**In Vitro** Anti-tumor Activity of Azulene Amide Derivatives

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Abstract. Background/Aim: There exist few research articles regarding the anticancer activity of azulene-related compounds. We investigated here the relative cytotoxicity of 10 azulene amide derivatives against cancer and normal cells. Materials and Methods: Cytotoxicity against four human oral squamous cell carcinoma (OSCC) cell lines and three human oral normal cells (gingival fibroblasts, periodontal ligament fibroblasts and pulp cells) was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Antitumor activity was evaluated by tumor-specificity (TS) (ratio of mean 50% cytotoxic concentration (CC50) against normal cells to that against OSCC cell lines) and potency-selectivity expression (PSE) (ratio of TS to CC50 against tumor cells). Apoptosis-inducing activity was evaluated by cleavage of poly ADP-ribose polymerase and caspase-3 with western blot analysis. Results. N-Propylguaiazulenecarboxamide [1] showed the highest TS and PSE values, compared to that of doxorubicin, and induced apoptosis in two OSCC cell lines. QSAR analysis demonstrated that their tumor-specificity of azulene amide derivatives was correlated with hydrophobicity and molecular shape. Conclusion: Compound [1] can be considered as a lead compound for manufacturing new anticancer drug candidates.

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Azulene, an isomer of naphthalene, has a dipole moment and a resonance energy with intermediate values between that of benzene and naphthalene, and is considerably more reactive, when compared with two arenes. Azulene gargle has been reported to reduce the incidence of postoperative sore caused by general anesthesia (1). Azulene sulfonate inhibited the capsaicin-induced plasma exudation in the pharyngeal mucosa of the rat (2). 6-Isopropyl-3-[4-(4-chlorophenylsulfonylamino)butyl]azulene-1-sulfonic acid sodium salt (KT2-962), thromboxane A receptor antagonist, significantly reduced the incidence of ventricular fibrillation during the ischemic period and also myocardial infarct size, possibly by its direct free radical scavenging properties (3). Guaiazulene, a lipophilic azulene derivative, protected rats from paracetamol hepatotoxicity via its antioxidant activity (4) and its inhibitory effect on some cytochrome P450 activities (5). However, there is a limited number of studies that investigated the cytotoxicity of guaiazulene against human leukemic cell lines (HL-60, K562) (6, 7), freshly prepared rat neuron cells, neuroblastoma cell lines (8) and human gingival fibroblasts (9).

In order to search for tumor-selective guaiazulene derivatives, we have synthesized ten azulene amide derivatives (Figure 1) and investigated their anticancer activity. Since previous reports have shown that anticancer agents induce apoptosis in clinical cancer tissues (10), we also investigated the induction of apoptosis by these compounds.

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-HCl (DXR) from Sigma-Aldrich Inc. (St. Louis, MO, USA); dimethyl sulfoxide (DMSO) from Wako Pure Chem. Ind., Osaka, Japan; Culture plastic dishes and plates (96-well) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA).
Protease and phosphatase inhibitors were purchased from Roche Diagnostics (Tokyo, Japan). Antibodies against cleaved caspase-3 (Cell Signaling Technology Inc., Beverly, MD, USA), PARP (Cell Signaling Technology Inc., Beverly, MD, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Trevigen, Gaithersburg, MD, USA) were used as primary antibodies. As secondary antibodies, α-rabbit IgG (DAKO, Tokyo, Japan) antibodies, which were conjugated with horseradish peroxidase, were used.

**Synthesis of alklyaminogroups.** N-propylguaiiazulenecarboxamide [1], N-butyrguaiiazulenecarboxamide [2], N-pentylguaiiazulenecarboxamide [3], N-(2-hydroxyethyl)guaiiazulenecarboxamide [4], N-(3-hydroxypropyl)guaiiazulenecarboxamide [5], N-(2-methoxyethyl)guaiiazulenecarboxamide [6], N-(3-methoxypropyl)guaiiazulenecarboxamide [7], N-(2-aminopropyl)guaiiazulenecarboxamide [8], N-(3-aminobutyl)guaiiazulenecarboxamide [9], N-(2-(2-aminoethoxy)ethoxyethyl)guaiiazulenecarboxamide [10] were synthesized, according to previous reports (11-14). 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 (15) 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% CO₂ atmosphere. HGF, HPLF and HPC at 12–20 population doubling level (PDL) were used in the present study.

**Assay for cytotoxic activity.** Cells were inoculated at 2×10³ cells/0.1 ml in a 96-microwell plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA). After 48 h, the medium was removed by suction with an aspirator and replaced with 0.1 ml of fresh medium containing different concentrations of the 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 the MTT method. In brief, the treated cells were incubated for another 2 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 560 nm of the cell lysate was determined using a microplate reader (Infinite F 50 R, TECAN, Kawasaki, Japan). The CC₅₀ was determined from the dose–response curve of triplicate samples.

