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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

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[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

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

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

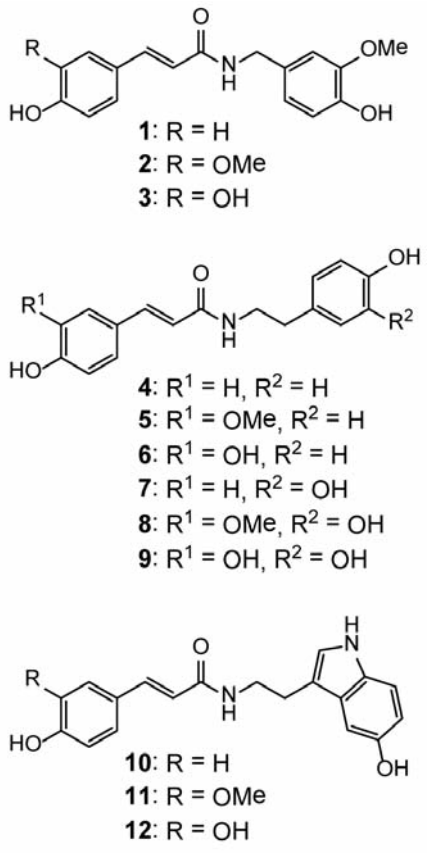

Figure 1. *Structure of phenylpropanoid amides.*

[(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),

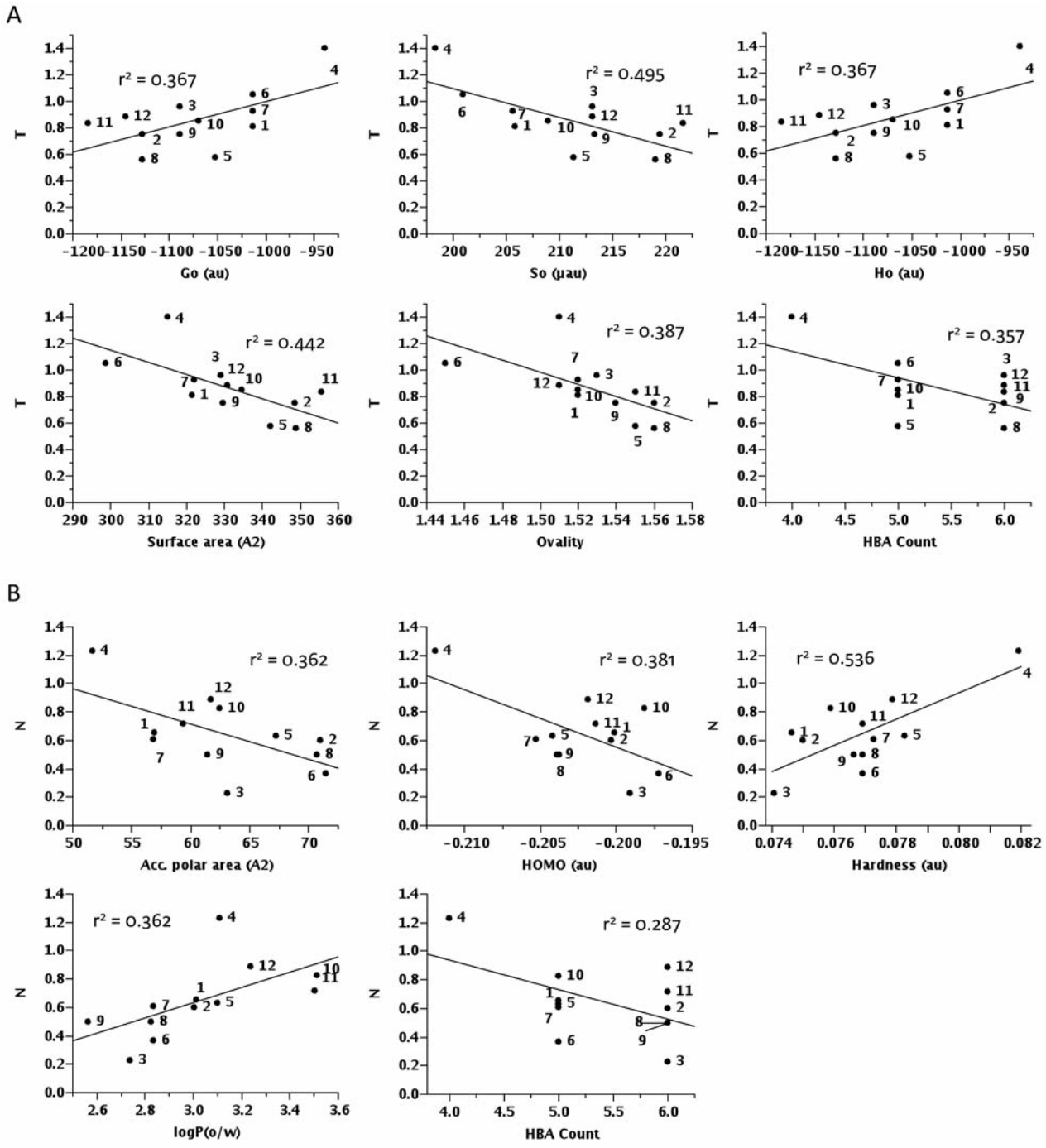

Figure 2. *Correlation coefficient of chemical descriptors and cytotoxicity against tumor cells (defined as T) (A) and normal cells (defined as N) (B). The mean (pCC_{50} i.e, the $-log\ CC_{50}$) values for normal cells and tumor cell lines were defined as N and T, respectively.*

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).

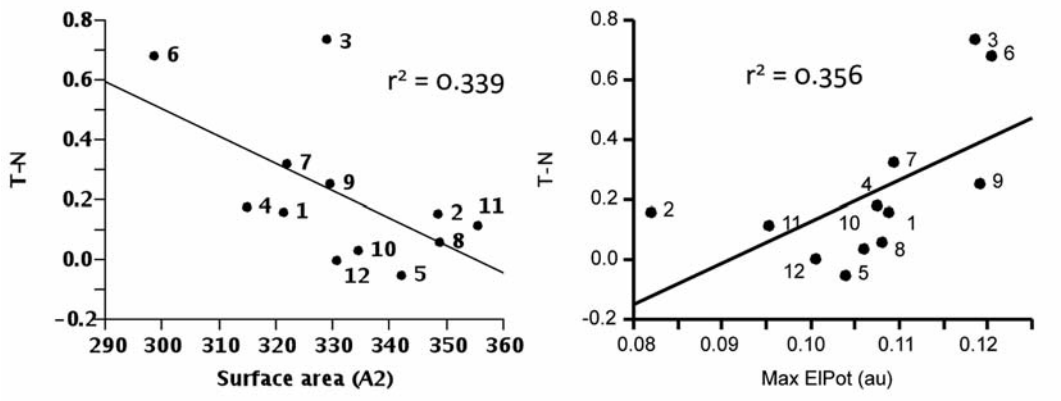

Figure 3. *Correlation coefficient of chemical descriptors and tumor selectivity, defined as T−N.*

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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General Policy. ANTICANCER RESEARCH (AR) will accept original high quality works and reviews on all aspects of experimental and clinical cancer research. The Editorial Policy suggests that priority will be given to papers advancing the understanding of cancer causation, and to papers applying the results of basic research to cancer diagnosis, prognosis, and therapy. AR will also accept the following for publication: (a) Abstracts and Proceedings of scientific meetings on cancer, following consideration and approval by the Editorial Board; (b) Announcements of meetings related to cancer research; (c) Short reviews (of approximately 120 words) and announcements of newly received books and journals related to cancer, and (d) Announcements of awards and prizes.

The principal aim of AR is to provide prompt publication (print and online) for original works of high quality, generally within 1-2 months from final acceptance. Manuscripts will be accepted on the understanding that they report original unpublished works on the cancer problem that are not under consideration for publication by another journal, and that they will not be published again in the same form. All authors should sign a submission letter confirming the approval of their article contents. All material submitted to AR will be subject to review, when appropriate, by two members of the Editorial Board and by one suitable outside referee. The Editors reserve the right to improve manuscripts on grammar and style.

The Editors and Publishers of AR accept no responsibility for the contents and opinions expressed by the contributors. Authors should warrantee due diligence in the creation and issuance of their work.

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Format. Two types of papers may be submitted: (i) Full papers containing completed original work, and (ii) review articles concerning fields of recognisable progress. Papers should contain all essential data in order to make the presentation clear. Reasonable economy should be exercised with respect to the number of tables and illustrations used. Papers should be written in clear, concise English. Spelling should follow that given in the “Shorter Oxford English Dictionary”.

