Taylor & Francis
Taylor & Francis Group

Journal of Enzyme Inhibition and Medicinal Chemistry

ISSN: 1475-6366 (Print) 1475-6374 (Online) Journal homepage: http://www.tandfonline.com/loi/ienz20

Synthesis and bioactivities of halogen bearing phenolic chalcones and their corresponding bis Mannich bases

Cem Yamali, Halise Inci Gul, Hiroshi Sakagami & Claudiu T. Supuran

To cite this article: Cem Yamali, Halise Inci Gul, Hiroshi Sakagami & Claudiu T. Supuran (2016): Synthesis and bioactivities of halogen bearing phenolic chalcones and their corresponding bis Mannich bases, Journal of Enzyme Inhibition and Medicinal Chemistry, DOI: 10.1080/14756366.2016.1221825

To link to this article: http://dx.doi.org/10.1080/14756366.2016.1221825

Published online: 04 Sep 2016.

Submit your article to this journal

Article views: 9

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at
http://www.tandfonline.com/action/journalInformation?journalCode=ienz20

Download by: [Cornell University Library] **Date:** 07 September 2016, At: 09:36

Journal of Enzyme Inhibition and Medicinal Chemistry

www.tandfonline.com/ienz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, Early Online: 1–7
© 2016 Informa UK Limited, trading as Taylor & Francis Group. DOI: 10.1080/14756366.2016.1221825

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Synthesis and bioactivities of halogen bearing phenolic chalcones and their corresponding bis Mannich bases

Cem Yamali[1], Halise Inci Gul[1], Hiroshi Sakagami[2], and Claudiu T. Supuran[3]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ataturk University, Erzurum, Turkey, [2]Division of Pharmacology, Meikai University School of Dentistry, Sakado, Japan, and [3]Neurofarba Department and Laboratorio di Chimica Bioinorganica, Università degli Studi di Firenze, Florence, Italy

Abstract

Phenolic bis Mannich bases having the chemical structure of 1-[3,5-bis-aminomethyl-4-hydroxyphenyl]-3-(4-halogenophenyl)-2-propen-1-ones (**1a-c**, **2a-c**, **3a-c**) were synthesized (Numbers 1, 2, and 3 represent fluorine, chlorine, and bromine bearing compounds, respectively, while a, b, and c letters represent the compounds having piperidine, morpholine, and *N*-methyl piperazine) and their cytotoxic and carbonic anhydrase (CA, EC 4.2.1.1) enzyme inhibitory effects were evaluated. Lead compounds should possess both marked cytotoxic potencies and selective toxicity for tumors. To reflect this potency, PSE values of the compounds were calculated. According to PSE values, the compounds **2b** and **3b** may serve as lead molecules for further anticancer drug candidate developments. Although the compounds showed a low inhibition potency toward hCA I (25–43%) and hCA II (6–25%) isoforms at 10 μM concentration of inhibitor, the compounds were more selective (1.5–5.2 times) toward hCA I isoenzyme. It seems that the compounds need molecular modifications for the development of better CA inhibitors.

Keywords

Carbonic anhydrase, chalcone, cytotoxicity, Mannich bases, phenol

History

Received 26 July 2016
Accepted 4 August 2016
Published online 31 August 2016

Introduction

Cancer is one of the most important diseases of death worldwide. It is fast progressing in twenty-first century and is predicted to affect 22 million people by 2030[1]. Although, several cancer chemotherapeutics are in market nowadays, their side effects are prompting many researchers to develop more safe, potent, and selective anticancer drugs.

Chalcones, 1,3-aryl-2-propen-1-ones, include α,β-unsaturated ketone moiety which have the ability to alkylate cellular thiols to lead to anticancer activity while they have little or no affinity for cellular amino and hydroxyl groups. This makes them free from the side effects which are associated with a number of alkylating agents used in cancer chemotherapy since α,β-unsaturated ketone moiety is a selective alkylator for thiol rather than hydroxy and amine groups[2–5]. Chalcones exhibit many important biological activities such as cytotoxic, antiinflammatory, antituberculosis, antifungal, antimalarial, antioxidant, antibacterial, carbonic anhydrase inhibitory, and antiulcerative activities[6–11].

Cytotoxic chalcones and their analogs that interact with thiols which are not found in nucleic acids may be devoid of the side effects of certain anticancer drugs which interact with the hydroxy and amino groups of DNA and RNA[2,11,12]. Mechanisms of anticancer effects of chalcones appear to be complex and frequently multimodal and include cell cycle disruption, inhibition of angiogenesis, inhibition of tubulin polymerization, induction of apoptosis, inhibition of topoisomerase, and inhibition of kinases[13–16].

Mannich bases are generally formed by the reaction between a compound containing a reactive hydrogen atom, formaldehyde, and mostly secondary amine[2,17]. The chemistry of Mannich bases has been the subject of wide and increasing interest, due to their biological activities such as analgesic, antimalarial, anticonvulsant, antipsychotic, cytotoxic, antiinflammatory, and antimicrobial activities[3–5,8,9,17–22]. In addition, the Mannich bases of several bioactive molecules such as Taxol (anticancer agent), SCH48461 (anticholesterol agent), Tramadol (analgesic), Osnervan (antiparkinson), and Moban (neuroleptic) were prepared and they showed increased bioactivities comparing to the parent compounds[23]. Doxorubicin causes multidrug resistance in tumor cells but the synthetic Mannich base analog of Doxorubicin showed significant activity against multi drug-resistant tumor cell lines[23]. The introduction of aminoalkyl Mannich side chain into Topotecan increased the water solubility of this anticancer agent[23].

Conversion of phenolic chalcones into their corresponding Mannich bases is often accompanied by increased bioactivity in *in vitro* and/or *in vivo* studies[2,9,14,17,22,24]. This situtation has been attributed to the deamination skill of a Mannich base to generate additional alkylation site to chalcones for additional nucleophilic attack by cellular thiols such as glutathione or cysteine to lead dramatic inrease by Mannich bases according to sequential cytotoxicity[2,15]. The theory of sequential cytotoxicity proposed by

Address for correspondence: Prof. Dr. H. Inci Gul. E-mail: incigul1967@yahoo.com

Dr. Dimmock states that the successive release of two or more cytotoxic compounds may cause greater toxicity to malignant tissue than normal cells[25].

