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RESEARCH ARTICLE

Carbonic anhydrase inhibition and cytotoxicity studies of Mannich base derivatives of thymol

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Abstract

Mannich bases of thymol were synthesized. The aminomethylation reaction was realised in the *ortho* position of the phenol for compounds **2** (dipropylamine), **3** (benzylamine), and **4** (dibenzylamine) while it was from *para* position for **1** (dimethylamine), **5** (piperidine), **6** (morpholine) and **7** (*N*-methylpiperazine). The carbonic anhydrase (CA, EC 4.2.1.1) inhibitory effects of the compounds were asssessed against hCA I and hCA II. All compounds moderately inhibited hCA I and hCA II. The cytotoxicity of the compounds against four human oral squamous cell carcinoma cell lines were compared those against three normal oral cells. Tumor specificity values were about 2 or slightly more for the compounds **2**, **3**, **4**, **5** and **6**. Compound **2** showed cytostatic activity against OSCC cell lines at 16 to 32-fold lower concentrations as compared with normal cells. This suggests that compound **2** can be considered as cytotoxicity enhancing drug candidate for further investigations.

Keywords

Carbonic anhydrase, cytotoxicity, Mannich bases, phenol, thymol

History

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Introduction

Carbonic anhydrase (CA, EC 4.2.1.1), a zinc-dependent metalloenzyme, catalyzes the reversible hydration of CO_2[1]. This enzyme plays an important role in physiologic and pathologic processes, such as pH and CO_2 homeostasis, electrolyte secretion, lipogenesis, ureagenesis and calcification[2]. Sixteen different isoforms are known in mammals which are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), membrane-bound (CA IV, CA IX, CA XII, CA XIV and CA XV), mitochondrial (CA VA and CA VB), or secreted (CA VI) proteins[2,3]. There are known diuretics, antiepileptic, antiglaucoma, anticancer drugs based on CA inhibitors (CAIs) and they target different human α-CA isoforms[2]. Recently, it has been showed that these enzymes are also present in protozoa, bacteria and fungi[1]. Therefore, these enzymes are the target of potential antibacterial, antimalarial and antifunfal agents[4].

Some primary sulfonamides with CA inhibitory action and their derivatives have been used for a long time in the treatment of some diseases because of their diuretic and antiglaucom effects[1,2]. Commercially available CAIs, such as acetazolamide, ethoxzolamide and dorzolamide are also known for their potential anticonvulsant[2], anticancer[3], analgesic[1] and antiinfective[2] activities. But the compounds mentioned above are not selective inhibitors of CAs and can cause some side effects[2].

The CA inhibitory effects of some natural products and their derivatives have been recently investigated[5]. It was reported that natural compounds carring phenol or polyphenol moiety had carbonic anhydrase inhibitory activity at micromolar concentration[6]. In addition, the compounds having phenol functional group are known to have anticancer, antibacterial, antiviral, antifungal and antioxidant activities[7–9].

Thymol, 2-isopropyl-5-methylphenol, is a bioactive natural phenolic compound which was isolated from *Thymus sulgaris*[10]. Thymol is known to show antioxidant[11–13], anticancer[14,15], antidepressant[16], antibacterial[17,18] and antifungal[19] activities.

Mannich bases are an important group of compounds in medicinal chemistry and may be synthsized by Mannich reaction. There are several type of Mannich bases, such as carbon Mannich bases and nitrogen Mannich bases[20]. Phenolic Mannich bases are a group of carbon Mannich bases[20]. CAs inhibition[9,21,22], cytotoxic[8,23–25], anticonvulsant[26–28], diuretic[29], antifungal[30] and antioxidant[10] activities of several Mannich bases had been reported.

The aims of this study were as follows:

(i) We aimed to synthesize several phenolic mono Mannich bases of thymol by using acyclic and cyclic amines having different chemical structure, properties and pKa values, which may govern the chemical reactions and bioactivities.
(ii) To investigate the CA inhibitory activity of the compounds against the human (h) isoforms hCA I and hCA II since it is

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a known phenol function, which is a group of carbonic anhydrase inhibitors. There is no study reporting the carbonic anhydrase inhibitory activities of mono Mannich bases of natural compound thymol.

