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ORIGINAL PAPER

Synthesis and investigation of anticancer, antibacterial activities and carbonic anhydrase, acetylcholinesterase inhibition profiles of novel (3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-acetyl-5-(aryl/heteroaryl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-diones

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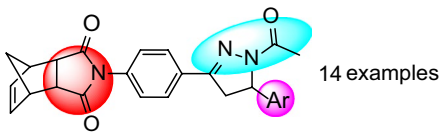
Abstract

A series of novel 1,3,5-trisubstituted pyrazoline derivatives, (3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-acetyl-5-(aryl/heteroaryl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-diones, were synthesized and evaluated for their antimicrobial and anticancer activities. In addition, the compounds were tested against acetylcholinesterase (AChE) enzyme and two physiologically relevant carbonic anhydrase I and II isozymes (hCA I and II). In this study, inhibition of hCA I and hCA II by the novel synthesized 1,3,5-trisubstituted pyrazolines was impressive, with K_i values in the range of 3.33–7.90 nM for hCA I and 2.07–8.47 nM for hCA II, while the K_i values of these compounds for AChE were recorded in the range of 9.61–48.42 nM, respectively. Two compounds can be investigated as the leader compounds because of their lowest K_i values to make further detailed CA inhibition studies.

Graphical abstract



IC_{50} = 4.22-6.86 nM for hCA I; AZA = 16.61 nM
IC_{50} = 3.01-5.45 nM For hCA II; AZA = 13.58 nM
IC_{50} = 22.08- 61.16 nM for AChE; Tacrine = 181.22 nM

Keywords Bioorganic chemistry · Heterocyclics · Enzyme inhibition · Chalcone · Antimicrobial · Anticancer

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Introduction

Heterocyclic compounds containing nitrogen atoms, which are found in the structure of natural and synthetic substances, are of growing importance [1]. Pyrazole, containing two adjacent nitrogen atoms in the structure, is the most typical examples of 5-membered ring heterocyclic compounds. Countless biologically and pharmacologically important compounds constitute from pyrazoles and their derivatives [2]. Pyrazole derivatives are known to exhibit many diverse biological activities such as antimicrobial, antifungal, antidepressant, immunosuppressive, anticonvulsive, antitumor, antiamibik, antibacterial, anti-inflammatory, analgesic, antitumor, anti-hypertensive, antipyretic, sedatives, and antidiabetic activities [3, 4]. Furthermore, recently, literatures revealed that the hydrazide–hydrazone (–CO–NH–N=CH–) moiety as a pharmacophore played a significant antitumor activity in some antitumor agents [5–8].

In addition, isoindole derivatives are essential for life [9]. Isoindole-1,3-dione derivatives have been the focus of attention for their many biological activities [10–14]. Chalcones, classes of promising anticancer agents, act curative for management of human cancers [15–17].

Carbonic anhydrases (CA, E.C.4.2.1.1) are ubiquitous Cd^{2+}, Zn^{2+}, or Fe^{2+} including metalloenzymes available in eukaryotes and prokaryotes. The CA superfamily includes seven separate and independent gene families including α-, β-, γ-, δ-, ζ-, n-, and θ-CAs [18, 19]. The α-CAs are found in prokaryotes, algae, the bacteria domain, vertebrates, cytoplasm of green plants with 16 isozymes actually defined in humans. Only the α-CAs are found in human. All mammals including humans generally contain α-CAs, which contain Zn^{2+} ions located in the active site of the enzyme [20, 21]. The β-CAs dominate in photosynthetic organisms and in some invertebrates, but they also were found in representatives of bacteria and fungi. On the other hand, the γ-CAs have been detected in some bacteria, Archaea and recently have been found in plants. The δ-CAs are recognized so far just in marine diatoms. Lately, ζ-CAs, which have Cd^{2+} ions in centre, are defined in marine diatoms [22–24]. Between their functions in physiological actions, CAs are involved in biomineralization both in vertebrates and invertebrates; however, the ensuing procedures are poorly understood recently [25–30]. In addition, as 1959, Goreau recorded the first witness of the involvement of CA in coral calcification [31–33]. Indeed, several CA isoforms are obtained in multitude critical physiological actions in human bodies, also their inhibition significantly by some compounds, is used medicinally for the therapy of epilepsy, obesity glaucoma, as diuretics or antitumor drugs/diagnostic factors [34–36].

