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Review

Search for New Type of Anticancer Drugs with High Tumor Specificity and Less Keratinocyte Toxicity

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Abstract. *Most current anticancer drugs have shown excellent therapeutic effects on human oral squamous cell carcinoma (OSCC), but they also produce potent cytotoxicity in normal oral keratinocytes. This review article summarizes our extensive research of tumor specificity and keratinocyte toxicity of nine groups of compounds synthesized in our laboratory. Among a total of 133 compounds, (E)-3-[2-(4-hydroxyphenyl)ethenyl]-6-methoxy-4H-1-benzopyran-4-one **[3]** (classified as 3-styrylchromones), (E)-3-[2-(4-chlorophenyl)ethenyl]-7-methoxy-2H-1-benzopyran [4] (classified as 3-styryl-2H-chromenes) showed the highest tumor specificity with the least keratinocyte toxicity. Compound **[3]** induced apoptotic cell death in a human OSCC cell line, possibly by down-regulating the glycerophospholipid pathway. Quantitative structure–activity relationship analysis demonstrated that the tumor specificities of **[3]** and **[4]** were well correlated with chemical descriptors related to their molecular size and lipophilicity. Chemical modification of these lead compounds by introduction of appropriate functional groups is a crucial step towards manufacturing new types of anticancer drugs with reduced keratinocyte toxicity.*

Previous studies have focused on the mechanism of apoptosis induction by anticancer drugs rather than the demonstration of their tumor specificity. Most anticancer drugs induce similar morphological changes to those observed during the developmental process (eliminating unnecessary tissues and harmful cells).

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Problems of Current Anticancer Drugs

It is well known that administration of anticancer agents induces skin toxicity (1-7). This prompted us to re-evaluate the cytotoxicity and tumor specificity of anticancer drugs. For this purpose, we established an *in vitro* assay system, using four human oral squamous cell carcinoma (OSCC) cell lines (Ca9-22, HSC-2, HSC-3 and HSC-4), three human mesenchymal normal oral cells [gingival fibroblasts (HGFs), pulp cells (HPCs), periodontal ligament fibroblasts (HPLFs)] and two human epithelial normal oral cells [buccal mucosal human oral keratinocytes (HOKs) and primary human gingival epithelial cells (HGEPs)] (Figure 1). Cells were incubated for 48 h with increasing concentrations of test agents, and the relative viable cell number was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Tumor-selectivity index (TS) was determined by dividing the mean of the concentration that reduced the viable cell number by 50% (CC_{50}) of each agent against normal cells by its mean CC_{50} against tumor cells (Figure 2A).

We first investigated the tumor specificity of anticancer drugs using OSCC cell lines and normal mesenchymal cells (MCs) (referred to as System 1). Many anticancer drugs, such as docetaxel, 5-fluorouracil (5-FU), methotrexate, mitomicin C, etoposide, daunorubicin, doxorubicin, SN-38 (active metabolite of irinotecan), camptothecin and gefitinib, showed excellent specificity (TS=10-1000) (Figure 2B) (8). This validated the present method for evaluating anticancer activity.

We next evaluated the tumor specificity of anticancer drugs using OSCC cell lines and normal epithelial cells (ECs.) (referred to as System 2). In this case, anticancer