(iii) To investigate the cytotoxic activities of thymol mono Mannich bases against four human oral squamous cell carcinoma cell lines: Ca9-22 (derived from the gingival tissue), HSC-2, HSC-3 and HSC-4 (derived from the tongue) as compared with three normal oral mesenchymal cells: HGF (human gingival fibroblasts), HPC (human pulp cells) and HPLF (human periodontal ligament fibroblasts). The results to be obtained may be useful for new drug candidate/s development.

Experimental

Materials

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were taken using a Varian Mercury Plus spectrometer (Varian Inc., Palo Alto, CA). Chemical shifts (δ) were reported in ppm. Melting points were determined using an Electrothermal 9100 (IA9100, Bibby Scientific Limited, Staffordshire, UK) instrument and are uncorrected. Liquid chromatography ion trap-time of flight tandem mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source, operating in both positive and negative ionization mode. Shimadzu's LCMS Solution software was used for data analysis.

Methods

*Synthesis of mono Mannich bases, **1–7***

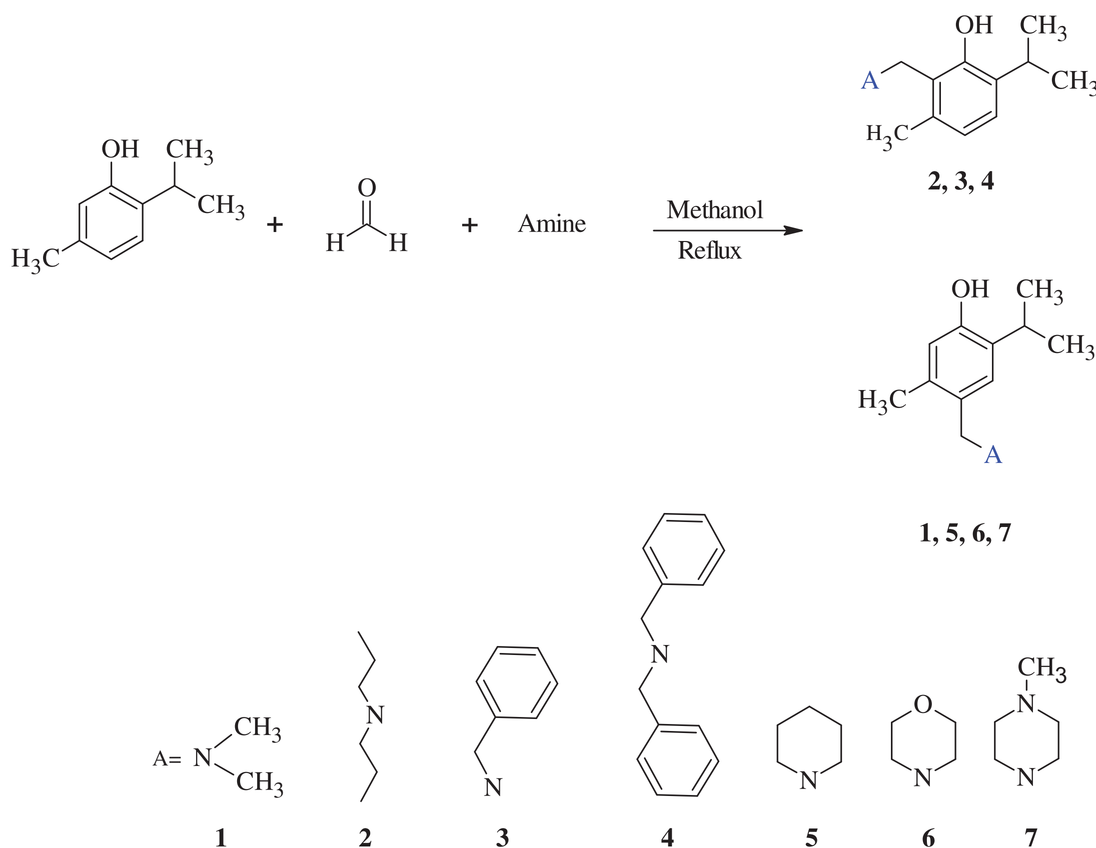
The mixture of thymol (6.6 mmol), formaline (6.6 mmol, 37%) and amine (6.6 mmol) [Dimethylamine (**1**), dipropylamine (**2**), benzylamine (**3**), dibenzylamine (**4**), piperidine (**5**), morpholine (**6**) and *N*-methylpiperazine (**7**)] was heated in methanol (15 ml) for 15-26 h (Scheme 1). Reactions were monitored by TLC. When the reaction was stopped, the solvent was reduced half of volume under vacuum. The content was kept at +4°C for 24 h. The solids obtained [for the compounds **5**, **6** and **7]** were crystallized from suitable solvents [it was dipropylether (**5**) and acetonitrile (**6** and **7**)]. On the other hand, the compounds **1**, **2**, **3** and **4** were purified by column chromatography on silica gel (SiO_2) using chloroform as a mobile phase.

