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Review

Selective Toxicity and Type of Cell Death Induced by Various Natural and Synthetic Compounds in Oral Squamous Cell Carcinoma

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Abstract. *This article reviews the selective toxicity and type of cell death induced in oral squamous cell carcinoma (OSCC) by hundreds of natural and synthetic compounds. Flavonoids, coumarins, tannins, ketones and other synthetic compounds showed low to moderate tumor-specific cytotoxicity against human OSCC cell lines as compared with normal human oral cells (gingival fibroblast, pulp cell, periodontal ligament fibroblast), whereas anthracyclines, nocobactins and cyclic α,β-unsaturated compounds showed much higher tumor-specific cytotoxicity. No strict relationship was found between the tumor-specific cytotoxicity and apoptosis induction. There was a considerable variation in drug-sensitivity among 5 OSCC cell lines. OSCC cell lines were generally resistant to apoptosis induction. The cytotoxic activity of antitumor agents is affected by various factors related to the compounds themselves, the cells and their environments. Systematization of the relationship between these factors and tumor-specificity may contribute in the quest for more active compounds.*

Oral squamous cell carcinoma (OSCC) is the fifth most common cancer worldwide, with the number of cases consistently increasing in developing countries. OSCC, like other types of cancer, is a genetic disease, resulting in the loss of differentiation, and possibly generated by the decline of apoptotic potential and immunity (1). Aggressive OSCC with a high score of malignancy showed reduced expression of the tumor suppressor gene phosphatase and tensin (PTEN) homologue deleted on chromosome 10 (2), p53 positivity and low apoptotic index (3), and increased expression of anti-apoptotic proteins, such as survivin (4) and Bcl-2 (5). Down-regulation of heat shock protein 27 enhanced the transformation of oral epithelial dysplasia into OSCC, possibly by impairing the protective mechanism against mutagenesis induced by environmental factors (6). Carcinogenesis of OSCC is related to the overexpression of prolyl isomerase Pin 1 (7), a stronghold for the therapy of Alzheimer's disease. These data suggest that OSCC may be produced by an imbalance of the regulation between cell survival and apoptosis.

During development, many unnecessary or harmful cells or tissues are destroyed by apoptosis [Type I programmed cell death (PCD)], characterized by condensation of the cytoplasm and chromatin, DNA fragmentation and cell fragmentation into apoptotic bodies. The apoptotic cells are removed and degraded by phagocytosis (8). Since many chemotherapeutic and chemopreventive agents have induced similar morphological changes in cancer cells, apoptosis-inducing activity has been considered as an essential criterion for antitumor agents by many researchers.

It was recently reported that several chemotherapeutic agents, however, induced non-apoptotic cell death (autophagy, paraptosis, mitotic catastrophe, necrosis) in cancer cell lines (9-12). Autophagy (Type II PCD) is a proteolytic system ubiquitously distributed in eukaryotic cells which transports and degrades cellular constituents (cytoplasmic proteins and organella) in its own endogenous

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lysosomal machinery. Autophagy is morphologically characterized by the accumulation of autophagic vesicles (autophagosomes and autophagolysosomes) and is often observed when massive cell elimination is demanded or when phagocytes do not have easy access to the dying cells (13). It is unclear whether autophagy directly executes cell death or exhibits the secondary effect of apoptosis (14). Paraptosis (15), characterized by swelling of vacuoles, mitochondria and the endoplasmic reticulum, is easily distinguished from apoptosis by its susceptibility to apoptosis signal-regulating kinase 1 (ASK1)-interacting protein (AIP1)/Alix, but not from autophagy (9). Mitotic catastrophe (15), induced by the breakup of the check-point mechanism, is characterized by the appearance of multinuclear cells, elevated permeability of the mitochondrial membrane and caspase activation. In contrast to apoptosis, however, mitotic catastrophe is not inhibited by caspase inhibitors or over-expression of Bcl-2 (9). Necrosis is induced by the extracellular environment and is characterized by swelling of the cells, membrane disruption, leakage of cellular components and inflammation.

The presence of multiple types of cell death suggests the importance of determining a definitive strategy for the exploration of new highly selective compounds for OSCC. The screening of highly selective compounds should be performed before the identification of the type of cell death (either apoptosis, necrosis or autophagy) and the elucidation of the cell death induction mechanism. However, there are few investigations on the relative toxicity of antitumor agents to both normal and malignant cells. This is true for polyphenols (16, 17), despite the vast literature on their antioxidant and apoptosis-inducing activities. Furthermore, as far as we know, there is no report on autophagic cell death induction in OSCC by antitumor agents.

