Evaluation of Cytotoxiciy and Tumor-specificity of Licorice Flavonoids Based on Chemical Structure

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Abstract. *Background: The mechanism of cytotoxicity induction by flavonoids has been studied by many investigators, but their tumor specificity is not clear. To address this point, 10 licorice flavonoids were subjected to quantitative structure-activity relationship (QASR) analysis with cytotoxicity assay with four human oral carcinoma and three normal cell lines. Materials and Methods: Cytotoxicity was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide method. Physico-chemical, structural, and quantum-chemical parameters were calculated based on the conformations optimized by the LowModeMD method. Results: Licurazid and isoliquiritigenin had the highest cytotoxicity against tumor cells, and liquiritin, isoliquiritin and licurazid had the highest tumor specificity, suggesting an antitumor potential for licurazid. Chalcones had slightly higher cytotoxicity and tumor specificity than flavanones. The number of sugar units in the molecule was somewhat negatively-correlated with cytotoxicity, but not with tumor specificity. Parameters that reflect the three-dimensional structure, molecular volume and number of phenolic OH groups were significantly correlated with cytotoxicity, but not with tumor specificity. On the other hand, solvation energy was significantly correlated with tumor specificity, but not with cytotoxicity. Conclusion: These physicochemical descriptors may be useful to estimate cytotoxicity or tumor specificity of structurally-related compounds to these licorice flavonoids.*

Low-molecular weight polyphenols, such as tannins and flavonoids, are known to exert antioxidant activity such as reduction of reactive oxygen species and radical scavenging activity (1-3). They also induced apoptotic or autophagic cell death in various cancer cell lines (4, 5), while they exhibited cytoprotective effects against neuronal and keratinocytic cell death (6, 7). However, whether their cytotoxicity is specific to cancer cells has not been well investigated.

To clarify this point, we investigated the cytotoxicity of 10 licorice flavonoids including liquiritin, liquiritigenin, liquiritin apioside, and neoisoliquiritin, against four human cancer cell lines (HSC-2, HSC-3, HSC-4, HL-60) and three human normal oral cells [gingival fibroblast (HGF), pulp cell (HPC), periodontal ligament fibroblast HPLF)]. Using these data, we performed a quantitative structure-activity relationship (QASR) analysis with a large number of chemical descriptors. The relationship between the chemical descriptors and tumor specificity was also analyzed.

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Key Words: Licorice flavonoids, QSAR, cytotoxicity, tumor-specificity index, chemical descriptor.

Materials and Methods

Materials. The following chemicals and materials were obtained from the indicated companies: RPMI-1640, 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), from Sigma Chemical Ind., St. Louis, MO, USA); dimethylsulfoxide (DMSO) from Wako Pure Chemical, Osaka, Japan; peplomycin sulfate from Santa Cruz Biotechnology, Santa Cruz, CA, USA; 96-microwell plates from Becton Dickinson, Franklin Lakes, NJ, USA; Sample compounds were dissolved in DMSO at 100 mM before use, and diluted with medium.

Preparation of flavonoid and chalcone derivatives. The air-dried roots of *Glycyrrhiza* inflate were extracted with MeOH under reflux. After removal of the solvent *in vacuo* to give the MeOH extract. The MeOH extracts were passed through a Diaion HP-20 column, eluting sequentially with H_2O, 50% EtOH and EtOH. The 50% EtOH eluate was chromatographed on a silica gel column ($CHCl_3$:MeOH:H_2O, 10:5:1) to give two fractions, A and B in order of elution. Fr. B eluate was chromatographed on an octa decyl silyl (ODS) column (MeOH:H_2O, 45:55) to give three fractions, B-1, B-2 and B-3 in the order of elution. Fr. B-1 was applied to high-performance liquid chromatography (HPLC) (YMC-Pack Pro C18, 250×20 mmI.D.;