Carbonic anhydrases (CAs) are a family of metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate anion and proton. All human CAs (hCAs) belong to the α-class and differ widely in their cellular localizations: CA I, II, III, VII, and XIII reside in cytosol, CA IV, IX, XII, and XIV are associated with cell membrane, and CA VA and CA VB occur in mitochondria, whereas CA VI is secreted in saliva and milk. CAs are involved in many physiological processes, such as pH homeostasis, ion transport, respiration and gluconeogenesis, bone resorption, renal acidification, formation of cerebrospinal fluid and gastric acid, and the inhibition of these CA isoenzymes may prevent or alleveiate various diseases such as glaucoma, neurological disorders including epilepsy and altitude disease, obesity, and cancer[26–31]. The CA inhibitory effects of several natural products and their analogs have been recently investigated.

It was reported that natural compounds carrying phenol or polyphenol moiety had carbonic anhydrase inhibitory activity at micromolar concentration[32,33]. In addition, the compounds having phenol functional group are known to show anticancer, antibacterial, antiviral, antifungal, and antioxidant activities[9,10,16,22].

Preperation of mono Mannich bases from phenolic chalcones increased the cytotoxicity comparing to their corresponding chalcones in our previous studies[9,14] in a supporting manner to sequential cytotoxicity hypothesis[25].

In this study, it was aimed to prepare phenolic bis Mannich bases having the chemical sturcture of 1-[3,5-bis-aminomethyl-4-hydroxyphenyl]-3-(4-halogenophenyl)-2-propen-1-ones by expectation to see dramatic increase in cytotoxicity of Mannich bases comparing to their corresponding chalcones according to sequential cytotoxicity hypothesis. It was also aimed to investigate the inhibitory effects of phenolic compounds synthesized on hCA I and II isoenzymes since phenolic group pharmacophore is effectively inhibits CA isoenyme of which have an important role in many biological process.

Experimental

Materials and methods

All chemicals and solvents used to synthesize Mannich bases were purchased from Merck (Germany) and Sigma-Aldrich (Germany). Synthesis of Mannich bases were carried out in a CEM Discover Microwave Synthesis System, 908010 (Matthews, NC). Melting points were determined using an Electrothermal 9100/IA9100 (Bibby Scientific Limited, Staffordshire, UK) instrument and are uncorrected. Chemical structures of the compounds were determined by ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectroscopies using a Varian Mercury Plus spectrometer (Varian Inc., Palo Alto, CA). Chemical shifts (δ) are reported in ppm, and coupling constants (J) are expressed in hertz (Hz); HRMS-ESI Mass spectra were recorded on HPLC-TOF Waters Micromass LCT Premier XE (Waters Corporation, Milford, MA). The reactions were monitored using silicagel HF254-366 TLC plates (E. Merck, Germany).

*General procedure for the synthesis of 1-(4-hydroxyphenyl)-3-(4-halogenophenyl)-2-propen-1-ones (**1**, **2**, **3**) (Scheme 1)*

Chalcone derivatives (**1**, **2**, **3**) were synthesized as described in literatures[2,8,10,21]. An aqueous solution of sodium hydroxide (10% w/v, 10 ml) was added to a solution of the appropriate aryl aldehyde (0.02 mol) and 4-hydroxyacetophenone (0.02 mol) in ethanol (6 ml). The reaction mixture was stirred at room temperature for 24 h, poured onto cold water (50 ml), and neutralized with hydrochloric acid solution (10% w/v). The precipitated solid product was recrystallized from ethanol/water. The chemical structures of the starting compounds chalcones were confirmed by ^{1}H NMR spectra and melting points of the compounds registered in literatures. Melting points of the compounds synthesized were in accordance with the literature report[2] (see Supplementary File).

*General procedure for the synthesis of 1-[3,5-bis-aminomethyl-4-hydroxyphenyl]-3-(4-halogenophenyl)-2-propen-1-ones by microwave irradiation (**1a–c**, **2a–c**, **3a–c**) (Scheme 1)*

A suitable amine and paraformaldehyde were heated in acetonitrile (15 ml) for 5 min at 80 °C, 150 Watt, and 13 barr. A solution of suitable chalcone in acetonitrile (10 ml) was added into the reaction mixture and heated for 40 min at 120 °C, 200 Watt, and 13 barr. The completion of reaction was monitored by TLC. Acetonitrile was removed under vacuum and the viscous residue was dissolved in hexane or diisopropyl ether, then the solution was left overnight at +4 °C (except compounds **1c** and **2c**). The solid was filtered, dried, and recrystallized from suitable solvent.

*1-[3,5-Bis(piperidin-1-yl)methyl-4-hydroxyphenyl]-3-(4-fluorophenyl)-2-propen-1-one (**1a**)*

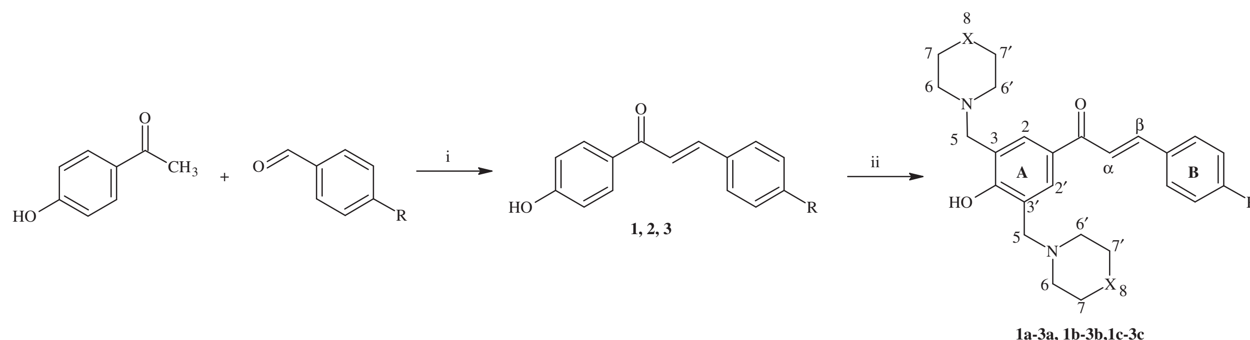
The compound **1** (0.5 g, 2.1 mmol), paraformaldehyde (0.4 g, 12.6 mmol), and piperidine (1,1 g, 12.6 mmol) were treated as described above. The crude product was recrystallized from acetonitrile to afford **1a** as a light yellow solid, yield 9%, mp 125–127 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.81 (s, 2H, H-2), 7.74 (d, $J = 15.7$ Hz, 1H, H_β), 7.64 (d, $J = 8.8$ Hz, 2H, B ring), 7.50 (d, $J = 15.7$ Hz, 1H, H_α), 7.1 (t, 2H, $J = 8.8$ Hz, B ring), 3.66 (s, 4H, H-5), 2.5 (s, 8H, $H_{6,6'}$), 1.65–1.59 (m, 8H, $H_{7,7'}$), 1.47 (s, 4H, H-8); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 189.1, 162.3, 142.3, 130.5, 130.4, 130.0, 128.9, 123.4, 122.1, 122.0, 116.4, 116.2, 59.6, 54.4, 26.0, 24.3; HRMS (ESI-MS) Calc. for $C_{27}H_{34}N_2O_2F$ $[M+H]^+$ 437.2604; found: 437.2607.