**Calculation of tumor-selectivity index (TS).** TS was calculated using the following equation: TS=mean CC₅₀ against three normal oral cells/mean CC₅₀ against OSCC cell lines [(D/B) in Table I] (16). Since both Ca9-22 and HGF cells were derived from the gingival tissue (17), the relative sensitivity of these cells was also compared [(C/A) in Table I]. We did not use human normal oral keratinocytes for tumor-selectivity assay, since many anticancer drugs showed potent specificity assay, since many anticancer drugs showed potent.

**Calculation of chemical descriptors.** 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 (19).

**Calculation of potency-selectivity expression (PSE).** PSE was calculated using the following equation: PSE=TS/CC₅₀ against tumor cells x100 (16) [that is, (D/B)² x100 (HGF, HPLF, HSC vs. Ca9-22, HSC-2, HSC-3, HSC-4) and (C/A)² x100 (HGF vs. Ca9-22 in Table I).

**Statistical treatment.** Each experimental value is expressed as the mean±standard deviation (SD) of triplicate or quadruplicate measurements. The relation among cytotoxicity, tumor specificity...
CC$_{50}$ value was determined by dose-response study, which was done in triplicate. Ca9-22, HSC-2, HSC-3 and HSC-4: Oral squamous cell carcinoma cell lines; HGF: human gingival fibroblasts; HPLF: periodontal ligament fibroblasts; HPC: pulp cells; CC$_{50}$: 50% cytotoxic concentration, DXR: doxorubicin; TS: tumor-selectivity index; PSE: potency-selectivity expression.

### Results

**Cytotoxicity.** Ten azulene amide derivatives used in this study were classified into four categories, having N-alkyl [1-3], hydroxylalkyl [4, 5], methoxyalkyl [6, 7] and aminooalkyl [8-10] groups at the end of carboxamide (Figure 1). We investigated their cytotoxic activity against four human oral squamous cell carcinoma (OSCC) cell lines (Ca9-22, HSC-2, HSC-3, HSC-4) and three normal oral cells (HGF, HPLF, HPC). The results of three independent experiments (each dose-response study was done in triplicate) are shown in Table I.

We first investigated their cytotoxicity against OSCC cell lines. Among N-propyl group, [1] having 2 methylene units...
showed the highest cytotoxicity (mean CC_{50}=>108, 13.8, 10.4 μM), and with the increase of methylene units to 3 or 4, their cytotoxicity declined (>367, >236, >386 μM; >369, >272, >386 μM), possibly due to the insolubility in culture medium. Hydroxylalkyl [4, 5], methoxyalkyl [6, 7] and aminoalkyl [8-10] groups showed intermediate range of cytotoxicity.

Cytoxicity of all compounds against normal cells was lower than that against OSCC cell lines.

Tumor-specificity (TS). TS was determined by dividing the mean CC_{50} value towards three normal cells by the mean CC_{50} value towards four OSCC cell lines (D/B, Table I). Considering that HGF is the normal cell corresponding to cancer cell Ca9-22 (both derived from gingival tissues), TS value was also generated by dividing the CC_{50} value towards HGF cells by the CC_{50} value towards Ca9-22 cells (C/A, Table I). Among ten compounds [1-10], [1] showed the highest tumor-specificity (TS=>3.7, >28.9, 37.0 in D/B; >27.7, >22.3, >53.6 in C/A in three independent experiments), followed by [7] (TS=4.3, 5.2, 16.5 in D/B; 3.7, 5.6, 7.7 in C/A) and [6] (TS=4.3, 4.0, 4.1 in D/B; 4.3, 3.3, 6.2 in C/A). These values are comparable with those of doxorubicin (TS=448.1, >5.7, >1 in D/B; 4.1, 1.1, >40.3 in C/A). TS values of other compounds were <4.6 in D/B and <3.2 in C/A (Table I).

**PSE value.** In order to identify the most promising compounds in terms of both good potencies and selectively cytotoxic, the potency-selectivity expression (PSE) values were calculated. [1] showed much higher PSE value (>≤34, ≥209.1, ≥356.1 in (D/B^2) ≤100; >191.6, >124.8, >717.3 in (C/A^2) ≤100), followed by [7] [9.6, 11.6, 44.7 in (D/B^2) ≤100; 8.3, 12.0, 21.2 in (C/A^2) ≤100] and [6] [6.6, 6.5, 6.1 in (D/B^2) ≤100; 6.6, 5.4, 11.2 in (C/A^2) ≤100]. PSE values of other compounds were ≤8.1 in (D/B^2) ≤100 and ≤5.3 in (C/A^2) ≤100.

Western blot analysis demonstrated that [1, 6, 7] induced the cleavage of poly ADP-ribose polymerase and caspase-3, suggesting the induction of apoptosis (21) (Figure 2).

**Computational analysis.** Since [2] and [3] showed very low cytotoxicity (CC_{50}>400 μM) in most cells, producing noise in QSAR analysis, we performed the QSAR analysis of eight azulene amide derivatives omitting [2] and [3], in regards to their cytotoxicity against tumor cells and normal cells. Among a total of 297 MOE descriptors, 18 descriptors correlated well with cytotoxicity and tumor specificity (Table III).