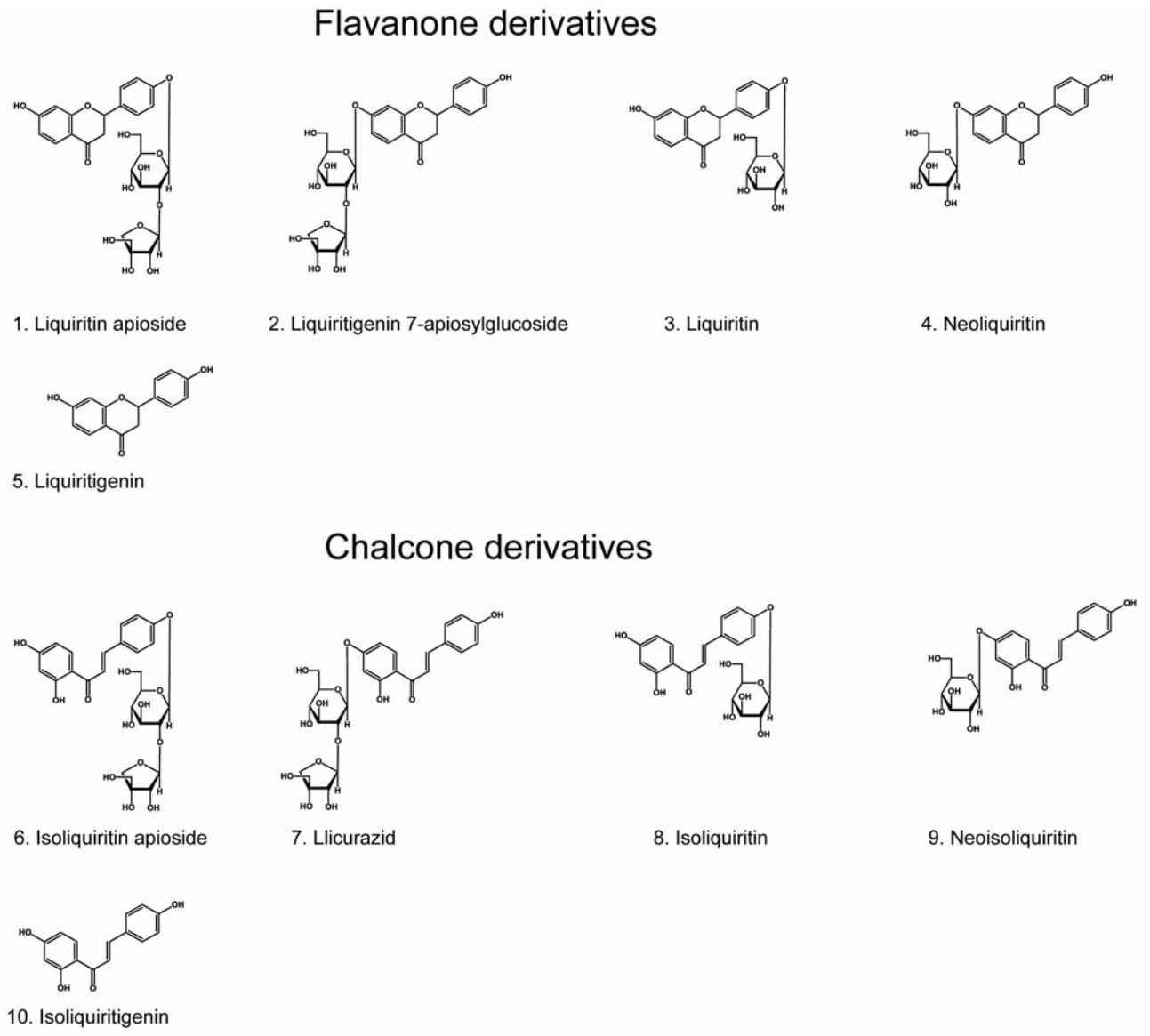


Figure 1. *Structures of licorice flavonoids*.

MeOH:H_2O, 40:60 and CH3CN:H_2O, 22:78) to give chromatographically pure compounds liquiritin apioside [**1**] (8), liquiritigenin 7-apiosylglucoside [**2**] (9), liquiritin [**3**] (10) and neoliquiritin [**4**] (10). Fr. B-2 was subjected to HPLC (YMC-Pack Pro C18, 250×20 mmI.D., MeOH:H_2O, 50:50, and CH_3CN:H_2O, 30:70) to give chromatographically pure compounds isoliquiritin apioside [**6**] (11), licurazid [**7**] (12), isoliquiritin [**8**] (10) and neoisoliquiritin [**9**] (10). Fr. B-3 was subjected to recycling HPLC (JAI-gel GS-310, 500×20 mmI.D., MeOH) to give chromatographically pure compounds liquiritigenin [**5**] (10) and isoliquiritigenin [10] (10). The structures of these compounds are shown in Figure 1.

