), 1.0 to 3.0 (C/A)]. Among them, **6** having caffeoyl group again showed the highest tumor-selectivity [TS>3.4 (D/B), 2.1 (C/A)], although **6** showed slightly lower cytotoxicity (CC_{50}=100 μM) than **4** (CC_{50}=47 μM).

Phenylpropanoid/dopamine **(7-9)** and serotonin **(10-12)** derivatives showed essentially no tumor-selectivity [TS=1.0-1.8 (D/B), 0.5-1.2 (C/A)], regardless of the presence or absence of caffeoyl group.

Anti-HIV activity. In contrast to higher anti-HIV activity of positive controls (dextran sulfate, curdlan sulfate, azidothymidine, 2',3'-dideoxycytidine) (SI=1789-15882),

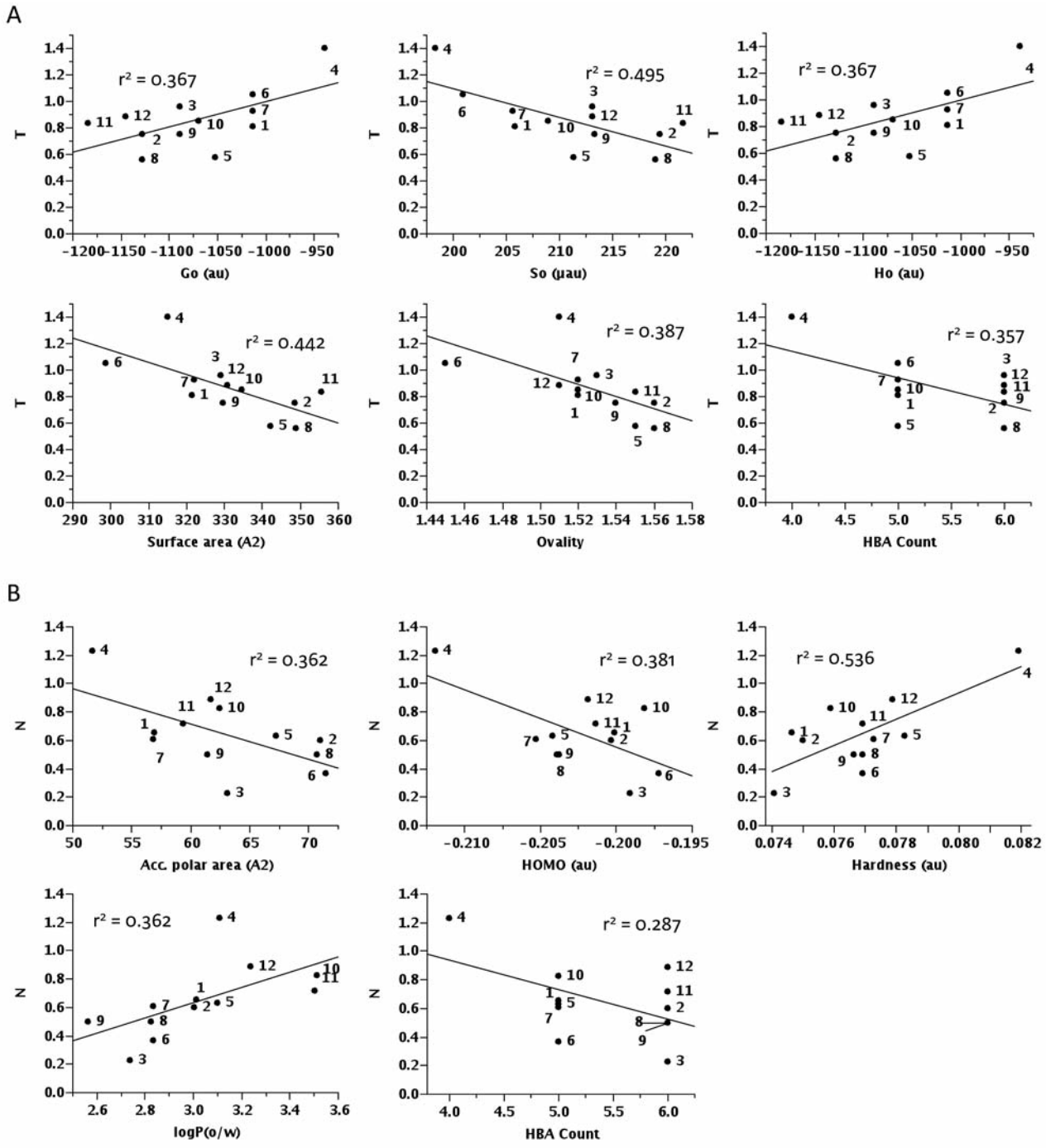

Figure 2. *Correlation coefficient of chemical descriptors and cytotoxicity against tumor cells (defined as T) (A) and normal cells (defined as N) (B). The mean (pCC_{50} i.e, the $-log\ CC_{50}$) values for normal cells and tumor cell lines were defined as N and T, respectively.*

none of the phenylpropanoid amides **1-12** were able to protect the cells from cytopathic effect of HIV infection (SI<1) (Table II). Based on these data, the following QASR analysis was focused on the cytotoxicity of phenypropanoid amides.