Considering this background, we report here the selective toxicity and type of cell death induced by various natural and synthetic compounds in OSCC cell lines, mostly based on our data. The tumor-specific cytotoxicity index (TS) was determined by the ratio of the mean 50% cytotoxic concentration (CC_{50}) against normal human oral cells [gingival fibroblast (HGF), pulp cell (HPC), periodontal ligament fibroblast (HPLF)] to that against human oral tumor cell lines [squamous cell carcinoma (HSC-2, HSC-3, HSC-4, NA, Ca9-22), submandibular gland carcinoma (HSG)]. The apoptosis induction was monitored *via* internucleosomal DNA fragmentation, the activation of caspase-3, -8 and -9, the expression of apoptosis-related proteins (Bcl-2, Bax, Bad) and the dysfunction of mitochondrial membrane (assayed with the MitoCapture method). Autophagy was monitored by determining autophagosome formation observed under transmission electron microscopy and fluorescent microscopy after staining with acridine orange or transfection of LC3-GFP staining.

Tumor-specific Cytotoxicity of Natural and Synthetic Compounds

The compounds analyzed were mostly isolated and identified by our groups. The cytotoxic activity and TS values of each group are listed in Table I.

Flavonoid-related phenols. Flavones showed weak cytotoxic activity against HSC-2 cells (CC_{50}=45-375 μg/ml) (Table I). Methoxymethyl ethers having a 5-hydroxyl group were inactive. Chrysin showed specific cytotoxicity to HSC-2 (CC_{50}=63 μg/ml) and HSG cell lines (CC_{50}=156 μg/ml), as compared to HGF (CC_{50}=345 μg/ml), yielding the TS value of 3.2. Since chrysin has both a hydrophilic A-ring and a hydrophobic B-ring (with no hydroxyl group), this selective action might be due to the presence of both hydrophilic and hydrophobic domains in the same molecule (18). Similarly, eugenol (4-allyl-2-methoxyphenol), which has both hydrophilic and hydrophobic groups in the molecule, showed weak cytotoxicity to HSC-2 and HSG cells (19). Many flavonoids have hydrophobic groups (such as a prenyl or geranyl group and 2,2-dimethylpyran ring) and hydrophilic groups (such as a hydroxyl group). Most pryranoflavones and their derivatives, and prenylated or geranylated flavones, were cytotoxic, but showed weak tumor specificity (TS=0.3-2.3). Pyranoflavones, which exhibited higher cytotoxic activity, have a hydrophobic group (3-prenyl or γ-hydroxyisoamyl group) and a hydrophilic group (2'-OH). This 2'-OH group is in the vicinity of a 2,2-dimethylpyran ring (D-ring). Prenylflavonols, which are also highly cytotoxic, have two domains which contain hydroxyl group at *ortho*-position of the prenyl group. This suggests that the presence of both hydrophobic and hydrophilic groups within the molecule is necessary for cytotoxic activity.

Licorice and *Morus* flavonoids in general have both hydrophilic and hydrophobic moieties. For example, gancaonins S and U have a hydrophilic B-ring and hydrophobic prenyl group(s) in the A-ring. Licochalcone B, a chalcone derivative without an isoprenoid group, showed the highest TS value of 31.7. Isoprenoid-substituted chalcone, prenylated isoflavone and genistein had higher cyotoxicity, but without high tumor-specificity, suggesting that the prenylation itself is not necessary for the tumor-specificity (20).

Flavonoids with isoprenoid substituents, sanggenol M, sanggenon C and sanggenon B, had lower TS values (2.3-2.7). More hydrophobic 2-arylbenzofurans exhibited much weaker cytotoxicity and low TS value (1.0-1.5) (21).

Among the benzophenones, compounds with two isoprenoid groups had higher cytotoxicity than the monoprenylated compound, but with small TS values [1.2-1.3]. For the xanthones, 1,3,5,6-tetrahydroxyxanthones, with a 1,1-dimethylallyl group at the C-2 position and a prenyl group on the B-ring, had the highest cytotoxicity. Xanthones gave marginal TS values (1.1->2.0) (22).