Cell culture. HL-60 cells (Riken, Tsukuba, Japan) were cultured at 37˚C in RPMI-1640 supplemented with 10% heat-inactivated FBS in a humidified atmosphere with 5% CO_2. Human oral squamous cell carcinoma (OSCC) cell lines (HSC-2, HSC-3, HSC-4) (Riken, Tsukuba, Japan) and normal oral cells (HGF, HPC, HPLF) [established from the periodontal tissues of the extracted tooth as previously reported (13) and used at 8-15 population doubling levels (PDL)] were cultured in DMEM supplemented with 10% heat-inactivated FBS.

Assay for cytotoxic activity. All of the cells were inoculated at 3×10^3 cells/well in 96-microwell plate (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 48 h, the medium was removed by suction with an aspirator, and replaced with different concentrations of the studied compounds. The cells were incubated for another 48 h, and the relative viable cell

Table I. *Cytotoxicity of licorice flavonoid-related compounds against human tumor and normal cells.*

Comp. no.	Comp. name	Estimated CC_{50} (mM)									
		Tumor cells				Normal cells			Average pCC_{50}		Tumor-specificity index
		HSC-2	HSC-3	HSC-4	HL-60	HGF	HPC	HPLF	T	N	(T-N)/(T+N)
Flavanone											
1	Liquiritin apioside	0.729	0.729	0.729	0.729	0.729	0.729	0.729	0.137	0.137	0.000
2	Liquiritigenin 7-apiosylglucoside	0.729	0.729	0.729	0.729	0.729	0.729	0.729	0.137	0.137	0.000
3	Liquiritin	0.373	0.165	0.306	0.067	0.956	0.956	0.956	0.725	0.020	0.947
4	Neoliquiritin	0.956	0.956	0.956	0.100	0.956	0.378	0.956	0.264	0.154	0.264
5	Liquiritigenin	0.148	0.191	0.336	0.047	0.566	0.273	0.538	0.838	0.360	0.399
Chalcone											
6	Isoliquiritin apioside	0.727	0.234	0.727	0.727	0.727	0.727	0.727	0.262	0.139	0.307
7	Licurazid	0.074	0.029	0.018	0.035	0.727	0.727	0.727	1.468	0.139	0.827
8	Isoliquiritin	0.103	0.390	0.174	0.184	0.956	0.956	0.956	0.723	0.020	0.947
9	Neoisoliquiritin	0.277	0.956	0.478	0.048	0.956	0.210	0.956	0.554	0.239	0.398
10	Isoliquiritigenin	0.016	0.037	0.039	0.008	0.164	0.098	0.144	1.690	0.879	0.316

CC_{50}, 50% cytotoxic concentration; pCC_{50}, – log CC_{50}; N, mean pCC_{50} values for normal cells; T, mean pCC_{50} values for tumor cell lines; HGF, gingival fibroblast; HPC, pulp cell; HPLF, periodontal ligament fibroblast.