*1-[3,5-Bis(morpholino-4-yl)methyl-4-hydroxyphenyl]-3-(4-fluorophenyl)-2-propen-1-one (**1b**)*

The compound **1** (0.5 g, 2.1 mmol), paraformaldehyde (0.5 g, 16.8 mmol), and morpholine (1.4 g, 16.8 mmol) were treated as described above. The crude product was recrystallized from diisopropyl ether/methanol to afford **1b** as a light yellow solid, yield 31%, mp 124–126 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.83 (s, 2H, H-2), 7.76 (d, $J = 15.4$ Hz, 1H, H_β), 7.65 (d, $J = 8.4$ Hz, 1H, B ring), 7.63 (d, $J = 8.8$ Hz, 1H, B ring),7.47 (d, $J = 15.7$ Hz, 1H, H_α), 7.1 (t, 2H, $J = 8.8$ Hz, B ring), 3.76 (s, 8H, $H_{7,7'}$), 3.71 (s, 4H, H-5), 2.57 (s, 8H, $H_{6,6'}$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 188.6, 161.4, 142.7, 130.5, 130.4, 130.4, 129.5, 122.6, 121.7, 116.4, 116.2, 67.0, 59.4, 53.5; HRMS (ESI-MS) Calc. for $C_{25}H_{30}N_2O_4F$ $[M+H]^+$ 441.2190; found: 441.2193.

*1-[3,5-Bis(4-N-methylpiperazin-1-yl)methyl-4-hydroxyphenyl]-3-(4-fluorophenyl)-2 propen-1-one (**1c**)*

The compound **1** (0.5 g, 2.1 mmol), paraformaldehyde (0.3 g, 8.4 mmol), and *N*-methylpiperazine (0.8 g, 8.4 mmol) were treated as described above. The contents of reaction flask were concentrated under vacuum and left overnight resulting in the formation of crystals. The solid was filtered, dried, and recrystallized from acetonitrile to afford **1c** as a light yellow solid, yield 24%, mp 158–160 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.81 (s, 2H, H-2), 7.75 (d, $J = 15.7$ Hz, 1H, H_β), 7.64 (d, $J = 8.8$ Hz, 1H, B ring), 7.63 (d, $J = 8.8$ Hz, 1H, B ring),7.47 (d, $J = 15.7$ Hz, 1H, H_α), 7.1 (t, 2H, $J = 8.7$ Hz, B ring), 3.71



Scheme 1. General synthesis of the chalcones and bis Mannich bases. Reagents and conditions: (i) NaOH (10%), EtOH, rt, 24h. (ii) Acetonitrile, paraformaldehyde, amine, 45 min, 120 °C, 200 Watt, 13 barr. R=F (**1**), Cl (**2**), Br (**3**), X= –CH2 (**1a**, **2a**, **3a**), –O– (**1b**, **2b**, **3b**), –NCH3 (**1c**, **2c**, **3c**).

(s, 4H, H-5), 2.59 (bs, 16H, -CH_2-piperazine), 2.29 (s, 6H, -CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 188.7, 161.7, 142.5, 130.5, 130.4, 130.3, 129.2, 122.9, 121.8, 116.4, 116.2, 58.9, 55.1, 52.9, 46.2; HRMS (ESI-MS) Calc. for $C_{27}H_{36}N_4O_2F$ $[M+H]^+$ 467.2822; found: 467.2835.

*1-[3,5-Bis(piperidin-1-yl)methyl-4-hydroxyphenyl]-3-(4-chlorophenyl)-2-propen-1-one (**2a**)*

The compound **2** (0.5 g, 1.9 mmol), paraformaldehyde (0.3 g, 11.4 mmol), and piperidine (0.9 g, 11.4 mmol) were treated as described above. The crude product was recrystallized from diisopropyl ether/methanol to afford **2a** as a light yellow solid, yield 29%, mp 139–140 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.80 (s, 2H, H-2), 7.72 (d, $J=15.4$ Hz, 1H, H_β), 7.66 (d, $J=8.4$ Hz, 2H, B ring), 7.54 (d, $J=15.7$ Hz, 1H, H_α), 7.38 (d, $J=8.4$ Hz, 2H, B ring), 3.66 (s, 4H, H-5), 2.49 (s, 8H, $H_{6,6'}$), 1.65–1.59 (m, 8H, $H_{7,7'}$), 1.47 (s, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 188.7, 162.4, 142.0, 136.2, 134.0, 130.0, 129.7, 129.4, 128.8, 123.3, 122.8, 59.6, 54.4, 26.0, 24.3; HRMS (ESI-MS) Calc. for $C_{27}H_{34}N_2O_2Cl$ $[M+H]^+$ 453.2309; found: 453.2325.