The significant role of CAs is holding the acidic tumour surrounding and in sensing, an essential feature of solid tumours, by their carbon dioxide (CO_2) hydration catalysis [37, 38]:

$$CO_2 + H_2O \overset{CA}{\Leftrightarrow} H_2CO_3 \Leftrightarrow HCO_3^- + H^+.$$

A wide range of chemical compounds has been reported to inhibit the CAs' activity. The classical CA inhibitors (CAIs) are primary benzene sulfonamides bearing various substituents, which bind to Zn^{2+} ions in a deprotonated form (RSO_2NH^-) by exchanging a water molecule. The majority of the clinically used CAIs, exemplified by primary sulfonamides, acetazolamide, dorzolamide, methazolamide, and brinzolamide [39, 40]. Sulfonamides have been used as drugs for treatment of glaucoma, obesity, epileptic seizures, altitude sickness, and as diuretics [41–43]. Clinically approved sulfonamides lack selectivity towards CA isoforms and result in various side effects. Hence, the availability of true isoform-selective inhibitors is essential from the standpoint of drug discovery strategies unencumbered by the need to tackle off-target effects [44].

Alzheimer's disease (AD) is the most current age-related neurodegenerative disorder in elderly people [45–47]. The danger of stricken to AD changes with age. AD commonly affects about 15 million people economical [48–50]. Acetylcholinesterase enzyme (AChE) is existent in the synapse between muscle cells and nerve and is accountable for decomposing acetylcholine molecule [51]. Investigating of AChE is momentous due to its relevance with AD, which is reflected by the actuality that different AD drugs inhibit AChE enzymes [52]. On this foundation, AChE inhibitors (AChEIs) have become the significant strategy for the extension of anti-AD factors [53]. The most considerable biochemical variation in AD patients is a diminution of acetylcholine (ACh) amounts in the cortex of the brain and hippocampus [54]. AChE is recognized in entire excitable tissue, whether muscle or nerve, in placental tissue and in most erythrocytes [55–57].

Literature surveys reveal that chalcone substituted benzenesulfonamides [58], indole-based chalcone analogues [59], urea substituted chalcone derivatives [60], and methanoisoidole containing thiazole derivatives [61] possess inhibition effects against erythrocyte carbonic anhydrase I and II isoenzymes.

In our previous study, we have reported that methanoisoindole-substituted chalcone derivatives exhibit potent anticancer activity and inhibition effects on hCA I, hCA II, and AChE enzymes [62]. In continuation of our studies, this study was designed to synthesize novel 1,3,5-trisubstituted pyrazoline derivatives containing methanoisoindol-1,3-dione and evaluated their anticancer, antimicrobial activity and AChE and hCA I and II inhibitory properties.

Results and discussion

Chemistry

The methanoisoindole-substituted chalcone derivatives **1a**–**1n** were synthesized according to our recently published procedure [62]. The 1,3,5-trisubstituted pyrazoline derivatives **2a**–**2n** were obtained by the addition of hydrazine hydrate to chalcone derivatives **1a**–**1n** in acetic acid at reflux conditions for 4 h (Scheme 1). A bright yellow to off-white product was formed and was several times washed with water, isolated by filtration, and re-crystallized from ethanol [63, 64]. Analytical and spectral data (FT-IR, ^{1}H, and ^{13}C NMR) for all the synthesized compounds were in full agreement with the proposed structure (Table 1). All of the compounds were also characterized for C, H, N, and S analyses.

In the ^{1}H NMR spectrum of compound **2b**, the aromatic protons gave the two AA'BB' systems as expected at 7.81–7.27 ppm ($J = 8.4$ Hz) and at 7.16–6.85 ppm ($J = 8.8$ Hz), respectively. In addition, the methylenic protons H7 gave the AB at 1.82–1.65 ppm ($J = 8.8$ Hz). Moreover, the H19 of the pyrazole ring resonates as doublet of doublet ($J = 11.6$, 4.4 Hz) at 5.56 ppm, the H18 protons gave the AB system at 3.72 ($J = 17.6$ Hz, 11.6 Hz) and 3.15 ($J = 17.6$ Hz, 4.4 Hz) ppm as doublet of doublet, respectively (see Fig. 1 for numbering).