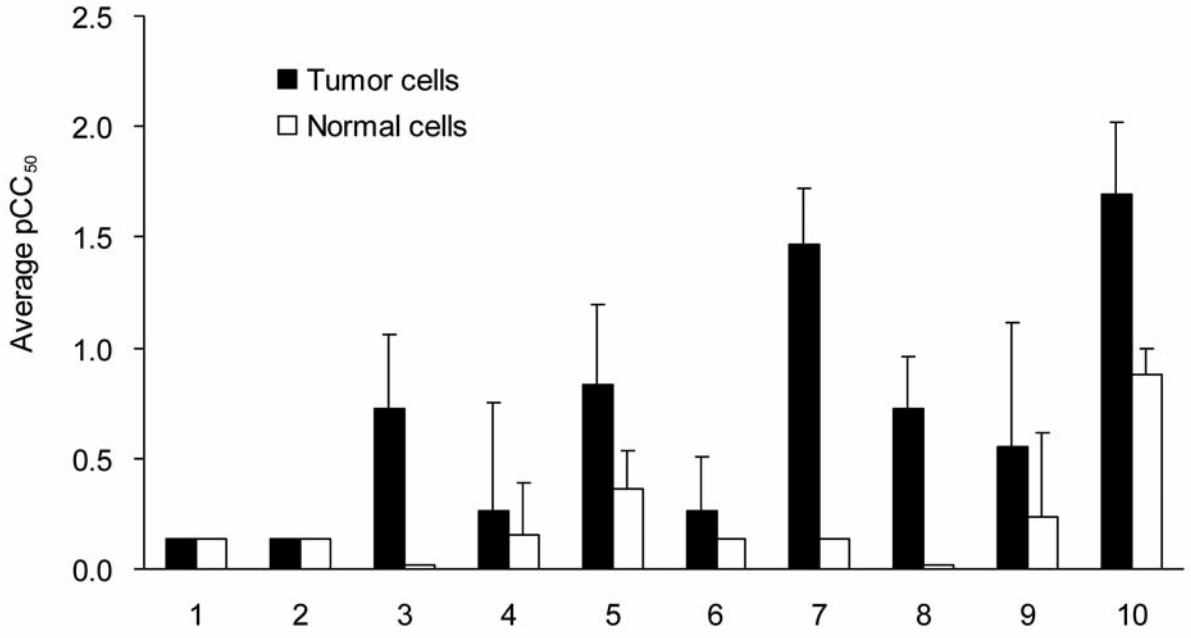


Figure 2. *Cytotoxicity of licorice flavonoids against human tumor and normal cells. The height and vertical bars represent mean + SD, respectively. Cytotoxicity was estimated as an average of –logCC_{50} (pCC_{50}). 1. liquiritin apioside; 2, liquiritigenin 7-apiosylglucoside; 3, liquiritin; 4, neoliquiritin; 5, liquiritigenin; 6, isoliquiritin apioside; 7, licurazid; 8, isoliquiritin; 9, neoisoliquiritin; 10, isoliquiritigenin.*

number was then determined by the MTT method, as previously reported (14). The 50% cytotoxic concentration (CC_{50}) was determined from the dose–response curve and the mean value of CC_{50} against each cell was calculated from three independent experiments.

Inverse logarithmic ratio. 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.

Calculation of the representative value for tumor specificity. Tumor specificity is defined by the balance between pCC_{50} values for normal (N) and tumor (T) cells. The following three parameters are available: (i) difference (T–N), (ii) ratio (T/N) and (iii) ratio of difference to their sum: (T–N)/(T+N). Of these, (T–N)/(T+N) produced the highest normality and correlation coefficient with chemical descriptors (data not shown). Furthermore, the ratio of T/N produced the same value, whenever both T and N had high or low values. On the other hand, the ratio of (T–N)/(T+N) produced a higher correlation coefficient for compounds with lower cytotoxicity (15). Based on these considerations, (T–N)/(T+N) was used for the following analyses as a tumor-specificity index.

Calculation of chemical descriptors. Each chemical structure was optimized by the LowModeMD method (16), a suitable search method for minimum energy conformers of flexible molecules such as glycosides, with Merck Molecular Force Field (MMFF94) in Molecular Operating Environment (MOE) 2011.10 (Chemical Computing Group Inc., Quebec, Canada). By using the optimized conformations, a number of chemical descriptors were obtained through semi-empirical molecular orbital calculations [Austin Model 1 (AM1), Parameterized Model Number 3 (PM3), and Parameterization Mothod 6 (PM6)], as well as physico-chemical parameters based on quantum chemical and molecular shape properties. Spartan10 for Windows (Wavefunction, Inc., Irvine, CA, USA) (17), Marvin view 5.3.1 (ChemAxon Kft., Budapest, Hungary) (18), MOE (19), and Dragon 6.0.2 (Talete srl., Milano, Italy) (20) were used for the calculations of 35, 27, 334 and 2,696 kinds of descriptors, respectively. These were selected by excluding calculation failures and lack of validation among the compounds. As a result, in total 3,092 kinds of chemical descriptors were extracted as useful parameters for QSAR analyses.

Statistical treatment. The relation among cytotoxicity, tumor specificity index, and chemical descriptors was investigated, using simple regression analyses by JMP Pro version 10.0.2 (SAS Institute Inc., Cary, NC, USA). Some data are expressed as mean and SD. Unpaired Student's *t*-test was used to examine significant differences in mean values. The significance level was set at $p<0.05$.

Results

Cytotoxicity. The original data for cytotoxicity use the sign of inequality such as '>'. For the convenience of analysis, these values were changed into forms suitable for the arithmetic calculation. Since '>400 μg/ml' is equal to '400~∞ μg/ml', we calculated the harmonic mean as follows: 1/(average (1/400,1/∞))=800 μg/ml as described previously (15), and converted to molar concentration (μmol/ml). As a result of the estimation by harmonic mean, the value became two-fold of the original value (Table I).