*1-[3,5-Bis(morpholino-4-yl)methyl-4-hydroxyphenyl]-3-(4-chlorophenyl)-2-propen-1-one (**2b**)*

The compound **2** (0.5 g, 1.9 mmol), paraformaldehyde (0.4 g, 15.2 mmol), and morpholine (1.3 g, 15.2 mmol) were treated as described above. The crude product was recrystallized from diisopropyl ether/methanol to afford **2b** as a yellow solid, yield 61%, mp 123–125 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.83 (s, 2H, H-2), 7.74 (d, $J=15.4$ Hz, 1H, H_β), 7.57 (d, $J=6.6$ Hz 2H, B ring), 7.51 (d, $J=15.4$ Hz, 1H, H_α), 7.39 (d, $J=6.6$ Hz, 2H, B ring), 3.75 (s, 8H, $H_{7,7'}$), 3.70 (s, 4H, H-5), 2.57 (s, 8H, $H_{6,6'}$); 13C NMR ($CDCl_3$, 100 MHz) δ 188.5, 161.5, 142.5, 136.4, 133.8, 130.5, 129.7, 129.4, 122.7, 122.5 122.4, 67.0, 59.4, 53.5; HRMS (ESI-MS) Calc. for $C_{25}H_{30}N_2O_4Cl$ $[M+H]^+$ 457.1894; found: 457.1895.

*1-[3,5-Bis(4-N-methylpiperazin-1-yl)methyl-4-hydroxyphenyl]-3-(4-chlorophenyl)-2-propen-1-one (**2c**)*

The compound **2** (0.5 g, 1.9 mmol), paraformaldehyde (0.2 g, 7.6 mmol), and *N*-methylpiperazine (0.7 g, 7.6 mmol) were treated as described above. The contents of reaction flask were concentrated under vacuum and left overnight resulting in the formation of crystals. The solid was filtered, dried, and recrystallized from acetonitrile to afford **2c** as a light brown solid, yield 60%, mp 185–187 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.79 (s, 2H, H-2), 7.70 (d, $J=15.4$ Hz, 1H, H_β), 7.55 (d, $J=8.4$ Hz, 2H, B ring), 7.49 (d, $J=15.7$ Hz, 1H, H_α), 7.36 (d, $J=8.4$ Hz, 2H, B ring), 3.68 (s, 4H, H-5), 2.49 (bs, 16H, -CH_2-piperazine), 2.27 (s, 6H, CH_3); 13C NMR ($CDCl_3$, 100 MHz) δ 188.6, 161.8, 142.3, 136.3, 133.9, 130.3, 129.7, 129.4, 122.9, 122.6, 58.9, 55.1, 52.9, 46.2; HRMS (ESI-MS) Calc. for $C_{27}H_{36}N_4O_2Cl$ $[M+H]^+$ 483.2527; found: 483.2515.

*1-[3,5-Bis(piperidin-1-yl)methyl-4-hydroxyphenyl]-3-(4-bromophenyl)-2-propen-1-one (**3a**)*

The compound **3** (0.4 g, 1.3 mmol), paraformaldehyde (0.2 g, 7.8 mmol), and piperidin (0.7 g, 7.8 mmol) were treated as described above. The crude product was recrystallized from diisopropyl ether/methanol to afford **3a** as a light yellow solid, yield 22%, mp 150–151 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.79 (s, 2H, H-2), 7.69 (d, $J=15.7$ Hz, 1H, H_β), 7.58–7.49 (m, 5H, B ring and H_α), 3.65 (s, 4H, H-5), 2.49 (s, 8H, $H_{6,6'}$), 1.64–1.58 (m, 8H, $H_{7,7'}$), 1.47 (s, 4H, H-8); 13C NMR ($CDCl_3$, 100 MHz) δ 188.7, 162.4, 142.1, 134.4, 132.3, 130.0, 129.9, 128.8, 124.5, 123.3, 122.9, 59.6, 54.4, 26.0, 24.3; HRMS (ESI-MS) Calc. for $C_{27}H_{34}N_2O_2Br$ $[M+H]^+$ 497.1804; found: 497.1806.

*1-[3,5-Bis(morpholino-4-yl) methyl-4-hydroxyphenyl]-3-(4-bromophenyl)-2-propen-1-one (**3b**)*

The compound **3** (0.4 g, 1.3 mmol), paraformaldehyde (0.3 g, 10.4 mmol), and morpholine (0.9 g, 10.4 mmol) were treated as described above. The crude product was recrystallized from diisopropyl ether/methanol to afford **3b** as a light yellow solid, yield 22%, mp 147–149 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.83 (s, 2H, H-2), 7.72 (d, $J=15.4$ Hz, 1H, H_β), 7.57–7.49 (m, 5H, B-ring and H_α), 3.77–3.74 (m, 8H, $H_{7,7'}$), 3.71 (s, 4H, H-5), 2.57 (s, 8H, $H_{6,6'}$); 13C NMR ($CDCl_3$, 100 MHz) δ 188.5, 161.5, 142.6, 134.3, 132.4, 130.5, 129.9, 129.4, 124.8, 122.7, 122.5, 67.0, 59.4, 53.5; HRMS (ESI-MS) Calc. for $C_{25}H_{30}N_2O_4Br$ $[M+H]^+$ 501.1389; found: 501.1384.

*1-[3,5-Bis(4-N-methylpiperazin-1-yl)methyl-4-hydroxyphenyl]-3-(4-bromophenyl)-2-propen-1-one (**3c**)*

The compound **3** (0.5 g, 1.7 mmol), paraformaldehyde (0.2 g, 6.8 mmol), and *N*-methylpiperazine (0.7 g, 6.8 mmol) were treated as described above. The crude product was recrystallized from diisopropyl ether/methanol to afford **3c** as a light green solid, yield 28%, mp 181–183 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.80 (s, 2H, H-2), 7.77 (d, $J=15.4$ Hz, 1H, H_β), 7.55–7.49 (m, 5H, B ring and H_α), 3.71 (s, 4H, H-5), 2.59–2.50 (m, 16H, -CH_2-piperazine), 2.29 (s, 6H, CH_3); 13C NMR ($CDCl_3$, 100 MHz) δ 188.6, 161.8, 142.4, 134.4, 132.4, 130.3, 129.9, 129.2, 124.6, 122.9, 122.7, 58.8, 55.1, 52.9, 46.2; HRMS (ESI-MS) Calc. for $C_{27}H_{36}N_4O_2Br$ $[M+H]^+$ 527.2022; found: 527.2023.