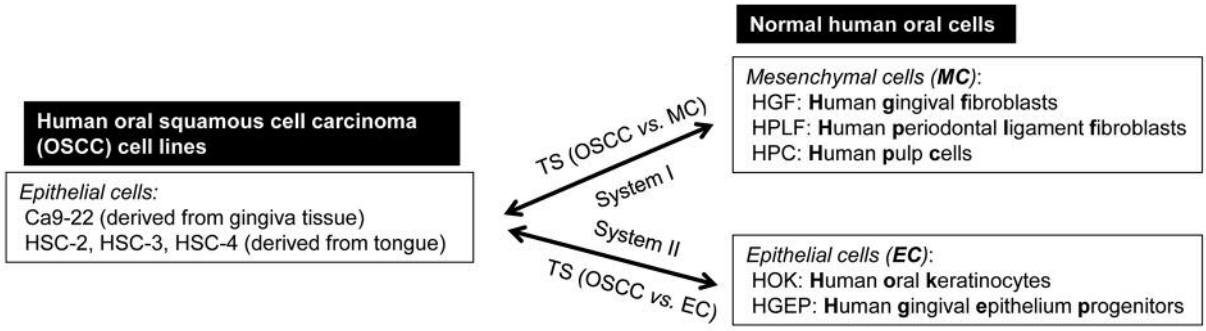


Figure 1. *Cells used for calculation of CC_{50} and tumor specificity (TS) value of previously investigated compounds.*

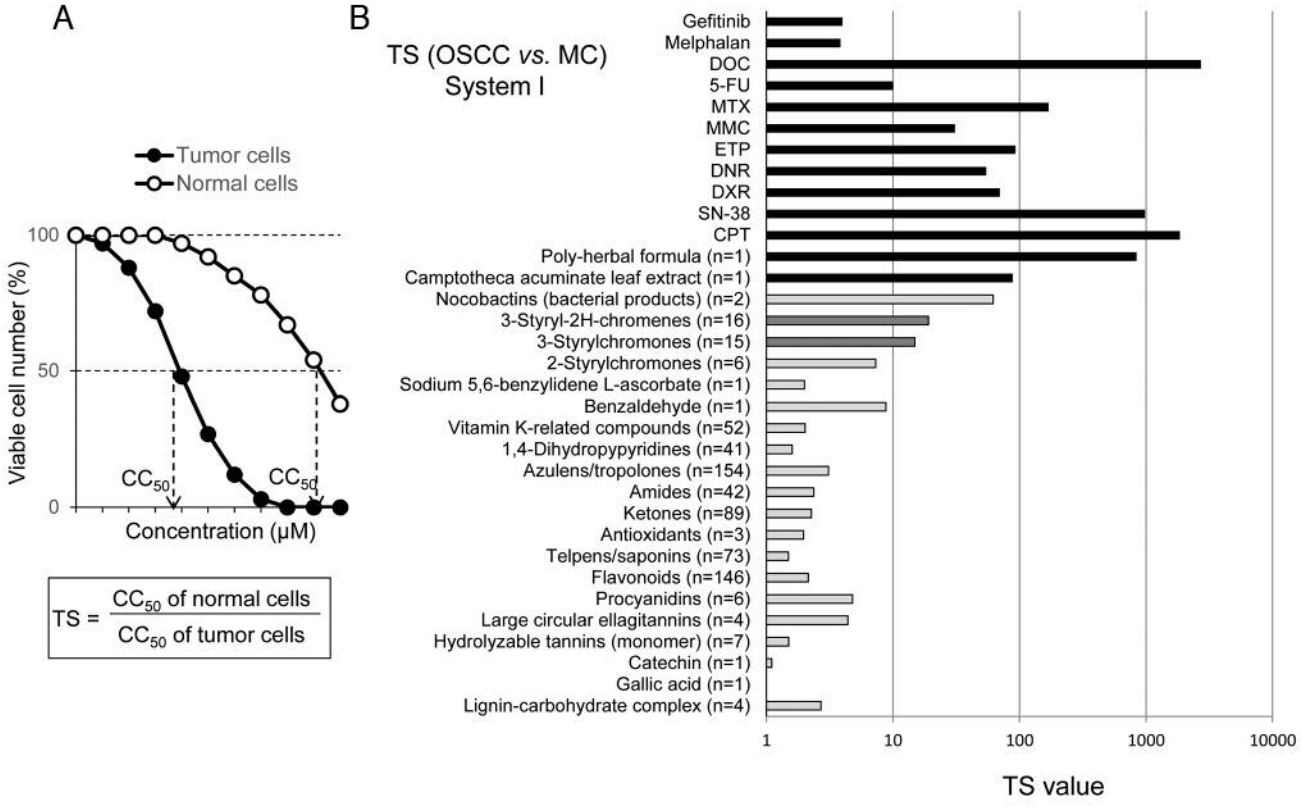


Figure 2. *A: Exemplary calculation of tumor specificity (TS) value using the concentration that reduced the viable cell number by 50% (CC_{50}) values for tumor and normal cells. B: Comparison of TS values for antitumor agents and various natural and synthetic compounds. Data are cited from reference (8).*