Table I. *Comparison of tumor-specificity.*

Group	TS (mean±S.D.)	n[a]	Ref.
Flavonoids			
Flavones, flavonols, isoprenylated flavonoids	1.2±0.6 (0.3～3.2[b])	36	18, 19
Flavonoids	4.0±7.0 (0.8～31.7)	18	20
	3.0±1.0 (1.4～4.0)	5	25
Isoprenylated flavonoids	2.3±0.4 (1.6～3.0)	11	21
2-Arylbenzofurans	1.2±0.2 (1.0～1.5)	6	21
Benzophenones	1.7±0.4 (1.2～2.3)	5	22
Xanthones	1.3±0.4	9	22
Anthraquinones	3.8±4.9 (1.0～18.6)	13	23
Phenylbutanone glucoside	2.4 (1.5～3.3)	2	23
Stilbene glucoside	1.8±0.8 (1.0～2.6)	3	23
Naphthalene glucosides	1.3 (1.1～1.4)	2	23
Isoflavones and isoflavanones	1.9±0.5 (1.1-2.8)	11	24
Stilbenes	3.0±1.2 (1.4～4.7)	6	25
Coumarins	2.4±3.0 (1.0～11.0)	23	27
Tannin-related compounds			
Procyanidins	4.8±2.3 (1.0～7.4)	6	28
Flavonoids	1.1±0.1 (1.0～1.2)	4	28
Monohydrolyzable tannins	1.5±0.5 (1.0～2.5)	7	29
Oligomeric hydrolysable tannins	1.4±0.2 (1.2～1.5)	3	29
Macrocyclic ellagitannins	4.4±2.7 (2.3～8.2)	4	29
Terpens			
Triterpenes	1.5±0.7 (0.7～2.8)	8	31
Triterpene aglycones, glycosides	1.6±0.5 (1.0～2.4)	10	32
Triterpene glycosides	1.2±0.2 (1.0～1.6)	11	32
Triterpenes, triterpene glycosides, chromones	1.0±0.1 (0.8～1.3)	20	32
Cycloartane glycosides	1.1±0.2 (0.9～1.4)	7	32
Furostaol glycosides	2.5±4.1 (0.4～17.0)	17	32
Ketones			
α,β-Unsaturated ketones	1.2±0.3 (0.6～1.9)	26	33
Cyclic α,β-unsaturated ketones	>229.0	1	37
α-Hydroxyketones	5.7±6.0 (1.0～17.6)	8	38
β-Diketones	1.8±1.4 (0.3～6.3)	22	39
Trifluoromethylketones	2.6±1.6	6	41
Azulenequinones	2.6±2.3 (1.0～10.2)	27	42
Tropolones	2.6±1.8 (1.0～9.9)	27	43
Bacterial products			
Anthracyclines	>167±89	4	54
Mitomycin C	>29	1	55
Bleomycin, peplomycin	>3.8	2	55
Nocobactines	62.0 (43.9～80.0)	2	56
Others Azulenes	1.7±1.0 (0.8～5.7)	27	44
Trihaloacetylazulenes	6.5±10.7	26	45
	1.6±0.6	20	46
Berberines	3.8 (3.6～4.0)	2	47
3,5-Dibenzoyl-1,4-dihydropyridines	>43.0 (>33～>53)	2	48
Styrylchromones	7.3±6.1 (1.1～17.4)	6	49
Isoxazole derivatives	1.2±0.2 (0.9～1.6)	24	32

[a]The number of compounds analyzed; [b]range.

Anthraquinones exhibited relatively higher TS values. Among them, emodin and aloe-emodin, without glycosylation, were the most potent (TS=8.5 and >18.6, respectively). On the other hand, other anthraquinone glycosides (TS=1.0->3.4), phenylbutane glucosides (TS=1.5-3.3), glucosides of a stilbene (resveratrol) (TS=1.0-2.6) and naphthalene glucosides (TS=1.1->1.4) were less active. These data suggest that the glycosyl moiety is not required for higher tumor-specificity expressed by anthraquinones (23).

Among 11 isoflavones and isoflavanones from *Sophora* species, tetrapterol G had the highest cytotoxic activity, followed by isophoranone, secundiflorol E, sophoraisoflavanone A, secundiflorol D, secundifloran, secundiflorol A, sophoronol, secundiflorol F, daidzein and genistein. These data suggest that compounds with two isoprenyl groups (one in the A-ring and the other in the B-ring) or α,α-dimethylallyl group at C-5' of the B-ring should have higher cytotoxic activity (24). The cytotoxic activity of these compounds reached its maximum level when log P was around 4 (24). Secundiflorol A had the highest TS value of 2.8, followed by genistein (TS=2.4). Tumor-specificity was not directly coupled with cytotoxic activity. Compounds with an intermediate magnitude of cytotoxic activity had higher TS values.

Stilbenes (resveratrol, piceatannol, rhaponticin), stilbene trimers (sophorastilbene A, (–)-ε-viniferin), a stilbene dimer ((+)-α-viniferin) (TS=1.4-3.6) and flavonoids (kaempferol, fisetin, quercetin, isoliquiritigenin, butein) (TS=1.4-4.7) gave lower TS values. These compounds induced activation of caspase-3, -8 and -9, and internucleosomal DNA fragmentation in HL-60 and HSC-2 cells (25). These data suggest that the stilbene structure is not an important factor in determining tumor specificity or apoptosis induction. Recently, resveratrol, piceatannol, fisetin, quercetin, isoliquiritigenin and butein have been shown to prolong the life-span of *Saccharomycess cerevisiae* through the activation of NAD-dependent protein deacetylase (26).