Biological activity

Cytotoxicity assay

The cytotoxicity of the compounds were assayed toward human oral squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4) (purchased from Riken Cell Bank, Tsukuba, Japan) and human normal oral cells (HGF, HPLF) (established from the first premolar tooth extracted from the lower jaw of a 12-year-old girl) as described with some minor modifications[14,16,21,22,34]. All cells were cultured in DMEM (Sigma-Aldrich Inc., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich). All test samples, positive controls such as Melphalan (Sigma-Aldrich), and 5-Fluorouracil (5-FU) (Kyowa, Tokyo, Japan) were dissolved in dimethylsulfoxide (DMSO) (Wako Pure Chem. Ind., Osaka) at 40 mM or 100 mM, respectively. Near confluent cells were incubated in 96-microwell (Becton Dickinson, Franklin Lakes, NJ) for 48 h with 1.6, 3.1, 6.3, 12.5, 25, 50, 100, or 200 μM of each test compound, Melphalan (3.1, 6.3, 12.5, 25, 50, 100, 200, or 400 μM), or 5-FU (7.8, 15.6, 31.3, 62.5, 125, 250, 500, or 1000 μM). Any cytotoxcity induced by DMSO (0.002, 0.004, 0.0078, 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5, or 1%) were subtraced from the corresponding treated groups. The viable cell numbers were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. In brief, the treated cells were incubated for another 3 h in fresh culture medium containing 0.2 mg/ml MTT (Sigma-Aldrich). Cells were then lysed with 0.1 ml of DMSO and the absorbance at 562 nm of the cell lysate was determined using a microplate reader (Sunrise Rainbow RC-R; TECAN, Männedorf, Switzerland). The CC_{50} value refers to the concentration of compounds in micromoles which kill 50% of the cells. CC_{50} values were determined from the dose–response curve, and the mean value of CC_{50} for each cell type was calculated from triplicate assays.

Carbonic anhydrase (CAs) enzyme inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity[35]. Phenol red (at a concentration of 0.2 mM) has been used as 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 percantage were obtained by using PRISM 3, as reported earlier[36], and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier[37]. The cell pellets were lysed, and hCA II and hCA I were purified through affinity chromatography using pAMBS resin.

Results and discussion

Condensation of 4-hydroxyacetophenone and appropriate aldehyde (4-flurobenzaldehyde in **1**, 4-chlorobenzaldehyde in **2**, 4-bromobenzaldehyde in **3**) afforded the unsaturated ketones, chalcones **1**, **2**, and **3**. These compounds reacted with various amines (piperidine in **1a**, **2a**, **3a**; morpholine in **1b**, **2b**, **3b**; *N*-methylpiperazine in **1c**, **2c**, **3c**) and paraformaldehyde to produce the corresponding bis Mannich bases (**1a**, **1b**, **1c**, **2a**, **2b**, **2c**, **3a**, **3b**, **3c**). Chemical structures of the compounds were confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS spectra. ^{1}H NMR spectra of the compounds confirmed that amino methylation occured in the ortho positions to aryl hydroxy group and olefinic double bond assumed the *E* configuration ($J = 15.4$–15.7 Hz) (see Supplementary File).

The cytotoxicity data of the compounds are shown in Table 1. The first question to be addressed was whether the compounds synthesized have antineoplastic properties. CC_{50} values of compounds were in the low micromolar range (<1.6–18.4 μM) toward HSC-2, HSC-3, and HSC-4 cell lines and this suggests that the compounds studied have antineoplastic properties. Cytotoxicities of the compounds were compared with reference compounds Melphalan which is clinically used an alkylating agent and 5-Fluorouracil (5-FU) which is pyrimidine antagonist. The compounds having higher cytotoxicities than reference compounds Melphalan and 5-FU in terms of times were as follows: The name of cell line; the compound (times potent as compared with reference compounds): HSC-2 cell line; **1** (1.6, 2.9), **1a** (1.6, 2.9), **1b** (1.2, 2.1), **1c** (-, 1.8), **2** (3.0, 5.4), **2a** (>5.3, >9.5), **2b** (>5.3, >9.5), **2c** (1.5, 2.8), **3** (2.4, 4.2), **3a** (3.0, 5.4), **3b** (>5.3, >9.5), **3c** (>5.3, >9.5). HSC-3 line; **1** (-, 4.3), **1a** (-, 5.9), **1b** (-, 6.2), **1c** (1.2, 9.1), **2** (1.1, 8.3), **2a** (1.9, 13.9), **2b** (2.9, 21.9), **2c** (1.5, 11.0), **3** (-, 6.1), **3a**, **3b**, **3c** (>3.5, >26.1). HSC-4 cell line;; **1** (2.1, 23.0), **1a** (-, 7.2), **1b** (1.1, 12.1), **1c** (3.8, 43.0), **2** (3.8, 43.0), **2a** (>7.4, >83.3), **2b** (>7.4, >83.3), **2c** (5.4, 60.6), **3** (1.8, 20.2), **3a** (4.8, 53.3), **3b** (6.0, 66.7), **3c** (4.4, 49.4).

When the cytotoxicities of Mannich bases **1a**, **1b**, **1c**, which were derived from 4-fluorochalcone (**1**) were compared to **1**, the compounds **1a** (1.4), **1b** (1.5), **1c** (2.1) toward HSC-3 cell line and **1c** (1.9) toward HSC-4 cell line were 1.4–2.1 times and 1.9 times more cytotoxic than **1**, respectively.

In addition, when the cytotoxicities of Mannich bases **2a**, **2b**, **2c**, which were derived from 4-chlorochalcone (**2**) were compared to **2**, it was noticed that **2a** and **2b** (>1.8 times) toward HSC-2 cell line, **2a** (1.7), **2b** (2.6), **2c** (1.3) toward HSC-3 cell line, **2a** and **2b** (>1.9 times), **2c** (1.4) toward HSC-4 cell line were >1.8, 1.3–2.6, 1.4- >1.9 times more cytotoxic than **2** toward HSC-2, HSC-3, and HSC-4 cell lines, respectively.