Manuscripts. Submitted manuscripts should not exceed fourteen (14) pages (approximately 250 words per double - spaced typed page), including abstract, text, tables, figures, and references (corresponding to 4 printed pages). Papers exceeding four printed pages will be subject to excess page charges. All manuscripts should be divided into the following sections:
(a) *First page* including the title of the presented work [not exceeding fifteen (15) words], full names and full postal addresses of all Authors, name of the Author to whom proofs are to be sent, key words, an abbreviated running title, an indication “review”, “clinical”, “epidemiological”, or “experimental” study, and the date of submission. (Note: The order of the Authors is not necessarily indicative of their contribution to the work. Authors may note their individual contribution(s) in the appropriate section(s) of the presented work); (b) *Abstract* not exceeding 150 words, organized according to the following headings: Background/Aim - Materials and Methods/Patients and Methods - Results - Conclusion; (c) *Introduction;* (d) *Materials and Methods/Patients and Methods;* (e) *Results;* (f) *Discussion;* (g) *Acknowledgements;* (h) *References*. All pages must be numbered consecutively. Footnotes should be avoided. Review articles may follow a different style according to the subject matter and the Author's opinion. Review articles should not exceed 35 pages (approximately 250 words per double-spaced typed page) including all tables, figures, and references.

Figures. All figures (whether photographs or graphs) should be clear, high contrast, at the size they are to appear in the journal: 8.00 cm (3.15 in.) wide for a single column; 17.00 cm (6.70 in.) for a double column; maximum height: 20.00 cm (7.87 in.). Graphs must be submitted as photographs made from drawings and must not require any artwork, typesetting, or size modifications. Symbols, numbering and lettering should be clearly legible. The number and top of each figure must be indicated. Colour plates are charged.

Tables. Tables should be typed double-spaced on a separate page, numbered with Roman numerals and should include a short title.

References. Authors must assume responsibility for the accuracy of the references used. Citations for the reference sections of submitted works should follow the standard form of “Index Medicus” and must be numbered consecutively. In the text, references should be cited by number. Examples: 1 Sumner AT: The nature of chromosome bands and their significance for cancer research. Anticancer Res 1: 205-216, 1981. 2 McGuire WL and Chamnes GC: Studies on the oestrogen receptor in breast cancer. In: Receptors for Reproductive Hormones (O' Malley BW, Chamnes GC (eds.). New York, Plenum Publ Corp., pp 113-136, 1973.

Nomenclature and Abbreviations. Nomenclature should follow that given in "Chemical Abstracts", "Index Medicus", "Merck Index", "IUPAC –IUB", "Bergey's Manual of Determinative Bacteriology", The CBE Manual for Authors, Editors and Publishers (6th edition, 1994), and MIAME Standard for Microarray Data. Human gene symbols may be obtained from the HUGO Gene Nomenclature Committee (HGNC) (http://www.gene.ucl.ac.uk/). Approved mouse nomenclature may be obtained from http://www.informatics.jax.org/. Standard abbreviations are preferable. If a new abbreviation is used, it must be defined on first usage.

Clinical Trials. Authors of manuscripts describing clinical trials should provide the appropriate clinical trial number in the correct format in the text.
For International Standard Randomised Controlled Trials (ISRCTN) Registry (a not-for-profit organization whose registry is administered by Current Controlled Trials Ltd.) the unique number must be provided in this format: ISRCTNXXXXXXXX (where XXXXXXXX represents the unique number, always prefixed by "ISRCTN"). Please note that there is no space between the prefix "ISRCTN" and the number. Example: ISRCTN47956475.

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Ethical Policies and Standards. ANTICANCER RESEARCH agrees with and follows the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" established by the International Committee of Medical Journal Editors in 1978 and updated in October 2001 (www.icmje.org). Microarray data analysis should comply with the "Minimum Information About Microarray Experiments (MIAME) standard". Specific guidelines are provided at the "Microarray Gene Expression Data Society" (MGED) website. Presentation of genome sequences should follow the guidelines of the NHGRI Policy on Release of Human Genomic Sequence Data. Research involving human beings must adhere to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, effective December 13, 2001. Research involving animals must adhere to the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society. The use of animals in biomedical research should be under the careful supervision of a person adequately trained in this field and the animals must be treated humanely at all times. Research involving the use of human foetuses, foetal tissue, embryos and embryonic cells should adhere to the U.S. Public Law 103-41, effective December 13, 2001.

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