*5-Methyl-4-[(dimethylamino)methyl]-2-(propan-2-yl)phenol, compound **1***

A viscous liquid, yield 15%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.99 (s, 1H), 6.37 (s, 1H), 3.33 (s, 2H), 3.20-3.15 (m, 1H), 2.31–2.20 (m, 9H), 1.23–1.20 (m, 6H).

*3-Methyl-2-[(dipropylamino)methyl]-6-(propan-2-yl)phenol, compound **2***

A viscous liquid, yield 6%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.99 (d, $J = 7.7$ Hz, 1H), 6.60 (d, $J = 7.7$ Hz, 1H), 3.75 (s, 2H), 3.33-2.26 (m, 1H), 2.49-2.45 (m, 4H), 2.21 (s, 3H), 1.62-1.53



Scheme 1. Synthesis of the Mannich bases **1–7**.

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(m, 4H), 1.21 (d, $J = 7.2$ Hz, 6H), 0.90 (t, $J = 7.2$ Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 156.1, 133.7, 133.5, 124.5, 120.6, 119.9, 55.7, 54.4, 26.6, 22.9, 19.8, 12.1. HRMS (ESI-MS) Calc. for $C_{17}H_{29}NO$ $[M+H]^+$ 264.2322; found 264.2346.

*3-Methyl-2-[(benzylamino)methyl]-6-(propan-2-yl)phenol, compound **3***

A viscous liquid, yield 10%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.39-7.28 (m, 5H), 7.01 (d, $J = 8.1$ Hz, 1H), 6.72 (d, $J = 8.1$ Hz, 1H), 3.91(s, 2H), 3.89 (s, 2H), 3.28–3.25 (m, 1H), 2.08 (s, 3H), 1.23 (d, $J = 6.6$ Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 151.6, 138.6, 133.9, 133.7, 129.3, 128.7, 127.6, 123.8, 121.8, 118.4, 81.6, 56.2, 48.8, 26.5, 22.9, 18.3. HRMS (ESI-MS) Calc. for $C_{18}H_{23}NO$ $[M+H]^+$ 270.1852; found 270.1855.

*3-Methyl-2-[(dibenzylamino)methyl]-6-(propan-2-yl)phenol, compound **4***

A viscous liquid, yield 6%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 11.40 (brs, 1H, –OH), 7.38–7.27 (m, 10H), 7.00 (d, $J = 7.9$ Hz, 1H), 6.63 (d, $J = 7.9$ Hz, 1H), 3.76 (s, 2H), 3.62 (s, 2H), 3.58 (s, 1H), 3.49 (d, $J = 8.4$ Hz, 2H), 3.35–3.33 (m, 1H), 2.24 (s, 2H), 1.24 (d, $J = 6.6$ Hz, 6H).