In the case of Mannich bases **3a**, **3b**, **3c** which were derived from 4-bromochalcone (**3**), cytotoxicities increased in Mannich bases comparing to **3**. Cytotoxicity was increased by n-fold (that is described in the parenthesis): **3a** (1.3), **3b**, and **3c** (>2.3) toward HSC-2 cell line, **3a**, **3b**, and **3c** (>2.3) toward HSC-3 cell line, **3a** (2.6), **3b** (3.3), **3c** (2.4) toward HSC-4 cell line.

The increases of cytotoxicity in Mannich bases comparing with the suitable chalcone which they are derived from can be explained by sequential cytotoxicity hypothesis which was suggested by Dimmock[25]. Sequential cytotoxicity hypothesis expresses that successive chemical attacks by two or more cytotoxic agents causes selective killing of neoplastic cells as compared with normal cells. Initial thiol attack could occur at one of the olefinic double bonds to be followed by a second thiol interaction which could be more damaging to neoplastic cells than normal tissues. This assumption depends on nonequivalent charges at the olefinic bonds in order to avoid synchronous attack at both olefinic carbon atoms. In general, these compounds were more than twice as cytotoxic as the corresponding chalcones/analogues[25]. The second aspect of these compounds to be considered is whether they are tumor-spesific cytotoxins since tumors are surrounded by different types of normal cells. Selectivity index (SI) figures were generated which are quotient of the average CC_{50} figure of a compound toward a spesific cell

Table 1. The cytotoxicity results of the compounds.

	CC_{50} (μM)													
	Tumor cell lines								Nonmalignant cells					
	HSC-2		HSC-3		HSC-4							Ave		
Compound	CC_{50}	SI	CC_{50}	SI	CC_{50}	SI	Ave CC_{50}	Ave SI	HGF CC_{50}	HPLF CC_{50}	CC_{50}	PSE	log P
1	5.2	9.2	9.7	4.9	5.8	8.2	6.9	7.5	51.2	44.3	47.8	108.7	3.35
2	2.8	14.5	5.0	8.1	3.1	13.1	3.6	11.9	40.2	40.7	40.5	330.6	3.75
3	3.6	11.2	6.8	5.9	6.6	6.1	5.7	7.7	40.5	39.8	40.2	135.1	4.02
1a	5.3	7.4	7.1	5.5	18.4	2.1	10.3	5.0	39.2	39.0	39.1	48.5	4.70
1b	7.2	7.5	6.7	8.0	11.0	4.9	8.3	6.8	39.6	68.1	53.9	81.9	2.44
1c	8.6	2.2	4.6	4.2	3.1	6.2	5.4	4.2	18.4	19.9	19.2	77.8	2.75
2a	<1.6	11.2	3.0	5.9	<1.6	11.1	<2	9.4	15.2	20.4	17.8	470.0	5.10
2b	<1.6	12.5	1.9	10.5	<1.6	12.5	<1.7	11.8	18.3	21.7	20.0	694.1	2.84
2c	5.5	2.6	3.8	3.7	2.2	6.4	3.8	4.2	6.3	21.8	14.1	110.5	3.15
3a	2.8	7.4	<1.6	12.9	2.5	8.2	2.3	9.5	17.9	23.2	20.6	413.0	5.37
3b	<1.6	12.3	<1.6	12.3	2.0	9.8	1.7	11.5	18.5	20.7	19.6	676.5	3.11
3c	<1.6	5.4	<1.6	5.4	2.7	3.2	2	4.7	8.0	9.1	8.6	235.0	3.42
Average	3.9	8.4	4.4	7.1	4.9	7.4	4.4	7.6	25.5	29.8	27.7	271.2	–
Melphalan	8.5	18.8	5.6	28.5	11.9	13.4	8.7	20.2	140	179	159.5	348.3	–
5-FU	15.2	65.8	41.7	24.0	133.3	7.5	63.4	>32.4	>1000	>1000	>1000	>51.2	–

The CC_{50} value refers to the concentration of compounds in micromoles which kill 50% of the cells. The SI value indicates the selective index, i.e. the quotient of the averaged CC_{50} value for nonmalignant cells (HGF and HPLF) divided by CC_{50} value for a specific tumor cell line (either HSC-2, HSC-3 or HSC-4). The PSE value indicates the potency selectivity expression which is the product of the averaged CC_{50} value and the averaged SI value for all three cell lines. log P values were calculated by Chem Draw Ultra 12.0 Software (Perkin Elmer, Waltham, MA).

line. The results in Table 1 reveal that SI values of greater than 1 were obtained and thus all compounds synthesized are tumor-specific antineoplastic agents[38].

The average selectivity index figures revealed that morpholine containing Mannich bases **1b** (with fluorine), **2b** (with chlorine), and **3b** (with bromine) had the highest average SI values comparing to the amines piperidine and *N*-methylpiperazine. The neoplasms differ in their selectivity to the compounds, which which was possibly caused by somewhat distinctive modes of action in each cell line.

Lead compounds should possess both marked cytotoxic potencies and selective toxicity for tumors. In order to identify such molecules, a potency selectivity expression (PSE) was devised which is the product of reciprocal of the average CC_{50} values toward HSC-2, HSC-3, and HSC-4 cells (a measure of potency) and the average SI figures toward these cell lines (a determination of tumor-selectivity) expressed as a percentage[16].

The PSE data of the compounds are presented in Table 1. PSE values increased in Mannich bases **2a** (1.4 times) with chlorine, **2b** (2.1 times) with chlorine and **3a** (3.1 times), **3b** (5.0 times), **3c** (1.7 times) with bromine comparing to the chalcones. Increases in cytotoxicity of Mannich bases can be atributited to the sequential cytotoxicity hypothesis. In the case of Mannich bases with fluorine derived from chalcone **1**, the compound had higher PSE value than the Mannich bases **1a**, **1b**, and **1c**. This may result from decreased solubility of its Mannich bases with fluorine in water comparing to its chalcone. Thus, the compounds **2a**, **2b**, **3a**, and **3b** seem to serve as lead molecules to develop new cytotoxic compounds because of their higher PSE values than the derived chalcones, and also remarkably high PSE values.