Coumarins. Coumarin itself and its 7-hydroxy-, 6-methoxy-7-hydroxy and 5,6-dimethoxy- derivatives were relatively non-toxic to all cell lines. Its 6,7-dihydroxy derivatives (esculetin) revealed a tumor cell line-specific cytotoxicity (TS>5.1) (27). Among the eight 4-methylcoumarin derivatives, the most potent and tumor-specific cytotoxicity was observed in the 6,7-dihydroxylated molecule only. Although the TS value for all four *ortho*-dihydroxycoumarins exceeded 5, such was not the case for *ortho*-dihydroxylated reference molecules such as caffeic acid and gallic acid. These data suggest that 6,7-dihydroxycoumarin represents a specific pharmacophore moiety suitable for designing tumor-specific cytotoxic agents. Higher tumor-specificity of 4-methyl (TS>8.3), 3,4-dimethyl (TS>9.3) and 3,4-cycloalkyl derivatives (TS>11.0) than 6,7-dihydroxycoumarin may be due to their higher lipophilicity. If such were the case, it should be possible to design more potent compounds by appropriate substitutions in the 3 and/or 4 positions of the molecule (27).

Tannin-related compounds. Among procyanidines and related flavonols from *Eriobotrya japonica* calli, procyanidin B-2 (MW578) (TS=5.8), procyanidin C-1 (MW866) (TS=>6.7) and procyanidin oligomers (MW3170) (TS>7.4) from intact leaves of *Eriobotrya japonica* had higher cytotoxicity and tumor-specific cytotoxicity against oral carcinoma than did catechin (MW 290) (TS=1.0), (–)-epicatechin (MW290) (TS=4.0) and (–)-epigallocatechin gallate (EGCG) (MW458) (TS=4.1). This suggests that a highly condensed structure contributes to an increase in tumor-specificity (28).

Among hydrolyzable tannins (which contain glucose in the core of the molecule), oligomeric hydrolyzable tannins (gemin A (MW1873), agrimoniin (MW1871), alienamin B (MW1854)) had an approximately two-fold higher cytotoxicity (CC_{50}=0.10-0.17 mM) than monomeric hydrolyzable tannins (pentagalloylglucose (MW941), tellimagrandin I (MW786), tellimagrandin II (MW939), pedunculagin (MW785), chebulagic acid (MW955), casuarinin (MW937), camelliatannin A (MW1056)) (CC_{50}=0.042-0.53 mM). Macrocyclic hydrolyzable tannins (oenothein B (MW1568), woodfordin C (MW1721), camelliin B (MW1721), woodfordin D (MW2506)) exhibited one order higher cytotoxicity (CC_{50}=0.026-0.060 mM) than monomeric hydrolyzable tannins (29). The tumor-specific cytotoxicity of macrocyclic hydrolyzable tannins (TS=2.7-8.2) was 2- or 3-fold higher than monomeric (TS=1.0-2.5) or oligomeric hydrolyzable (TS=1.2-1.5) tannins (29). On the other hand, gallic acid (MW170) (TS=1.1), methylgallate (MW184) (TS=1.3), ellagic acid (MW302) (TS=1.0) and chlorogenic acid (MW354) (TS=1.7) had much lower tumor-specific cytotoxicity (29, 30). Taken together, these data suggest that a more condensed structure, with glucose in the molecule (hydrolyzable tannins) or without (condensed tannins), increases the tumor-specificity.

Terpenoids. Generally, terpenoids had low tumor-specificity. Oleanolic acid showed some tumor-specific cytotoxicity (TS>2.8), due to its lower toxicity to normal cells (31). The 22α-methoxyfurostanol monodesmosides showed selective cytotoxic activities against the tumor cell lines with CC_{50} values ranging from 0.2 to 24 μM, with the highest tumor-specificity (TS=7.4, >17,4). The presence of a hydroxyl group at the C-1β position may enhance the cytotoxicity. Compounds with saturated C-25[27] are far less cyotoxic, indicating that the C-25[27] olefinic group is of importance for potent cytotoxic activity. On the other hand, ursolic acid, 2α-hydroxyursolic acid, maslinic acid, tormentic acid, 2α,19α-dihydroxy-3-oxo-urs-12-en-28-oic acid, 3-O-*cis* and *trans*-*p*-coumaroyltormentic acid (mixture), hyptadienic acid exhibited much lower tumor-specificity (TS=0.7-2.2) (31). Another 62 terpenoids showed little or no tumor-specificity (TS=0.5-2.4) (32).