Computational analysis. Cytotoxicity of phenylpropanoid amides against tumor cells (defined by T) correlated with G˚ (r^2=0.367), S˚ (r2=0.495), H˚ (r^2=0.367), molecular surface area (r^2=0.442), oval ellipticity (r^2=0.387) and HBA count (r^2=0.357) (Figure 2A).

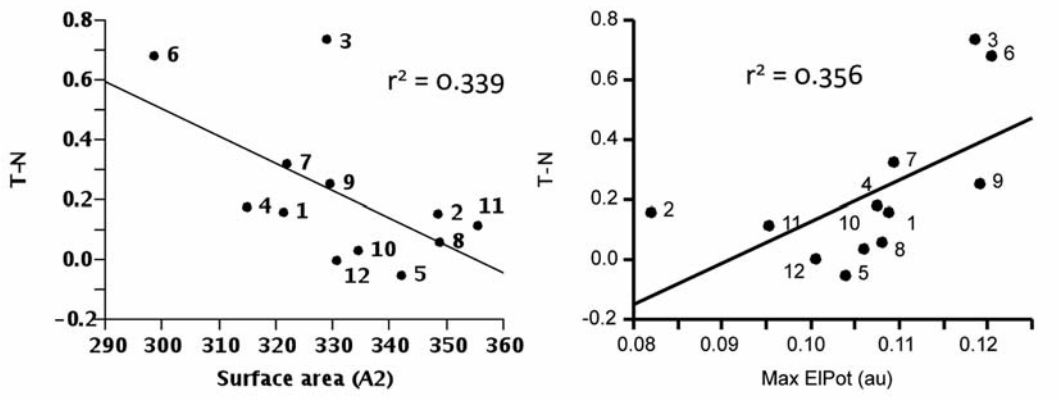

Figure 3. *Correlation coefficient of chemical descriptors and tumor selectivity, defined as T−N.*

On the other hand, cytotoxicity of phenylpropanoid amides against normal cells (defined by N) was correlated with water-accessible polar surface area (r^2=0362), HOMO energy (r^2=0.381), chemical hardness (r^2=0.536), lipophilicity (r^2=0.362) and HBA count (r^2=0.287) (Figure 2B).

Tumor selectivity of phenylpropanoid amides (defined by T−N) correlated with surface area and the maximum electrostatic potential (Figure 3).

Discussion

The present study demonstrated for the first time that phenylpropenoid amides showed moderate cytotoxicity and tumor selectivity, but no detectable anti-HIV activity. Among them, *N*-caffeoyl derivatives coupled with vanillylamine (**3**) and tyramine (**6**) showed relatively higher tumor selectivity (Figure 1, Table I), however such higher tumor selectivity was almost completely eliminated by replacing these amines with serotonin moiety (**12**), possibly due to the increase of surface area or decrease of electrostatic interaction (the maximum electrostatic potential) (Figure 3). We also found that phenylpropanoid amides coupled with dopamine (**7-9**), having a catechol moiety, had little or no tumor selectivity.

Previous study with phenylpropanoid amides coupled with octopamine or dopamine demonstrated that antioxidant activity, but not tyrosinase-inhibitory activity, depends on the presence of catechol moiety in the molecule (2). However, catechols are known to exert both anti-oxidant and pro-oxidant actions under different experimental conditions (16, 17), and catechol present in the *N*-caffeoyl moiety of the same molecule may affect the biological activity. Phenylpropanoid amides brasiliamide A and B (6), *N-p*-coumaroylserotonin and *N*-feruloylserotonin (7) showed antimicrobial and antifungal activities, respectively. Recently, *p*-coumaric acid derivatives showed potent tyrosinase-inhibitory activity (4). These data suggest the possibility that different combinations of phenylpropanoid and phenylethylamine or phenylmethylamine moieties may produce totally new biological activities.

QSAR analysis provided several useful parameters for estimating the cytotoxicity against normal cells or tumors cells. Electrostatic interaction-related descriptors such as polar surface area and chemical hardness may be involved in the expression of cytotoxicity of phenylpropanoid amides

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

Phenylpropanoid amides	CC_{50} (μM)	EC_{50} (μM)	SI
1	196.84	>400	<1
2	226.11	>400	<1
3	121.51	>400	<1
4	30.19	>400	<1
5	192.25	>400	<1
6	158.56	>400	<1
7	49.75	>400	<1
8	51.16	>400	<1
9	43.74	>400	<1
10	220.69	>400	<1
11	193.13	>400	<1
12	47.16	>400	<1
Positive controls			
Dextran sulfate (μg/ml)	620.5	0.05	12363
Curdlan sulfate (μg/ml)	>1000	0.18	>5523
Azidothymidine (μM)	232.87	0.015	15882
2',3'-Dideoxycytidine (μM)	2145.33	1.2	1789

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

against normal cells, whereas free energy, surface area and ellipticity may affect cytotoxicity against tumor cells. This suggests that the molecular size, shape and electrostatic interaction may be involved in cytotoxicity induction by phenylpropanoid amides.

In conclusion, the present study demonstrates there are many chemical descriptors specific to T or N. Multivariate statistics with these chemical descriptors may be useful for estimation of tumor selectivity.

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