The solubility of any compound is the important determinant of bioactivity. Solubility of the compound should be optimum between lipid and water phases for optimum bioactivity. The indicator of solubility for the compound is partition coefficient (P) or its logaritmic value logP. According to log P values calculated for the morpholine containing Mannich bases, **1b** (with fluorine), **2b** (with chlorine), **3b** (with bromine) had the highest PSE values among the series having different halogens. When the log P value of the compound was the lowest, the PSE value of the compound was the highest (Table 1). This suggests that solubility of the compounds among the series **1a–c**, **2a–c**, **3a–c** may effect the cytotoxicity.

Table 2. Inhibitory effects of the compounds synthesized on hCAI and II isoenzymes.

	Inhibition %		
Compound No.	hCA I	hCA II	Selectivity for hCA I
1	34	17	2.0
2	30	11	2.7
3	43	9	4.8
1a	31	6	5.2
1b	26	16	1.6
1c	26	16	1.6
2a	29	19	1.5
2b	30	20	1.5
2c	28	13	2.2
3a	28	25	1.1
3b	29	17	1.7
3c	25	17	1.5
AZA	86	96	0.9

Acetazolamide (AZA) was used as a reference compound. Inhibitor concentration was 10^{-5} M for all compounds.

The CA inhibitory effects of the compounds were evaluated against hCA I and hCA II isoenzymes. The inhibition percentages (% inhibition at 10^{-5} M concentration of inhibitor) of the compounds synthesized on hCA I and hCA II isoenzymes are shown in Table 2. According to Table 2, the percentages were in the range of 25–43% for hCA I and of 6–25% for hCA II. In addition, the compounds synthesized were more selective (1.1–5.2 times) toward hCA I isoenzyme than hCA II isoenzyme. The compound **3** which is a chalcone having bromine was more effective on hCA I (43%) compared to its bis Mannich derivatives (25–29%). On the other hand, the compound **3a** which is a bis Mannich base with piperidine was more effective on hCA II (25%) than its corresponding chalcone **3** (9%). In general, chalcones (**1**, **2**, and **3**) were more effective inhibitors than bis

Mannich bases (**1a–c**, **2a–c**, **3a–c**) toward hCA I isoenzyme while bis Mannich bases had superior inhibitory activity than chalcones toward hCA II isoenzyme. Desreases in CA inhibitory potential in bis Mannich bases are probably due to the fact that in the *ortho* position to the OH moiety there are two rather bulky functionalities (aminoalkyl groups) which may interfere with the binding of the compound to the enzyme active sites.

Conclusion

Phenolic bis Mannich bases having the chemical structure of 1-[3,5-bis-aminomethyl-4-hydroxyphenyl]-3-(4-halogenophenyl)-2-propen-1-ones (**1a–c**, **2a–c**, **3a–c**) were successfully synthesized and evaluated in terms of their cytotoxic and carbonic anhydrase inhibitory effects. According to the PSE values, bis Mannich bases having piperidine **2a** (PSE = 470) and **3a** (PSE = 413) and having morpholine **2b** (PSE = 694.1) and **3b** (PSE = 676.5) moities attract attention with high PSE values among the compounds. Especially **2b** with chlorine and **3b** with bromine halogen and morpholine moity in both compound may serve as lead molecules for further anticancer investigations because of the highest PSE values. On the other hand, although the compounds showed a low inhibition potency toward hCA I (25–43%) and hCA II (6–25%) isoforms at 10 μM concentration of inhibitor, the compounds were more selective (1.5–5.2 times) toward hCA I isoenzyme. So, the compounds need molecular modifications for the development of better CA inhibitors.

Acknowledgements

The authors thank to Tanc M. for his kind assistance during the measuring hCA I and II inhibitions. The authors are thankful to Dr. Murat Sukuroglu (Gazi University, Ankara, Turkey) for HRMS spectra and to Department of Chemistry, Faculty of Science, Ataturk University, Turkey for NMRs spectra.

Declaration of interest

The authors declare that they have no conflict of interest.

Funding

This work was financially supported by Research Foundation of Ataturk University, Erzurum, Turkey (Project Number: 2015-322).