agents showed cytotoxicity to both of these cell types to comparable extents, producing a much lower TS value (9). It was unexpected that doxorubicin would induce apoptosis [loss of cell surface microvilli, chromatin condensation, nuclear fragmentation (Figure 3A) and caspase-3 activation (Figure 3B)] in HOKs (9). It is therefore imperative to explore new anticancer drugs with less keratinocyte toxicity.

In Search of Antitumor Agents with Less Toxicity to Keratinocytes

We have synthesized a total of 133 compounds, which are classified into nine groups, by introducing various functional groups into distinct backbone structures (Figure 4). The most potent compound in each group was: (2*E*)-3-(3,4-dihydroxy-

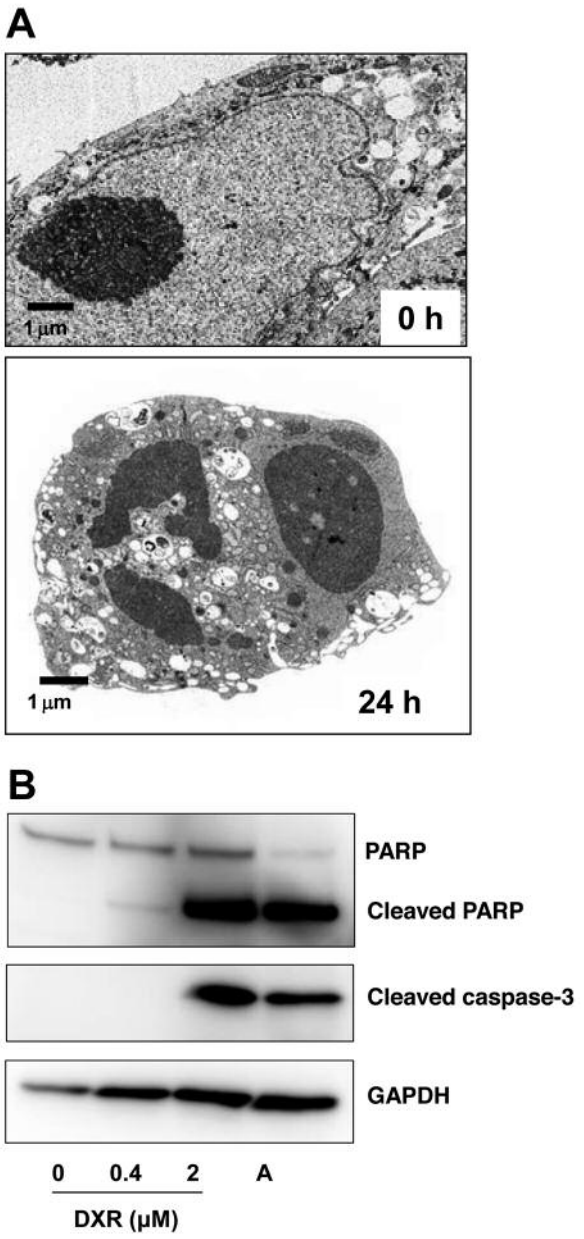


Figure 3. *Keratinocyte toxicity induced by doxorubicin (DXR). HSC-2 cells were incubated for 0 or 24 h with 10 μM doxorubicin (A), or with different concentrations of doxorubicin (B), and then processed for observation under transmission electron microscopy (A) or subjected to western blot analysis (B). Cited from (9) with permission. PARP: Poly (ADP-ribose) polymerase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.*