For the comparison, the CC_{50} value was converted to the pCC_{50}, as described above (Figure 2). It should be noted that the higher a pCC_{50} value of a compound, the higher its cytotoxicity. The order of cytotoxicity against tumor cells (HSC-2, HSC-3, HSC-4, HL-60) (evaluated by mean pCC_{50} values of tumor cells, T) was in the order of isoliquiritigenin >licurazid >liquiritin >liquiritin >isoliquiritin >neoisoliquiritin >neoliquiritin >isoliquiritin apioside >liquiritin apioside, liquiritigenin 7-apiosylglucoside. On the other hand, the order of cytotoxicity against normal cells (HGF, HPC, HPLF) (mean pCC_{50} values of normal cells, N) was in the order of isoliquiritigenin >liquiritin >neoisoliquiritin >neoliquiritin >isoliquiritin apioside, licurazid >liquiritin apioside, liquiritigenin 7-apiosylglucoside >liquiritin >isoliquiritin.

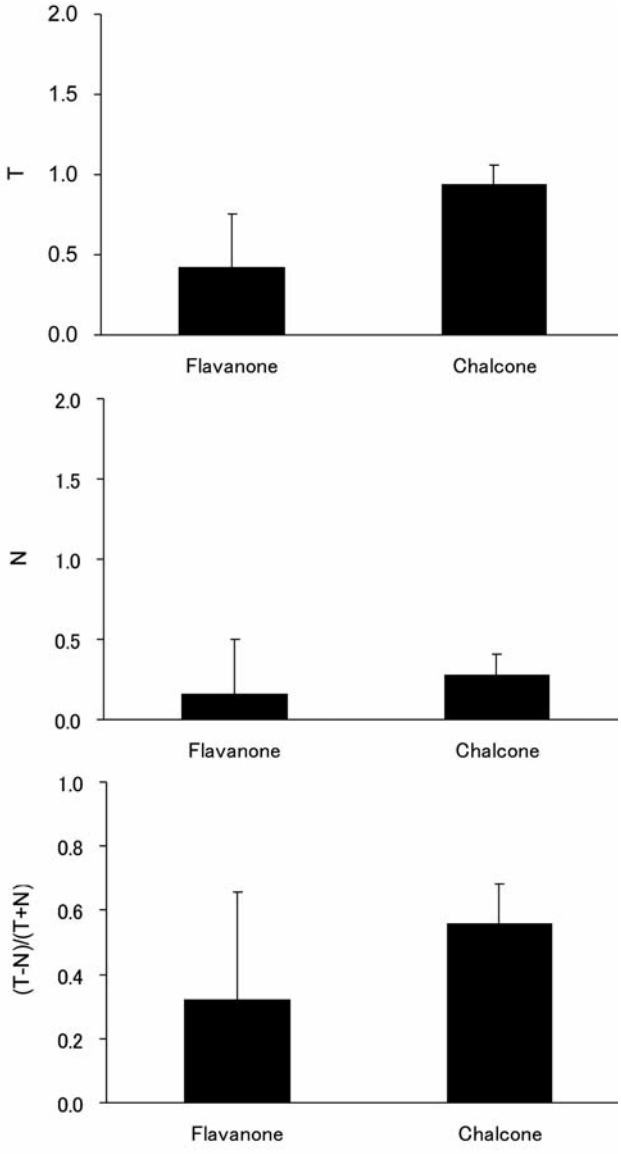
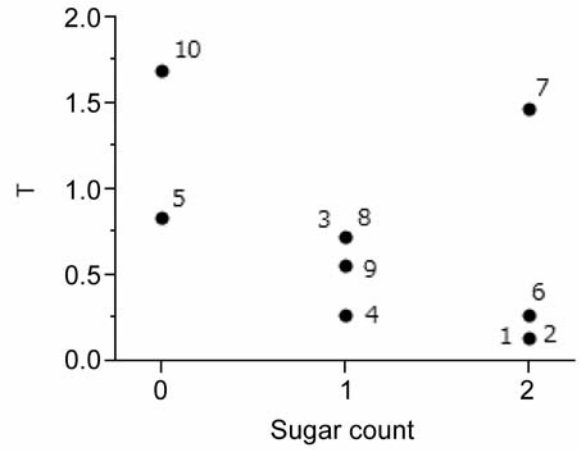
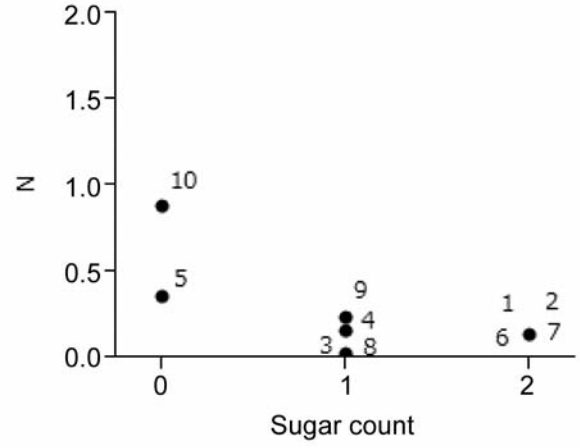