Ketones. Among 26 α,β-unsaturated ketones, seven simple cycloalkenones showed higher cytotoxicity. The cytotoxic activity declined with the introduction of methyl group at C-3 (β) or the addition of *N*-acetyl-L-cysteine (NAC), an antioxidant, to the culture medium. This suggests that the

cytotoxicity of α,β-unsaturated ketones is generated by the interaction between the C-3 and SH group of targeted molecules (the so-called "non-sterically hindered Michael acceptor"). The introduction of an exocyclic double bond enhanced the cytotoxicity. 4,4-Dimethyl-2-cyclopenten-1-one showed some tumor-specificity (TS=1.9) whereas other unsaturated lactones were moderately tumor-specific (TS=1.0-1.4). 4,4-Dimethyl-2-cyclopenten-1-one reduced the expression of anti-apoptotic protein Bcl-2 protein, but failed to induce activation of caspase-3, -8 or -9 and internucleosomal DNA fragmentation. On the other hand, less active compounds induced these markers (33). This suggests that tumor-specific cytotoxicity does not always correlate with the apoptosis-inducing activity. Recently, codeinone, an oxidative metabolite of codeine containing a α,β-unsaturated ketone backbone, induced cell death in HL-60 and HSC-2 cells, without induction of caspase activation or internucleosomal DNA fragmentation (34). Morphinone, an oxidative metabolite of morphine, similarly induced non-apoptotic cell death, accompanied by a significant reduction of mitochondrial size (35). It is not clear at present whether this resulted from the perturbation of mitochondrial morphogenesis or the fragmentation of mitochondria as recently reported in *Bax*/*Bak* double knockout mouse embryonic fibroblast (36).

Cyclic α,β-unsaturated ketones, such as 3-arylidene-1-(4-nitrophenylmethylene)-3,4-dihydro-1H-naphthalen-2-ones exhibited unusually high tumor-specificity (TS>229) (37). Using this compound as a lead compound, we are pursuing more active derivatives.

Among 8 α-hydroxyketones, deferiprone (TS>17.6), mimosine (TS>7.9), tropolone (TS>4.1) and hinokitiol (TS=10.7) had the highest tumor-specific cytotoxicity. On the other hand, maltol (TS=1.0), kojic acid (TS=1.4), 3-methyl-1,2-cyclopentanedione (TS=1.3), 1,2-cyclohexanedione (TS=1.2) had lower tumor-specificity. Deferiprone induced the decline in the intracellular concentration of Bcl-2, the activation of caspase-3, -8 and -9, and finally led to internucleosomal DNA fragmentation. These activities were diminished by the addition of a non-cytotoxic concentration of $FeCl_3$. In contrast, the DNA fragmentation and caspase activation induced by maltol were both enhanced more than 6 to 7-fold in the presence of $FeCl_3$ (38).

Among 23 β-diketones, 3-formylchromone was the most tumor-specific (TS=6.3), followed by (+)- and (–)-3-(trifluoroacetyl)camphor (TS=4.4), 4,4,4-trifluoro-1-phenyl-1,3-butanedione (TS=3.4) and (–)-3-(trifluoroacetyl)camphor (TS=3.3); others including curcumin were much less active (TS=0.9-2.2). 3-Formylchromone induced internucleomal DNA fragmentation, the activation of caspase-3, -8 and -9, the dysfunction of mitochondrial membrane and the decline of Bcl-2 protein expression. However, (–)-3-(trifluoroacetyl) camphor failed to induce DNA fragmentation and caspase activation. This indicates that tumor-specificity and apoptosis induction do not always correlate with each other, and that antioxidant activity may be responsible for redox-regulation (39). The cytotoxic activity of curcumin was significantly inhibited by the addition of an equimolar concentration of $FeCl_3$; to account for this a chelate complex of curcumin and $FeCl_3$ was proposed using the semiempirical molecular orbital method (CAChe) (40).

Among 6 trifluoromethyl ketones, a-diketone (TS>3.5), and two α-hydroxyketones exhibited some tumor-specificity (TS=4.5, 4.0) (41). These compounds induced autophagic cell death (formation of autophagosome), but not apoptosis (internucleosomal DNA fragmentation, caspase activation) (41).

Among 27 azulenequinone derivatives, 3-phenoxy-1,5-azulenequinone (TS>8.5) and 7-isopropyl-3-(4-methylanilino)-2-methyl-1,5-azulenequinone (TS=10.2) had highest tumor-specificity and apoptosis-inducing activity (caspase activation) (42). Among 27 tropolone derivatives, 5-aminotropolone was the most tumor-specific (TS=9.9) and had the highest apoptosis-inducing activity (43).