In vitro antimicrobial activity

All newly synthesized compounds were screened for their in vitro antimicrobial activities by disc-diffusion method using Mueller–Hilton agar medium against *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC-6633, which are Gram-positive bacteria, *Proteus vulgaris* KUEN 1329, *Pseudomonas aureginosa* ATCC 9027, *Escherichia coli* AÜ tıp which are Gram-negative bacteria, and *Candida albicans* ATCC 1213 which are fungus. In these tests, SCF (sulbactam/cefoperazone) was used as standard [62].

Recently, we have reported to synthesis and investigation of their antibacterial activities of series carbothioamide substituted-4,5-dihydropyrazole derivatives. While, most of the compounds show no activity against some microorganism, the compounds containing methoxy, chloro group and thienyl ring show good activity against most of the microorganism with 16–19 mm of inhibition zone, SCF: 25 mm [78]. In this study, all compounds show moderate-to-good activity against almost all microorganisms.

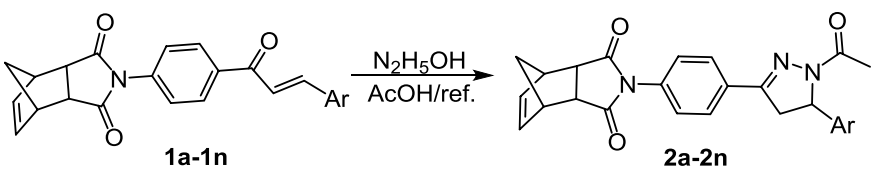
According to the test results obtained from Table 2, most of the compounds **2a**–**2n** shown inhibition activity against all used microorganisms. However, the most active compound is **2h** against *Staphylococcus aureus* ATCC 29213 with 15 mm inhibition zone (SCF: 25 mm). In addition, compounds **2c**, **2e**, and **2g** demonstrated good activity (15 mm) against *Proteus vulgaris* KUEN 1329 (SCF: 20 mm). Compounds **2g**, **2i**, **2k**, **2m**, and **2n** showed the most effective activity against *Bacillus subtilis* ATCC 6633 with (15 mm), (SCF: 21 mm). Compounds **2j**, **2k**, and **2l** exhibited remarkable activity against *Pseudomonas aeruginosa* ATCC 9027 with (15 mm) (SCF: 20 mm). The most active compounds are **2f** (17 mm), **2j**, and **2l** (16 mm) against *Candida albicans* ATCC 1213 (SCF: 19 mm). All compounds except **2a**–**2c** exhibited remarkable activity against *Escherichia coli* AÜ tip with 14–15 mm of inhibition zone (SCF: 26 mm).

Anticancer activity results against C6 cells

The anticancer activities of compounds **2a**–**2n** were investigated against C6 cell lines using the BrdU Cell Proliferation Assay (BCPA) [62]. The results were compared with 5-FU that was used as positive control for widely using in the treatment of cancer and given in figure (in supplementary material).