phenyl)-*N*-[2-(4-hydroxyphenyl)ethyl]-2-propena-mide [**1**] (among 12 phenylpropanoid amides) (10), (2*E*,4*E*)-*N*-[2-(3,4-dihydroxyphenyl)ethyl]-5-(3,4-methylenedioxyphenyl)-2,4-pentadienamide [**2**] (among 12 piperic acid amides) (11), (*E*)-3-[2-(4-hydroxyphenyl)ethenyl]-6-methoxy-4*H*-1-benzopyran-4-one [**3**] (among 15 3-styrylchromones) (12, 13), (*E*)-3-[2-(4-chlorophenyl)ethenyl]-7-methoxy-2*H*-1-benzopyran [**4**] (among 16 3-styryl-2*H*-chromenes) (14), (*Z*)-*N*-[2-(3,4-dihydroxyphenyl)ethyl]-9-octadecenamide [**5**] (among 18 oleoylamides) (15), (3*E*)-2,3-dihydro-3-[(3,4-dihydroxyphenyl)methylene]-7-methoxy-4*H*-1-benzopyran-4-one [**6**] (among 17 3-benzylidenechromanones) (16), (2*E*)-1-(2,4-dimethoxyphenyl)-3-(4-methoxyphenyl)-2-propen-1-one [**7**] (among 15 chalcones) (17), (2*E*,4*E*)-5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid (4-methoxyphenyl)methyl ester [**8**] (among 11 piperic acid esters) (18) and (2*Z*)-2-[(4-hydroxyphenyl)methylene]-3(2*H*)-benzofuranone [**9**] (among 17 aurones) (19).

Among these nine compounds, [**3**] and [**4**] had the highest TS values (69.0 and 59.9, respectively), as assessed with System I [OSCC *vs*. MCs]. Their TS values (59.9-69.0) were comparable with that of doxorubicin (63.7±95.0) and 5-FU (13.1±21.1), but greatly exceeded that of resveratrol (TS=2.4), a stilbene with anticancer activity (20) (Figure 4).

Compounds [**3**] and [**4**] also had the highest TS values when assessed with System II (OSCC *vs*. ECs: 204.5 and >85.1, respectively) (Figure 4). Their TS values were much higher than those of doxorubicin (1.7±1.9) and 5-FU (1.4±1.1) (Figure 4).

Treatment of HSC-2 cells with [**4**] induced mitochondrial vacuolization and inhibition of autophagy (as evidenced by loss of microtubule-associated protein 1A/1B-light chain 3 (LC3)-II at an early stage, followed by the induction of apoptosis (as evidenced by cleavage of poly (ADP-ribose) polymerase and caspase-3). Compound [**4**] increased the intracellular levels of diethanolamine and cytidine diphosphate-choline, whereas it reduced the level of choline, suggesting down-regulation of the glycerophospholipid pathway (13).

Estimation of TS by Chemical Descriptors

Six descriptors that correspond greatly with cytotoxicity against normal cells (N) and tumor cells (T), and with tumor specificity (T−N) of nine groups of compounds are listed in Table I. Generally, these descriptors did not overlap with each other. Tumor specificity of 3-styrylchromones was well corrected with molecular size (12). T−N can be estimated by molecular diameter (largest value in the distance matrix defined by the elements Dij), vsurf_DD23 (interaction with hydrophobic probe assumed surrounding the molecule) and R3 OH (4'-hydroxy substitution in the phenyl group of styryl moiety) as: T−N=0.607(±0.169)diameter − 0.121 (±0.035)vsurf_DD23 + 1.11 (±0.235)R3OH − 7.17 (±2.26), with n=15, R^2=0.764, Q^2=0.570. s=0.308 (Figure 5A).

Tumor selectivity of 3-styryl-2*H*-chromenes correlated well with six descriptors (std_dim3, BCUT_SLOGP_1, vsurf_D4, vsurf_R, vsurf_D5 and E_oop) which reflect structure connectivity and conformation, hydrophobicity, surface rugosity and out-of-plane potential energy (Table I). The T−N value of [**4**] can be estimated using two descriptors (vsurf_R and E_oop)

Table I. *Chemical descriptors that correlate with 50% cytotoxic concentration (CC_{50}) against OSCC (T), normal oral cells (N) and tumor specificity (T−N) of previously investigated compounds.*