Synthetic compounds. Among 27 azulene derivatives, 2-acetylaminoazulene (TS>3.6), diethyl 2-chloroazulene-1,3-dicarboxylate (TS>5.7) and methyl 7-isopropyl-2-methoxyazulene-1-carboxylate (TS=2.4) had the highest tumor-specificity (44). Chlorination of azulene resulted in the elevation of both cytotoxicity and tumor-specificity, while fluorination of the same compound was not so effective (45).

There was no apparent difference between the cytotoxic activity of 2-methoxyazulenes and 2-ethoxyazulenes (46). Trichloroacetylazulenes generally gave higher cytotoxicity and tumor-specificity as compared with the corresponding trifluoroacetylazulenes. Substitution of chlorine, bromine or iodine at the C-3 position further enhanced their cytotoxicity to four tumor cell lines. Among 20 trihaloacetylazulene derivatives, 1-trichloroacetyl-3-bromo-2-methoxyazulene and 1-trichloroacetyl-3-chloro-2-ethoxyazulene had the highest tumor specificity (TS=>3.5 and >2.5, respectively) (46).

Berberine exhibited some tumor-specificity (TS=3.6-4.0) (47). Two 3,5-dibenzoyl-1,4-dihydropyridines had higher tumor-specificity, but only weakly induced apoptosis markers (DNA fragmentation, caspase activation) (48). All 6 styrylchromones had higher cytotoxic activity against tumor cell lines than against normal cells. Styrylchromones, with one to three methoxy groups, had higher tumor-specificity and water solubility (TS=5-17) and induced DNA fragmentation and caspase-3, -8 and -9 activation (49). Twenty four 3-acetyl- and 3-benzoylisoxazole derivatives exhibited much lower tumor specificity (TS=0.9-1.6) (32).

Flavopiridol, a synthetic flavone, induced apoptosis (sub-G1 DNA content, DNA fragmentation, PARP cleavage) *via* activation of *Bcl-x* in OSCC cell lines (50).

Antitumor antibiotics. Doxorubicin, (*8S-cis*)-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyrano-syl)-oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione), is an anthracycline antibiotic isolated from *Streptomyces peucetius* var *caesius* and has the same anthraquinone chromophore and glycoside structure as does daunomycin. The cytotoxicity of doxorubicin appears to be due to its ability to intercalate with DNA (especially with guanine residues) (51), interact with plasma membranes (52) and take part in the oxidation-reduction reactions (53). Doxorubicin has been used for the treatment of cancer of the bladder, breast (in combination with other anticancer agents) and prostate, but is, however, suspected to be a human carcinogen. Doxorubicin, used as a positive control in our screening system, exhibited the highest cytotoxic activity (TS=255.0) (54). It activated caspase-3, -8 and -9 in both HSC-2 and HL-60 cells, but only induced internucleosomal DNA fragmentation in HL-60 cells. Western blot analysis showed that doxorubicin did not significantly change the intracellular concentration of Bcl-2, Bax or Bad in HL-60 cells. Real time PCR analysis showed that HPC cells (normal) expressed the highest amount of mdr1 mRNA, followed by HSC-2 (tumor) > HGF (normal) > HSC-3 (tumor) > HPLF (normal) > HSG (tumor) > HL-60 (tumor). Electron spin resonance (ESR) spectroscopy shows that doxorubicin produced no discernible radical under alkaline conditions (pH 7.4 to 10.5) except at pH 12.5, and did not scavenge O_2^-, NO or DPPH radicals (54). These data suggest that doxorubicin induces tumor-specific cytotoxicity and some, but not all apoptosis markers possibly *via* a radical-independent mechanism, and that mdr1 expression in the tumor cells seems to be unrelated to the tumor-specificity of doxorubicin (54). Combinational treatment using doxorubicin and either one of methotrexate, nocobatcin, vitamin C, sodium 5,6-benzylidene-L-ascorbate (SBA) or sodium fluoride (NaF) produced additive cytotoxicity rather than synergistic action (Suzuki *et al.*, manuscript in preparation). Other anthracyclines, such as mitoxantrone (TS>259) and daunorubicin (TS>164), produced comparable tumor-specificity with doxorubicin (Suzuki et al., manuscript in preparation). Idarubicin exhibited slightly lower cytotoxicity and tumor-specificity (TS=47). On the other hand, the tumor-specificity of other groups of antitumor antibiotics such as mitomycin C (TS>29), bleomycin (TS>3.7) and peplomycin (TS>4.0) was much lower (55). This confirms the antitumor potential of anthracylines for the treatment of OSCC.