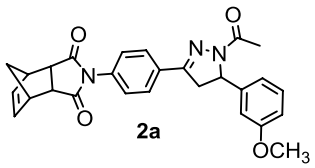
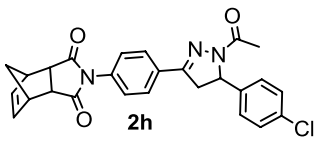
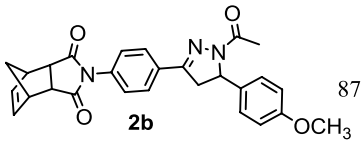
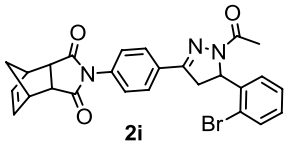
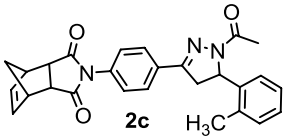
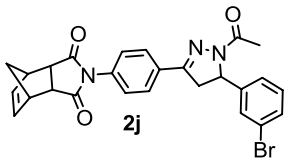
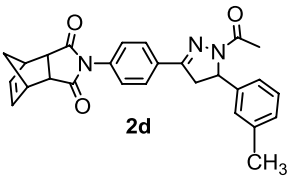
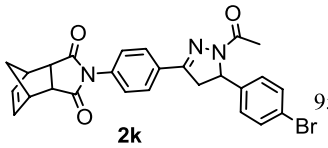
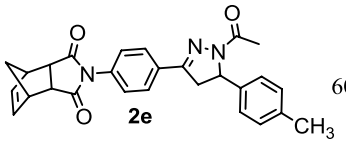
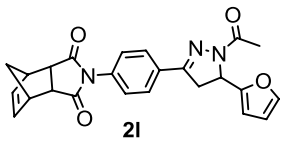
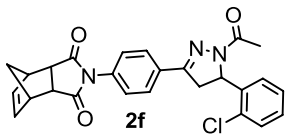
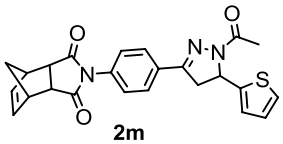
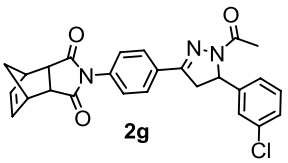
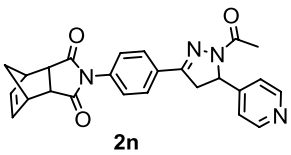
According to figure (in supplementary material) and Table 3, all compounds except **2l**–**2n** showed promising antiproliferative activity at all used concentrations. Compounds **2c**, **2d**, **2e**, **2f**, **2g**, **2j**, and **2k** showed very high activity with the IC_{50} values range of 33.46–95.04 µM compared to 5-FU (IC_{50}: 116.80 µM) at all concentrations. While the methoxy substituted compounds **2a** and **2b** shown lower activity than that of 5-FU, the methyl substituted compound **2c**–**2e** exhibited higher activity than 5-FU. Similarly, chloro- and bromo-substituted compounds **2f**–**2k** except **2i** (4-Br) exhibited

Scheme 1



Ar = **a**) 3-CH_3OPh, **b**) 4-CH_3OPh, **c**) 2-CH_3Ph, **d**) 3-CH_3Ph, **e**) 4CH_3Ph, **f**) 2-ClPh, **g**) 3-ClPh, **h**) 4-ClPh, **i**) 2-BrPh, **j**) 3-BrPh, **k**) 4-BrPh, **l**) 2-furyl, **m**) 2-thienyl, **n**) 4-pyridyl

Table 1 Synthesis of 1,3,5-trisubstituted pyrazoline derivatives **2a**–**2n**

Entry	Product	Yield /%	M. P. /°C	Entry	Product	Yield /%	M. P. /°C
1	 **2a**	83	125-128	8	 **2h**	91	124-128
2	 **2b**	87	117-127	9	 **2i**	93	198-202
3	 **2c**	78	201-203	10	 **2j**	83	124-127
4	 **2d**	69	205-208	11	 **2k**	95	128-132
5	 **2e**	60	120-124	12	 **2l**	75	246-248
6	 **2f**	86	226-229	13	 **2m**	90	248-250
7	 **2g**	50	150-154	14	 **2n**	55	270-272

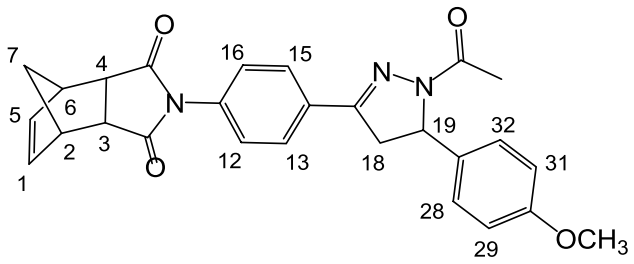


Fig. 1 Numbering scheme for compound **2b**

very high activity. In addition, furan, thiophene, and pyridine ring containing compounds **2l**–**2n** demonstrated very low activity. The most active compounds are **2j** (3-Br, IC_{50}: 33.46 µM), followed by **2c** (2-CH_3, IC_{50}: 50.05 µM), **2h** (4-Cl, IC_{50}: 53.32 µM), **2f** (2-Cl, IC_{50}: 54.60 µM), **2e** (4-CH_3, IC_{50}: 60.46 µM), **2g** (3-Cl, IC_{50}: 84.25 µM), **2k** (4-Br, IC_{50}: 88.85 µM), **2d** (3-CH_3, IC_{50}: 95.04 µM), and the others.