*5-Methyl-4-[(piperidin-1-yl)methyl]-2-(propan-2-yl)phenol, compound **5***

A white solid, yield 12%, m.p. 148 °C, 145 °C[31]. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.99 (s, 1H), 6.26 (s, 1H), 3.32 (s, 2H), 3.16 (q, $J = 6.9$ Hz 1H), 2.42 (brs, 4H), 2.18 (s, 3H), 1.60–1.55 (m, 4H), 1.43 (brs, 2H), 1.21 (d, $J = 6.9$ Hz, 6H).

*5-Methyl-4-[(morpholino-4-yl)methyl]-2-(propan-2-yl)phenol, compound **6***

A white solid, yield 16%, m.p. 152 °C, 152 °C[10]. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.00 (s, 1H), 6.48 (s, 1H), 3.69 (brs, 4H), 3.38 (s, 2H), 3.16–3.13 (m, 1H), 2.44 (brs, 4H), 2.26 (s, 3H), 1.22 (d, $J = 6.9$ Hz, 6H).

*5-Methyl-4-[(N-methylpiperazin-1-yl)methyl]-2-(propan-2-yl)phenol, compound **7***

A white solid, yield 29%, m.p. 159 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.00 (s, 1H), 6.45 (s, 1H), 3.38 (s, 2H), 3.21–3.14 (m, 1H), 2.47 (brs, 8H), 2.29 (s, 3H), 2.24 (s, 3H), 1.22 (d, $J = 6.9$ Hz, 6H).

Biological activity

Carbonic anhydrase enzyme assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity[32]. Phenol red (at a concentration of 0.2 mM) has been used as an indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition percentages were obtained by using PRISM 3, as reported earlier[33], and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier[34]. The cell pellets were lysed, and hCA II and hCA I were purified through affinity chromatography using pAMBS resin.

Cytotoxicity evaluation

The cytotoxicity of the compounds were assayed towards human oral squamous cell carcinoma (Ca9-22, HSC-2, HSC-3, HSC-4) and human normal oral cells (HGF, HPLF, HPC) as described[35] with some minor modifications. In brief, all cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). The following concentrations of the compounds in dimethylsulfoxide (DMSO) were added to the medium and incubated at 37°C for 48 h: compounds **1–7** (0.32, 1, 3.2, 10, 31.6, 100, 316, 1000 μM) and Melphalan (3.12, 6.25, 12.5, 25, 50, 100, 200, 400 μM). The media that contained the same concentration of DMSO (0.0078, 0.156, 0.03125, 0.0625, 0.125, 0.25, 0.5 or 1%) were used as controls, since DMSO above 0.25% is cytotoxic. The viable cell numbers were determined by the MTT method. The CC_{50} values were determined from dose-response curves.

Results and discussion

The compounds investigated here were synthesized by the conventional heating method. The mixture of thymol, formalin solution and amine was refluxed in methanol; the reagents had the same mole ratios. Although the same experimental procedure was applied for the synthesis of phenolic mono Mannich bases of thymol, the aminomethylation reaction took place at the *ortho* position of phenol in the case of the reactions for compounds **2**, **3** and **4**, in which secondary or primary aliphatic amines were used. On the other hand, aminomethylation reaction took place at *para* position of phenol for the reactions of the compounds **1**, **5**, **6** and **7**. Secondary cyclic amines were used for the reactions of **5**, **6** and **7**. Compounds obtained with the secondary cyclic amines were solid apart from the others. Unique pattern of reaction was observed when the dimethyl amine (for compound **1**) was used as an amine component. Although compound **1** is aliphatic and non-cyclic amine like dipropylamine (**2**), benzylamine (**3**) and dibenzylamine (**4**), it gave different reaction from compounds **2** to **4**. Aminomethylation reaction took place at *para* position of phenol function in a similar way with secondary cyclic amines. Actually, to occupy the place of aminomethylation, i.e. Mannich reaction at *ortho* or *para* positions of phenol is an expected scientific fact, since hydroxyl group is a first-class substituent and direct substituent which will come to *ortho* or *para* position by itself. What are the substituents on nitrogen and how the substituents are located on it seems to be of great importance to direct aminomethylation process of thymol to *ortho* or *para* position of phenol function. Aminomethylation took place at *para* position of phenol function when the substituents on nitrogen were two methyls or bigger than this in size and these were acceptable on the condition that the amine used was in cyclic form in which free rotation was prevented as in the case of piperidine, morpholine and *N*-methylpiperazine. On the other hand, aminomethylation took place at *ortho* position of phenol function when the two substituents on nitrogen had longer chain than methyl (dipropylamine) and the hydrogens of methyl substituents located on nitrogen were replaced by phenyl rings (dibenzylamine). *Ortho* aminomethylation also occurred when the substituent was benzylamine. In this case, there is one methyl substituent on nitrogen and one of its hydrogens was replaced by phenyl (benzylamine). The common point for the *ortho* substitution of