References

1. Mahapatra DK, Bharti SK, Asati V. Anti-cancer chalcones: structural and molecular target perspectives. Eur J Med Chem 2015;98: 69–114.
2. Dimmock JR, Kandepu NM, Hetherington M, et al. Cytotoxic activities of Mannich bases of chalcones and related compounds. J Med Chem 1998;41:1014–26.
3. Gul HI, Ojanen T, Hanninen O. Antifungal evaluation of bis Mannich bases derived from acetophenones and their corresponding piperidinols and stability studies. Biol Pharm Bull 2002;25: 1307–10..
4. Gul M, Gul HI, Das U, Hanninen O. Biological evaluation and structure-activity relationships of bis-(3-aryl-3-oxo-propyl)-methylamine hydrochlorides and 4-aryl-3-arylcarbonyl-1-methyl-4-piperidinol hydrochlorides as potential cytotoxic agents and their alkylating ability towards cellular glutathione in human leukemic T cells. Arzneimittelforschung 2005;55:332–7.
5. Gul M, Atalay M, Gul HI, et al. The effects of some Mannich bases on heat shock proteins HSC70 and GRP75, and thioredoxin and glutaredoxin levels in Jurkat cells. Toxicol in Vitro 2005;19:573–80.
6. Bandgar BP, Gawande SS, Bodade RG, et al. Synthesis and biological evaluation of simple methoxylated chalcones as anticancer, anti-inflammatory and antioxidant agents. Bioorg Med Chem 2010;18:1364–70.
7. Singh P, Anand A, Kumar V. Recent developments in biological activities of chalcones: a mini review. Eur J Med Chem 2014;85: 758–77.
8. Yamali C, Tugrak M, Gul HI, et al. The inhibitory effects of phenolic Mannich bases on carbonic anhydrase I and II isoenzymes. J Enzyme Inhib Med Chem 2016. [Epub ahead of print]. doi: 10.3109/14756366.2015.1126715.
9. Gul HI, Yerdelen KO, Gul M, et al. Synthesis of 4′-hydroxy-3′-piperidinomethylchalcone derivatives and their cytotoxicity against PC-3 cell lines. Arch Pharm (Weinheim) 2007;340:195–201.
10. Bilginer S, Unluer E, Gul HI, et al. Carbonic anhydrase inhibitors. Phenols incorporating 2- or 3-pyridyl-ethenylcarbonyl and tertiary amine moieties strongly inhibit Saccharomyces cerevisiae β-carbonic anhydrase. J Enzyme Inhib Med Chem 2014;29:495–9.
11. Dimmock JR, Elias DW, Beazely MA, Kandepu NM. Bioactivities of chalcones. Curr Med Chem 1999;6:1125–49.
12. Das U, Gul HI, Alcorn J, et al. Cytotoxic 5-aryl-1-(4-nitrophenyl)-3-oxo-1,4-pentadienes mounted on alicyclic scaffolds. Eur J Med Chem 2006;41:577–85.
13. Ivanova Y, Momekov G, Petrov O, et al. Cytotoxic Mannich bases of 6-(3-aryl-2-propenoyl)-2(3H)-benzoxazolones. Eur J Med Chem 2007;42:1382–7.
14. Yerdelen KO, Gul HI, Sakagami H, Umemura N. Synthesis and biological evaluation of 1,5-bis(4-hydroxy-3-methoxyphenyl)penta-1,4-dien-3-one and its aminomethyl derivatives. J Enzyme Inhib Med Chem 2015;30:383–8.
15. Gul HI, Cizmecioglu M, Zencir S, et al. Cytotoxic activity of 4′-hydroxychalcone derivatives against Jurkat cells and their effects on mammalian DNA topoisomerase I. J Enzyme Inhib Med Chem 2009;24:804–7.
16. Bilginer S, Gul HI, Mete E, et al. 1-(3-aminomethyl-4-hydroxyphenyl)-3-pyridinyl-2-propen-1-ones: a novel group of tumor-selective cytotoxins. J Enzyme Inhib Med Chem 2013;28:974–80.
17. Roman G. Mannich bases in medicinal chemistry and drug design. Eur J Med Chem 2015;89:743–816.
18. Gul HI, Calis U, Vepsalainen J. Synthesis and evaluation of anticonvulsant activities of some bis Mannich bases and corresponding piperidinols. Arzneimittelforschung 2002;52:863–9.
19. Gul HI, Gul M, Erciyas E. Toxicity of some bis Mannich bases and corresponding piperidinols in the brine shrimp (*Artemia salina*) bioassay. J Appl Toxicol 2003;23:53–7.
20. Gul HI, Das U, Pandit B, Li PK. Evaluation of the cytotoxicity of some mono-mannich bases and their corresponding azine derivatives against androgen-independent prostate cancer cells. Arzneimittelforschung 2006;56:850–4.
21. Tugrak M, Yamali C, Sakagami H, Gul HI. Synthesis of mono Mannich bases of 2-(4-hydroxybenzylidene)-2,3-dihydroinden-1-one and evaluation of their cytotoxicities. J Enzyme Inhib Med Chem 2016;31:818–23.
22. Gul HI, Yamali C, Yasa AT, et al. Carbonic anhydrase inhibition and cytotoxicity studies of Mannich base derivatives of thymol. J Enzyme Inhib Med Chem 2016. [Epub ahead of print]. doi: 10.3109/14756366.2016.1140755.
23. Subramaniapillai SG. Mannich reaction: A versatile and convenient approach to bioactive skeletons. J Chem Sci 2013;125:467–82.
24. Xiaowu Dong TL, Jing C, Huazhou Y, Yongzhou H. Microwave-assisted Mannich reaction of 2-hydroxy-chalcones. Synt Comm 2009;39:733–42.
25. Dimmock JR, Kandepu NM, Nazarali AJ, et al. Sequential cytotoxicity: a theory evaluated using novel 2-[4-(3-aryl-2-propenoyloxy)phenylmethylene]cyclohexanones and related compounds. J Med Chem 2000;43:3933–40.
26. Supuran CT. Carbonic anhydrases: Novel therapeutic applications for inhibitors and activators. Nat Rev Drug Discov 2008;7: 168–81.
27. Supuran CT. How many carbonic anhydrase inhibition mechanisms exist? J Enzyme Inhib Med Chem 2016;31:345–60.
28. Supuran CT, Scozzafava A, Casini A. Carbonic anhydrase inhibitors. Med Res Rev 2003;23:146–89.
29. Supuran CT. Carbonic anhydrases as drug targets – an overview. Curr Top Med Chem 2007;7:825–33.
30. Supuran CT. Structure and function of carbonic anhydrases. Biochem J 2016;473:2023–32.
31. Bayram E, Senturk M, Kufrevioglu OI, Supuran CT. In vitro inhibition of salicylic acid derivatives on human cytosolic

carbonic anhydrase isozymes I and II. Bioorg Med Chem 2008;16: 9101–5.
32. Innocenti A, Beyza Ozturk Sarikaya S, Gulcin I, Supuran CT. Carbonic anhydrase inhibitors. Inhibition of mammalian isoforms I-XIV with a series of natural product polyphenols and phenolic acids. Bioorg Med Chem 2010;18:2159–64.
33. Gorlach A, Kietzmann T. Superoxide and derived reactive oxygen species in the regulation of hypoxia-inducible factors. Meth Enzymol 2007;435:421–46.
34. Sakagami H, Shimada C, Kanda Y, et al. Effects of 3-styrylchromones on metabolic profiles and cell death in oral squamous cell carcinoma cells. Toxicol Rep 2015;2:1281–90.
35. Khalifah RG. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. J Biol Chem 1971;246:2561–73.
36. Maresca A, Temperini C, Pochet L, et al. Deciphering the mechanism of carbonic anhydrase inhibition with coumarins and thiocoumarins. J Med Chem 2010;53:335–44.
37. D'Ambrosio K, Smaine FZ, Carta F, et al. Development of potent carbonic anhydrase inhibitors incorporating both sulfonamide and sulfamide groups. J Med Chem 2012;55:6776–83.
38. Robles-Escajeda E, Das U, Ortega NM, et al. A novel curcumin-like dienone induces apoptosis in triple-negative breast cancer cells. Cell Oncol (Dordr) 2016;39:265–77.

Supplementary materials available on line