Nocobactins NA-a (NBNAa) and NA-b (NBNAb) are mycobactin-like siderophores, which may play a role in the uptake of iron from the proteins of the host by chelation of ferric ion (Fe^{3+}). These compounds exhibited high tumor specificity index (TS=80.0 and 43.9, respectively) (56). The addition of an equimolar concentration of $FeCl_3$ almost completely abrogated the cytotoxicity and changed the absorption spectra of nocobactins. Mass spectrometry and the semiempirical molecular orbital method (CAChe) demonstrated that one molecule of nocobatin produces a chelated complex with one atom of iron, thus diminishing its cytotoxicity (56). Nocobactins are promising compounds for further study of antitumor potential *in vivo*, although their biological activity is significantly affected by Fe^{3+} concentration in both intracellular and extracellular milieu.

Plant extracts. A lignin carbohydrate complex had no apparent cytotoxicity to HGF cells, but was cytotoxic against oral tumor cell lines (HSC-2, HSG). However, due to lower cytotoxic activity against these cells (CC_{50}>1000 μg/mL), the accurate calculation of a TS value was impractical (57). Lignin stimulated the cytotoxic action of sodium ascorbate (58) and cytokines (TNF) (59).

Poly-herbal extracts of Himalaya (HD-12, DLH-3073) exhibited highly tumor-specific cytotoxicity to tumor cell lines (TS=>1070, >106, respectively). These extracts produced radicals under alkaline condition and scavenged O_2^-. The tumor specificity and antioxidant properties suggest their medicinal efficacy (60). The identification of the active principle is essential.

Type of Cell Death

Whether certain compounds induce apoptosis or non-apoptotic cell death depends on the type of cells. Vitamin C, hydrogen peroxide, etoposide, TNF, hyperthermia (43˚C, 30-60 min), UV irradiation (6 J/m^2) induced internucleosomal DNA fragmentation in human myelogenous leukemic cell lines (HL60, ML-1, U-937, THP-1) (Group I), but not in human erythroleukemia (K-562), T-cell leukemia (MOLT4) (61), glioblastoma (T98G, U87MG) or human glioma cell lines (KG-1-C) (Group II) (62). This may partially result from the higher sensitivity of chromatin DNA of Group I cells to endonuclease attack than that of Group II, as revealed in the isolated nucleus system (63). Human glioblastoma cell lines (M059J, M059K, U373-MG, T98G) were committed to autophagy (characterized by autophagosome formation, the accumulation of Agp8p/Aut7p and LC3 (ATG 8 homolog) in autophagosome, and the inhibition of cell death by 3-methyladenine, an autophagic inhibitor), upon exposure to radiation (^{137}Cs) (64), arsenic trioxide (65), ceramide (66) or temozolomide (a new alkylating agent) (67). We found that most of the compounds listed in Table I failed to induce internucleosomal DNA fragmention in OSCC cell lines (HSC-2, HSC-3, HSC-4). This indicates that whether cells are committed to apoptosis or non-apoptotic cell death depends on the type of cell. In fact, anthracyclines, with the highest tumor-specificity, induced non-apoptotic cell death in glioblastoma (68), acute myeloblastic leukemia (69), cardiomyocytes (70) and breast cancer cells (71). In general, OSCC cells are relatively resistant to Fas-mediated apoptosis;

this may be due to a lower expression of FAS (5) or the cellular FLICE-inhibitory protein (c-FLIP) (72). A cyclooxygenase (COX)-2 inhibitor (NS398) induced G0/G1 arrest, but no apoptosis in OSCC cells (73). We recently found that while 1-trichloroacetyl-3-bromo-2-methoxyazulene and 1-trichloroacetyl-3-chloro-2-ethoxyazulene induced apoptotic cell death (caspase-3, -8 and -9 activation, internucleosomal DNA fragmentation) in HL-60 cells, they induced autophagic cell death characterized by lower activation of caspases, a lack of DNA fragmentation, vacuolization and autophagosome formation as detected with acridine orange and LC3-GFP fluorescence (46), suggesting the diversity of cell death type induced in human tumor cell lines.

Another factor that determines the type of cell death is the kind of compounds that is used. For example, epigallocatechin gallate (EGCG) induced apoptosis cell death (characterized by DNA strand break detected with the TUNNEL method, and the appearance of degradation products of cytokelatin 18), whereas 5,6-benzylidene-L-ascorbate (SBA) did not (74). α,β-Unsaturated ketones (2-cyclohexen-1-one, 2-cyclopenten-1-one, 4,4-dimethyl-2-cyclopenten-1-one, 2-cyclohepten-1-one, α-methylene-γ-butyrolactone, 5,6-dihydro-2H-pyran-2-one, methyl 2-oxo-2H-pyran-3-carboxylate, codeinone, morphinone) (33, 34) and 3,5-dibenzoyl-1,4-dihydropyridines (48) activated caspase only marginally in HL-60 cells. Unexpectedly, 3,5-benzoyl-1,4-dihydropyridines enhanced the expression of Bcl-2 anti-apoptotic protein. This unbalanced Bcl-2 expression suggests the occurrence of autophagic cell death, since autophagic cell death is not always inhibited by Bcl-2 (75).