Figure 3. *Comparison between flavanone and chalcone groups for the cytotoxicity and specificity. The estimated 50% cytotoxic concentration against tumor (T) and that against normal cells (N), and the tumor-specificity index, estimated as the ratio of (T-N)/(T+N) are shown. The height and vertical bars represent the mean and SD, respectively.*





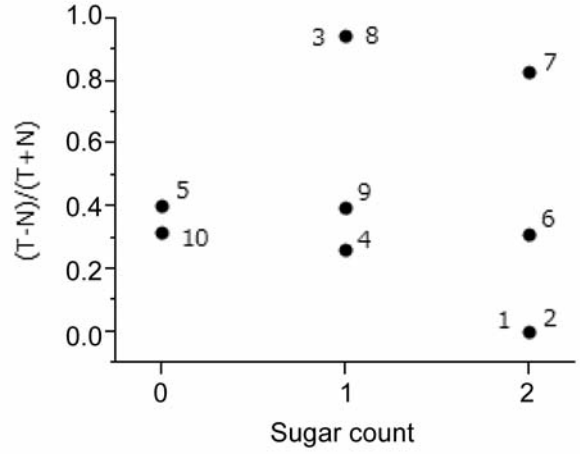


Figure 4. *Effect of the number of sugar units on the cytotoxicity and tumor specificity of licorice flavonoids.*

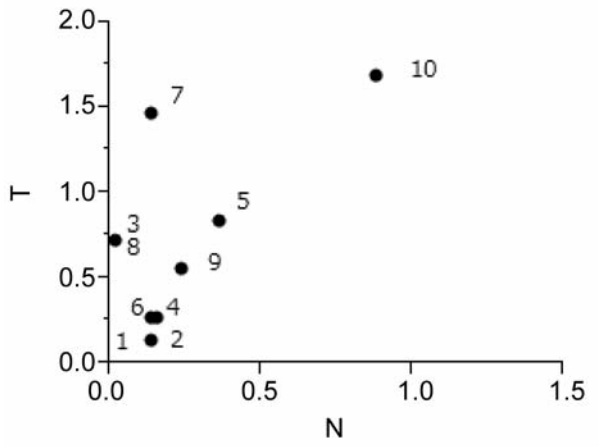


Figure 5. *Scatter plot of the estimated 50% cytotoxic concentration (CC_{50}) against tumor cells (T) and that against normal cells (N).*

Comparison between flavanones and chalcones. Since flavanones [**1-5**] have considerably different steric structures from chalcones [**6-10**], these two groups of compounds were compared with respect to their cytotoxicity and tumor specificity. Although the chalcone group exhibited slightly higher cytotoxicity and tumor specificity than the flavanone group, the differences were not significant ($p>0.05$) (Figure 3).

Effect of the number of sugar units. With an increase in the number of sugar units, the cytotoxicity against tumor cells declined ($r^2=0.707$, $p<0.005$) except licurazid. Significant negative correlation was observed for normal cells ($r^2=0.404$, $p<0.05$). However, there was no significant correlation between the tumor specificity and the number of sugar units (Figure 4).

Tumor specificity. The cytotoxicity (pCC_{50} value) of the 10 flavonoids against tumor cells (N) and the one against normal cells (T) was scatter-plotted (Figure 5). Compounds in the upper left region had higher tumor specificity. When the tumor-specificity index was calculated by the ratio of (T–N)/(T+N), liquiritin and isoliquiritin had the highest tumor-specificity (0.947), followed by licurazid (0.827) > liquiritin (0.399) > neoisoliquiritin (0.398) > isoliquiritigenin (0.316) > isoliquiritin apioside (0.307) > neoliquiritin (0.264) > liquiritin apioside, liquiritigenin 7-apiosylglucoside (0.000) (Figure 6).