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phenol function was that substituents present on nitrogen have the ability of free rotation.

^{1}H NMR spectra of the *ortho* substituted thymols gave two doublets for the hydrogens located at 4 and 5 position of phenol function, while ^{1}H NMR spectra of the *para* substituted thymol derivatives gave two singlets for the hydrogens located at 3 and 6 position of phenol function as reported in the ''Experimental'' section. The compounds **2** and **3** were reported for the first time. Chemical structure of the compounds were confirmed by ^{1}H NMR and/or melting points for the literature registered compounds (**1, 4, 5, 6** and **7**). ^{1}H NMR, ^{13}C NMR and HRMS for the compounds **2** and **3** reported here for the first time.

The CA inhibition (% inhibition at 0.1 μM concentration of inhibitor) of the Mannich bases prepared here is shown in Table 1. This percentage was in the range of 18–26% for hCA I and of 28–33% for hCA II. Contrary to other phenols investigated earlier[5,6,9], the Mannich bases reported here show poor inhibitory activity against these two CA isoforms. This is probably due to the fact that in the *ortho* position to the OH moiety there is one or two rather bulky functionalities, which may interfere with the binding of the compound to the enzyme active site.

The cytotoxic activity of the compounds were compared between four human oral squamous cell carcinoma (OSCC) cell lines (Ca9-22, HSC-2, HSC-3, HSC-4) and three human normal oral cells (HGF, HPLF, HPC). The results are presented in Table 2. Tumor-specificity (TS) value reflects the selectivity of the compounds against cancer tissues rather than normal ones. In this study, two types of TS values were calculated. First, TS was calculated by dividing the mean CC_{50} value of each compound against three human oral normal cells (Column D) by the mean CC_{50} value against four human OSCC cell lines (Column B). Second, TS was also calculated by dividing the value CC_{50} of each compound against HGF cells (Column C) by the CC_{50} value against Ca9-22 cell lines (Column A), both cells being originated from the same tissue (gingiva). Tumor-specificity values are presented at Table 2. All compounds showed lower TS values than reference drug melphalan by these two types of criteria. According to TS values obtained by first calculation method, the order of the potency of TS values of the compounds was as follow: The compound number (TS value): **5** (2.2) > **6** (2.0) > **3** (1.9) > **2** (1.8). The calculation of TS value for **4** was difficult due to much lower cytotoxicity of this compound to both malignant and non-malignant cells. When the second calculation was considered, compound **4** showed TS value greater than 2.1 due to the scale over of CC_{50} value, while the compound **5** (TS=2.6) had higher TS value than the compounds **2** and **3,** which showed comparable tumor-specificity (TS = 2.2). On the other hand, compound **2** showed some selectivity against human oral squamous cell lines, especially at lower concentration. The growth of all four OSCC cell lines declined at 7.8–15.6 μg/ml, whereas the growth of all three normal cells declined at 16–32-fold higher concentration (250 μg/ml) (Figure 1 and Table 3). The TS values may be significantly affected by the type of growth inhibition, either cytotoxic or cytostatic.

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Table 1. hCA I and hCA II inhibition percentage of the Mannich bases **1**–**7**.