Category	T	N	T-N	Ref.
Phenylpropanoid amide	G^o	Acc. Polar area	Surface area	10
	S^o	HOMO	Max ElPot	
	H^o	Hardness		
	Surface area	Log P (o/w)		
	Ovality	HBA coung		
	HBA count			
Piperic acid amides	PEOE_VSA_NEG	vsurf_IW7	vsurf_IW8	11
	PEOE_VSA_FPOS	vsurf_EWmin1	PEOE_VSA_POL	
	PEOE_VSA_FNEG	vsurf_HB7	PEOE_VSA+4	
	log P (o/w)	vsurf_W7	PEOE_VSA_PPOS	
	a_hyd	vsurf_HB6	PEOE_VSA_PNEG	
	log S	PEOE_VSA_FPOS	a_nO	
3-Styrylchromones	OMe at R1	vsurf_DD23	OH at R3	12, 13
	OH at R3	G1u	vsurf_DD23	
			G2u	
3-Styryl-2*H*-chromenes	chi1v	std_dim2	std_dim3	14
	KierFlex	E_tor	SCUT_SLOGP_1	
	KierA1	E_oop	vsurf_D4	
	SMR_VSA7	std_dim3	vsurf_R	
	KierA3	vsurf_A	vsurf_D5	
	Weight	SCUt_SMR_1	E-oop	
Oleoylamides	PEOE_VSA-0	lip_don	AM1_dipole	15
	PM3_HOMO	E_tor	MNDO_dipole	
	PM3_IP	SlogP_VSA0	PM3_HOMO	
	SlogP_VSA0	Q_VSA_PPOS	PM3_IP	
	lip_don	BCUT_PEOE_0	dipole	
	PEOE_VSA+2	b_single	log(o/w)	
3-Benzylidenechromanones	RDF095i	Mor03v	Mor3m	16
	RDF095u	Mor03m	Mor03v	
	RDF095e	Mor09m	SpMAD_AEA	
	vsurf_IW6	G1u	vsurf_HB7	
	vsurfID7	Mor03p	R3m+	
	vsurf_ID1	R3m+	Mor25v	
Chalcones	HATS6p	CAT3D_10_DA	R6p	17
	vsurf_IW6	RDF010s	R5v+	
	R6v	Q_VSA_PNEG	L3m	
	R6p	PEOE_VSA-6	VE2_H2	
	h-logS	B10[O-O]	L3s	
	RDF035u	F10[O-O]	HATS6p	
Piperic acid esters	R5i+	CATS3D_17_AA	vsurf_DW13	18
	R5u+	rsynth	HATS5i	
	R2e+	CATS3D_13_AL	TDB08u	
	SPH	CATS2D_04_AL	TDB08i	
	JGI10	CATS3D_16_AA	TDB08e	
	Dp	JG10	MATS5v	
Aurones	E1u	vsurf_IW5	HATS6p	19
	RDF010s	MLOGP	R6p	
	CATS3D_04_DL	MLOGP2	R6v	
	Mor05i	HATS2p	Gm	
	vsurf_HB4	GATS1e	G3m	
	vsurf_CW4	O%	HATS6v	