Correlation between each chemical descriptor and cytotoxicity index. Three descriptors, BCUT_SMR_0 (combined steric-electronic descriptor), R4v+ (reflects three-dimensional structure and molecular volume) and nArOH (reflects the number of phenolic OH groups) were significantly correlated with the cytotoxicity of the 10 licorice flavonoids against both tumor ($r^2=0.451$, 0.681, 0.565) and normal cells normal cells ($r^2=0.917$, 0.603, 0.520) (Figure 7). R4v+ had the highest correlation with T ($r^2=0.681$), whereas BCUT_SMR_0 had the highest correlation with N ($r^2=0.917$). However, these descriptors did not correlate with tumor specificity. On the other hand,

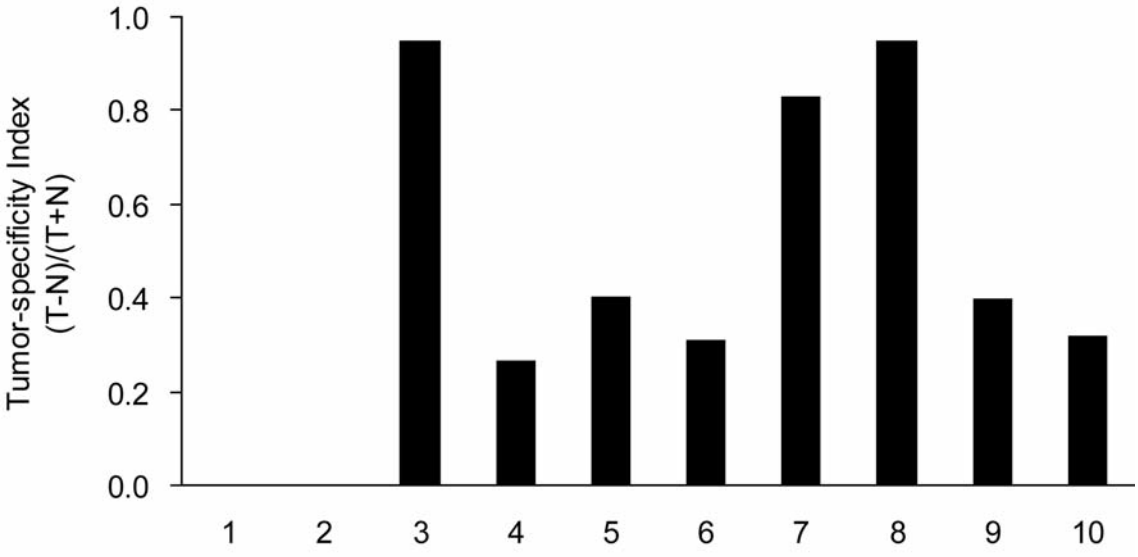


Figure 6. *Tumor specificity of licorice flavonoids. The tumor-specificity index was estimated as the ratio of (T-N)/(T+N), whereas N and T were the estimated 50% cytotoxic concentrations of each compound against normal and tumor cells, respectively. 1. Liquiritin apioside; 2, liquiritigenin 7-apiosylglucoside; 3, liquiritin; 4, neoliquiritin; 5, liquiritigenin; 6, isoliquiritin apioside; 7, licurazid; 8, isoliquiritin; 9, neoisoliquiritin; 10, isoliquiritigenin.*

solvation energy had the highest correlation with tumor specificity (r^2=0.659), although it was not apparently correlated with cytotoxicity (Figure 7).

Discussion

In contrast to liquiritin and isoliquiritin, biological activities of licurazid have been limited to just one article that reported the inhibition of tube formation from vascular endothelial cells (12). The present study demonstrated for the first time that licurazid had comparable tumor specificity with liquiritin and isoliquiritin, and the cytotoxicity of licurazid against tumor cells was approximately twice as much as that of iquiritin and isoliquiritin. Further study is needed to evaluate the antitumor potentiality of licurazid.

The present study also demonstrated that three descriptors, BCUT_SMR_0, R4v+ and nArOH, showed good correlation with the cytotoxicity of licorice flavonoids (r^2=0.451–0.917), but not with their tumor specificity. From a structural point of view, the presence of phenolic OH groups contributes to their cytotoxicity. On the other hand, solvation energy was well-correlated with tumor specificity (r^2=0.659), but not cytotoxicity. These data suggest that the descriptors that can be used for the estimation of cytotoxicity and those for tumor specificity may be not common to each other.

We have reported that water-accessible surface had the highest correlation coefficient with the tumor specificity of a total of 38 1,2,3,4-tetrahydroisoquinoline derivatives, regardless of the method of calculation (15). The types of physicochemical descriptors that correlate with tumor specificity may differ with the type of compound analyzed. More specifically, electrostatic interaction between flavonoids and protein(s) might be an important factor by which they act differentially towards normal cells and tumor cells. The present study suggests the applicability of physicochemical descriptors in the estimation of tumor specificity of structurally related compounds.