Compound	Inhibitor concentration, M	% Inhibition hCA I	% Inhibition hCA II
1	10^{-7}	26	32
2	10^{-7}	19	32
3	10^{-7}	19	29
4	10^{-7}	18	28
5	10^{-7}	23	33
6	10^{-7}	21	32
7	10^{-7}	23	30
Acetazolamide	10^{-7}	80	80

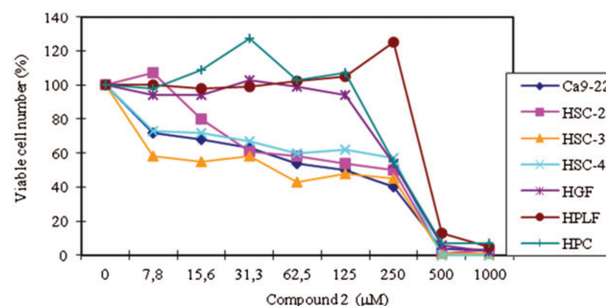

Figure 1. Viable cell number percentange of human OSCC and normal cells treated with increasing concentrations with compound **2**.

Table 2. Cytotoxic activities of the Mannich bases **1**–**7**.

	CC_{50} (μM) Human OSCC cell lines Ca9-22	HSC-2	HSC-3	HSC-4	mean	Human oral normal cells HGF	HPLF	HPC	mean	TS	TS
Compound	(A)				(B)	(C)			(D)	(D/B)	(C/A)
1	734	698	632	719	696 ± 45	726	785	791	767 ± 36	1.1	1
2	125	250	54	281	178 ± 106	271	417	276	321 ± 83	1.8	2.2
3	98	150	102	99	112 ± 25	220	207	203	210 ± 9	1.9	2.2
4	467	>1000	>1000	>1000	>867 ± 267	>1000	>1000	>1000	>1000	<1.2	>2.1
5	321	419	335	394	367 ± 47	836	851	722	803 ± 71	2.2	2.6
6	728	417	61	428	409 ± 273	753	970	766	830 ± 122	2	1
7	575	632	53	679	485 ± 291	747	800	747	765 ± 31	1.6	1.3
Melphalan	27.8	11.2	10.9	11.1	15.3 ± 8.4	144	168	198	170 ± 27	11.1	5.2

CC_{50} values refer to the concentrations of the compounds in micromoles which reduce the viable cell number by 50%. Human oral squamous cell carcinoma (OSCC) cell lines used are Ca9-22 (derived from gingiva), HSC-2, HSC-3, HSC-4 (derived from tongue). Normal oral cells used are human gingival fibroblasts (HGF), human periodontal ligament fibroblasts (HPLF), and human pulp cells (HPC). Tumor-specificity (TS) value is calculated by dividing the mean CC_{50} value of each compound against normal cells by the mean CC_{50} value against OSCC. CC_{50} value was determined from the growth curves plotted at different concentrations of each compounds in triplicate wells.

Table 3. Cytotoxic activities of the compound **2** against human OSCC and normal oral cells.

	Viable cell number (%)*						
	Human OSCC cell lines				Human oral normal cells		
Concentration (μM)	Ca9-22	HSC-2	HSC-3	HSC-4	HGF	HPLF	HPC
0	100	100	100	100	100	100	100
7.8	72	107	58	73	94	100	98
15.6	68	80	55	72	94	98	109
31.3	63	61	58	67	103	99	127
62.5	54	58	43	60	99	102	103
125	50	54	48	62	94	105	107
250	40	50	45	57	54	125	55
500	4	1	1	0	6	13	7
1000	3	3	1	0	2	5	7

*Each value represents the cell number determined by triplicate assays.

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Conclusion

The chemical structure and tumor-specific cytotoxicity of compounds **2** and **3** are reported in this study for the first time. At the same experimental condition *ortho* and *para* aminomethylation occurred depending on the nature of amine used. The compounds were slightly more selective against hCA II rather than hCA I. Based on the cytostatic property of compound **2,** it is important to consider the possibility that this compound may enhance the cytotoxicity of popular chemotherapeutic agents.

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Declaration of interest

The authors report no conflict of interest and are responsible for the contents and writing of the paper.

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