Red	Molecular size
Blue	Electrostatic
Green	Lipophilicity

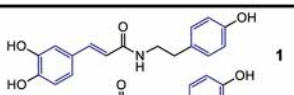
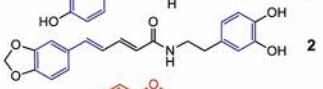
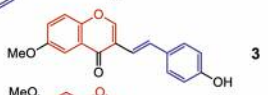
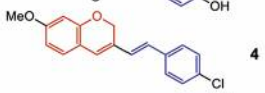
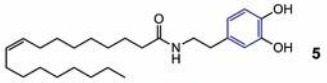
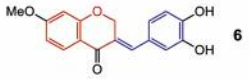
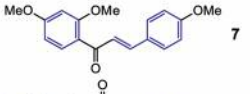
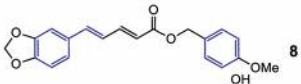
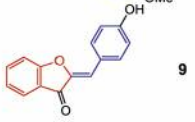
Classification	Number of compounds tested	Most potent compound	TS value OSCC *vc.* MC (System I)	TS value OSCC *vs.* EC (System II)	Ref.
Phenylpropanoid amides	12	 1	>3.4		10
Piperic acid amides	12	 2	>10.7		11
3-Styrylchromones	15	 3	69.0	204.5	12.13
3-Styryl-2*H*-chromenes	16	 4	59.9	>85.1	14
Oleoylamides	18	 5	15.5	2.3	15
3-Benzylidenechromanones	17	 6	>55.2	0.5	16
Chalcones	15	 7	>8.6		17
Piperic acid esters	11	 8	>10.5		18
Aurones	17	 9	>9.7		19
	Total number (133)				
Doxorubicin			63.7 ±95.0	1.7 ±1.9	
5-FU			13.1±21.1	1.4±1.1	
Resveratrol			2.4		

Figure 4. *Nine groups of compounds synthesized by chemical modification of back-bone structures present in naturally occurring compounds. OSCC: Oral squamous cell carcinoma; TS: tumor specificity; MC: mesenchymal cells; EC: epithelial cells.*

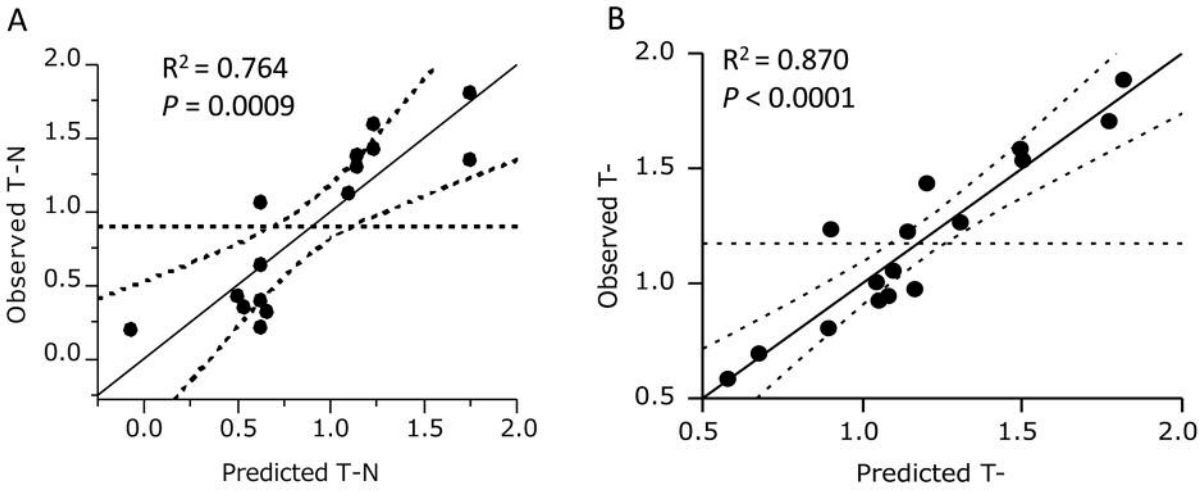


Figure 5. *Multiple regression models for the estimation of the difference in cytotoxicity between tumor and normal cells (T−N) of 3-styrylchromones (A) and 3-styryl-2H-chromenes (B). Data are cited from references (12) and (14).*

as: T−N=32.1(±4.39)vsurf_R + 121(±17)E_oop − 46.1(±6.2), with n=16, R^2=0.870, Q^2=0.821, s=0.145 (Figure 5B).

Future Directions

In our work, we have demonstrated that [**3**] and [**4**] are two new compounds that showed the highest TS and potency-selectivity expression values among the compounds tested, and they exhibited much less keratinocyte toxicity compared to doxorubicin and 5-FU (Figure 4). Chemical modification of these lead compounds by introduction of appropriate functional groups is a crucial step towards manufacturing new types of anticancer drugs with reduced keratinocyte toxicity.

Conflicts of Interest

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

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