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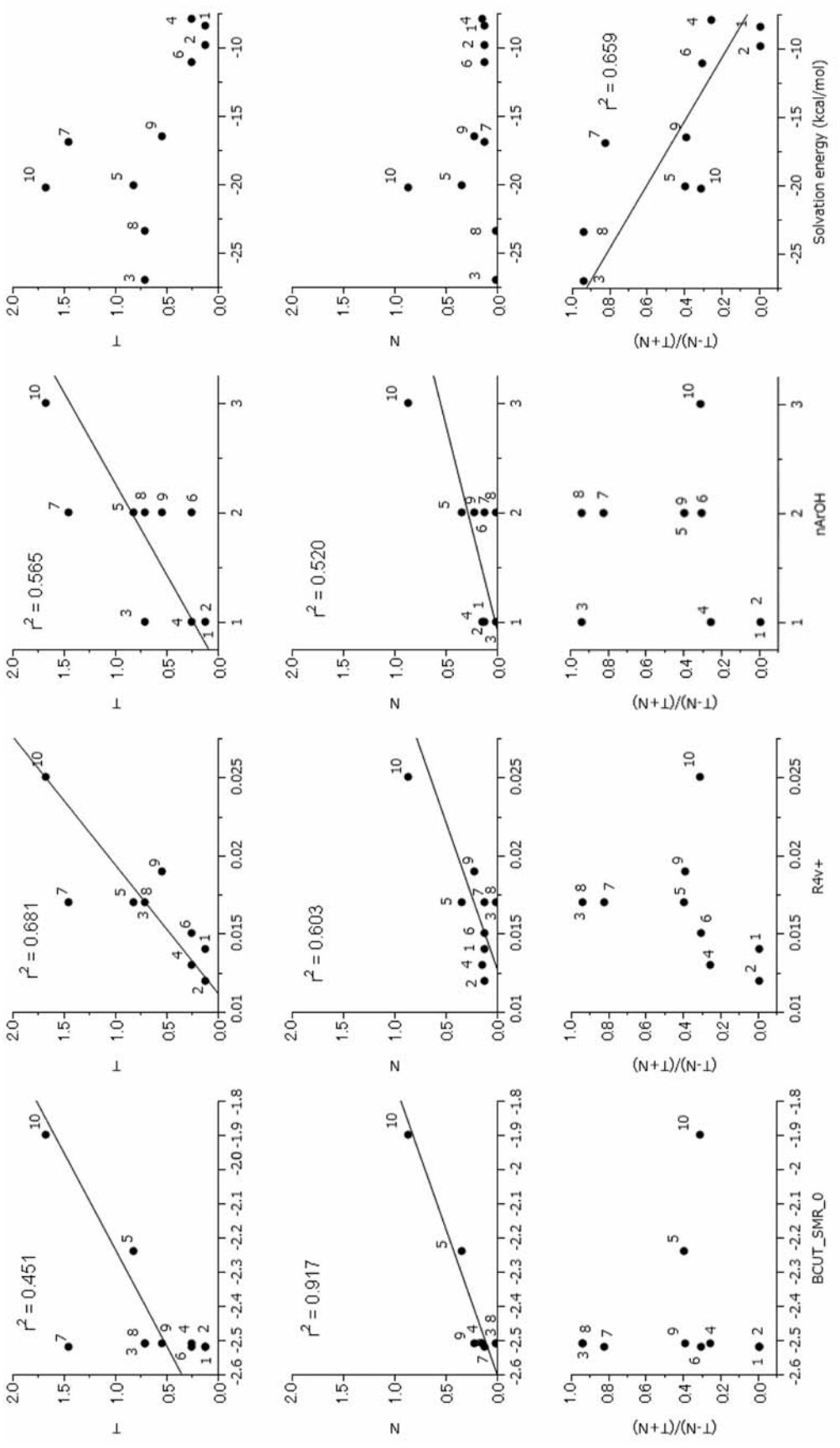

Figure 7. *Relationship of descriptors to cytotoxicity and to tumor-specificity index. -LogCC$_{50}$ (pCC$_{50}$) values were used to evaluate tumor specificity. The relationship between the tumor-specificity index (T-N)/(T+N) and each chemical descriptor was recorded, in which N and T mean average pCC$_{50}$ values for normal and tumor cell lines, respectively.*

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