Other Considerations

The growth of tumor OSCC cells tends to be inhibited under hypoxic conditions, due to the induction of apoptosis (cytochrome c release from mitochondria), which was reversed by overexpression of hypoxia-inducible factor-1α (HIF-1α) (76). Vitamin C, which induces hypoxia (77), may selectively damage tumor cells. We recently found that NaF induced apoptotic cell death in HL-60 (caspase activation, internuclesomal DNA fragmentation) and incomplete apoptosis (accumulation of Bad-GFP protein in the mitochondrial fraction of nuclear periphery, without the induction of internucleosomal DNA fragmentation) (78). We found that external pressure, such as centrifugal force and water pressure (used as *gausi*-orthodontic force), enhanced the cytotoxicity of NaF to both tumor and normal cells (79, 80).

There was a considerable variation (approximately 10～20-fold) in drug-sensitivity of 5 OSCC cell lines (55). The sensitivity to mitomycin C is in the following order (from sensitive to resistant): HSC-2 (CC_{50}=3.5 μM) > HSC-3 (CC_{50}=9.7 μM) > Ca9-22 (CC_{50}=16.4 μM) > HSC-4 (CC_{50}=18.0 μM) > NA (CC_{50}=37.8 μM). The sensitivity to bleomycin is in the following order: HSC-2 (CC_{50}=4.6 μM) > HSC-3 (CC_{50}=6.3 μM) > HSC-4 (CC_{50}=77.4 μM) > NA (CC_{50}=91.6 μM) > Ca9-22 (CC_{50}=111.6 μM). The sensitivity to peplomycin is in the following order: HSC-2 (CC_{50}=9.9 μM) > HSC-3 (CC_{50}=25.2 μM) > NA (CC_{50}=143.2 μM) > HSC-4 (CC_{50}=175.8 μM) > Ca9-22 (CC_{50}=216.9 μM).

Table II. *Factors affecting cytotoxicity and tumor-specficity.*

Origin	Factor
Compound	Coexistence of hydrophilic and hydrophobic groups
	Presence of an isoprenyl group
	Presence of a halogen
	Polycyclic structure
	Highly condensed structure
	Lipophilicity
Cell	Expression of multidrug resistant protein
	Expression of drug metabolizing enzyme
Environment	Serum type
	Metal ion presence
	Oxygen concentration
	External pressure

Morphological observation is a useful technique to distinguish autophagy from apoptosis. On the other hand, molecular biological observation for this purpose has not yet been established. The natural polyamines putrescine, spermidine and spermine are involved in cell growth and the maintenance of cell viability (81). Following inhibition of ornithine decarboxylase, a key enzyme of polyamine biosynthesis, numerous links have been identified between the polyamine and apoptotic pathways (81). We recently found that the intracellular concentration of putrescine declined at the early stage (first 0-3 hours after treatment) of apoptosis induced in HL-60 cells by ascorbic acid, SBA (82), EGCG or etoposide (83), or mastic (Chios Gum) (84), while that of other polyamines (spermidine, spermine) remained almost unchanged. The decline of putrescine seems to be coupled to apoptosis, since putrescine levels did not change significantly during autophagic cell death induced in HSC-4 cells by either 1-trichloroacetyl-3-bromo-2-methoxyazulene or 1-trichloroacetyl-3-chloro-2-ethoxyazulene (46). At present, whether the decrease of putrescine at an early stage of apoptosis is due to the reduced synthesis or enhanced degradation of this polyamine is not clear. Further studying with a variety of cells and inducers under a wide range of incubation times is necessary to confirm whether putrescine is truly an apoptosis marker or not.

Conclusion

This review reveals that many factors affect cytotoxicity and tumor-specificity (Table II). Factors in the test compounds include the co-existance of both hydrophilic and

hydrophobic groups in the same molecule, the presence of an isoprenyl group, a halogen and/or a polycyclic structure, a highly condensed structure, and lipophilicity. In the cells themselves, the expression of multidrug resistant proteins and drug metabolizing enzymes play an important role. Factors in the environment include the type of serum, oxygen concentration, metallic ion presence/concentration, external pressure. Systematization of the relationship between various factors mentioned above and tumor-specificity may contribute to the quest for more active compounds.

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