Cytotoxicity against human OSCC cell lines was correlated with dens (Molecular density) (r^2=0.835, p=0.0015), density (Molecular density) (r^2=0.824, p=0.0018), vsurf_R (Surface rugosity and shape) (r^2=0.764, p=0.0045), h_logP (Hydropobicity) (r^2=0.689, p=0.0108), h_logS (Water-solubility) (r^2=0.683, p=0.0114), vsurf_HB3 (Hyrogen bond and shape) (r^2=0.681, p=0.0117) (Figure 3).

Cytotoxicity against human normal oral mesenchymal cells was correlated with h_pstrain (Strain energy) (r^2=0.878,
p=0.0006), h_logD (Hydrophobicity) ($r^2=0.859, p=0.0009$), vsurf_Wp5 (Polarity and shape) ($r^2=0.818, p=0.0020$), h_pKb (Dissociation constant) ($r^2=0.817, p=0.0020$), vsurf_IW1 (Hydrophilicity and shape) ($r^2=0.815, p=0.0021$) and vsurf_Wp4 (Polarity and shape) ($r^2=0.809, R=0.0024$) (Figure 4).

Tumor specificity was correlated with vsurf_ID1 (Hydrophobicity and shape) ($r^2=0.931, p=0.0001$), vsurf_ID5 (Hydrophobicity and shape) ($r^2=0.918, p=0.0002$), vsurf_ID4 (Hydrophobicity and shape) ($r^2=0.869, p=0.0007$), vsurf_CW4 (shape) ($r^2=0.852, p=0.0011$), vsurf_ID3 (Hydrophobicity and shape) ($r^2=0.823, p=0.0019$) and vsurf_CW3 (shape) ($r^2=0.818, p=0.0020$) (Figure 5).

**Discussion**

The present study demonstrated that among ten azulene amide derivatives, N-propylguaiazulenecarboxamide [1] showed the highest in vitro antitumor activity, based on its greatest TS and PSE values and apoptotic-induced activity.

Table III. Properties of descriptors that significantly affect the cytotoxicity against tumor cells (T), normal cells (N) and tumor-specificity (T-N).

| Descriptors | Meaning | $r^2$ | p-Value |
|-------------|---------|-------|---------|
| T dens      | Molecular density | 0.835 | 0.0015 |
| T density   | Molecular density | 0.824 | 0.0018 |
| T vsurf_R   | Surface rugosity and shape | 0.764 | 0.0045 |
| T h_logP    | Hydrophobicity      | 0.689 | 0.0108 |
| T h_logS    | Water-solubility    | 0.683 | 0.0114 |
| T vsurf_HB3 | Hydrogen bond and shape | 0.681 | 0.0117 |
| N h_pstrain | Strain energy       | 0.878 | 0.0006 |
| N h_logD    | Hydrophobicity      | 0.859 | 0.0009 |
| N vsurf_Wp5 | Polarity and shape  | 0.818 | 0.0020 |
| N h_pKb     | Dissociation constant | 0.817 | 0.0020 |
| N vsurf_IW1 | Hydrophilicity and shape | 0.815 | 0.0021 |
| T-N vsurf_ID1 | Hydrophobicity and shape | 0.931 | 0.0001 |
| T-N vsurf_ID5 | Hydrophobicity and shape | 0.918 | 0.0002 |
| T-N vsurf_ID4 | Hydrophobicity and shape | 0.869 | 0.0007 |
| T-N vsurf_CW4 | Shape            | 0.852 | 0.0011 |
| T-N vsurf_ID3 | Hydrophobicity and shape | 0.823 | 0.0019 |
| T-N vsurf_CW3 | Shape            | 0.818 | 0.0020 |
Figure 3. Determination of coefficient between chemical descriptors and cytotoxicity of eight azulene amide derivatives 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.

Figure 4. Determination of coefficient between chemical descriptors and cytotoxicity of eight azulene amide derivatives against normal cells (defined as N). The mean (pCC_{50} i.e., the −log CC_{50}) values for normal cells were defined as N.
against two of OSCC cell lines. It was unexpected that [2] and [3], which have longer methylene units, showed much lower cytotoxicity against OSCC cell lines. This may be due to the insolubility of these compounds in the culture medium. By omitting these two compounds, we found a good association of TS value and hydrophobicity. If the solubility of [2] and [3] is improved, TS value of these compounds may be much improved.

QSAR analysis demonstrated that [1] exhibited the highest value of 6 chemical descriptors (hydrophobicity and molecular shape) that correlated with TS value (Figure 5). We also confirmed that [6, 7], that ranked at the second and third position of TS values, also had high scores in these parameters (Figure 5).

On the other hand, [8, 9, 10], having an amino group at the terminal and intermediate score of hydrophobicity (Figure 3), showed the highest cytotoxicity against normal cells (Figure 4) and lowest tumor specificity (Figure 5).

Taken together, the present study suggests that compound [1] can be considered as a lead compound for manufacturing new anticancer drug candidates.

**Conflicts of Interest**

The Authors confirm that there are no known conflicts of interest associated with this publication and there was no significant financial support for this work that could have influenced its outcome.

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