Biochemistry results

CA isoforms catalyse a very plain reaction, the interconversion between bicarbonate ion (HCO_3^-), and CO_2 [65, 66].

Table 2 Antimicrobial activities of compounds **2a**–**2n**

Entry	Product	*S. aureus* ATCC 29213	*P.vulgaris* KUEN 1329	*B.subtilis* ATCC 6633	*P.auregi-nosa* ATCC 9027	*C.albicans* ATCC 1213	*E. coli* AÜ tip
1	**2a**	–	12	10	12	14	7
2	**2b**	–	12	13	10	10	–
3	**2c**	8	15	14	9	7	–
4	**2d**	14	13	10	12	12	14
5	**2e**	13	14	10	9	12	15
6	**2f**	11	10	10	10	17	14
7	**2 g**	12	14	15	10	14	15
8	**2 h**	15	12	10	10	15	14
9	**2i**	12	13	15	13	15	15
10	**2j**	13	13	10	15	16	14
11	**2 k**	12	12	15	15	14	14
12	**2 l**	13	13	10	15	16	14
13	**2 m**	13	13	15	12	15	15
14	**2n**	13	12	15	12	15	14
	SCF	25	20	21	20	19	26

Table 3 IC_{50} values of **2a**–**2n** against C6 cell (μM)

Compounds	IC_{50}	Compounds	IC_{50}
2a	156.00	**2 h**	53.32
2b	144.12	**2i**	154.06
2c	50.05	**2j**	33.46
2d	95.04	**2k**	88.85
2e	60.46	**2l**	362.04
2f	54.60	**2m**	–
2 g	84.25	**2n**	324.00
5-FU	116.80	5-FU	116.80

– Not determined

They are also involved in significant physiological actions related with transport of HCO_3^-/CO_2 among metabolizing lungs and tissues and respiration, biosynthetic reactions including lipogenesis, gluconeogenesis, ureagenesis, CO_2 homeostasis and pH, electrolytes spatter in a diversity of organs or tissues, calcification, and bone resorption [67, 68]. Many of these isoforms are aims of inhibitors with diverse therapeutic function [69–71]. The main clinical inhibitors of CA isoforms are ethoxzolam, acetazolamide, and methazolamide [72, 73]. Recently, it has been recognized that two CA isoforms (CA IX and XII) are significantly associated with cancer, where they are involved in tumour advancement and pH regulation [74, 75]. In addition, to the important role of CA inhibitors (CAIs) as antiglaucoma drugs and diuretics, it has newly determined that they have conceivable as antiobesity, anticonvulsant, and anti-infective drugs [76, 77]. More amazing to us, studies have recognized that several of benzofuran compounds such as tacrine and donepezil are capable to inhibit AChE enzyme, or have the susceptibility of decreasing aggregated β-amyloid (Aβ) in the brain [22, 27]. Inhibition results against two catalytically active hCA isoenzymes, that is, cytosolic hCA I and II and AChE enzymes with the 1,3,5-trisubstituted pyrazolines **2a**–**2n**, as well as the standard sulfonamide AZA are shown in Table 4.

(1) Cytosolic hCA I isoform is recognized in the human body and can be obtained in gastrointestinal tract and high concentrations in the blood [53, 77]. To explain inhibitory results, researchers frequently use a half-maximal inhibitory concentration (IC_{50}) value. In addition, a more appropriate measure is the inhibitor constant (K_i) value. In this work, cytosolic hCA I isoform was potently inhibited by novel 1,3,5-trisubstituted pyrazolines derivatives **2a**–**2n**. As shown in Table 4, IC_{50} values were in the range of 4.22-6.86 nM towards CA I. In addition, K_i values were recognized in ranging between 3.33 ± 0.77 and 7.90 ± 1.74 nM (Table 4). The results determined from this work obviously shown that novel 1,3,5-trisubstituted pyrazolines **2a**–**2n** had effective inhibition profile against cytosolic isoform hCA I. In addition, it was reported that some similar novel carbothioamide substituted-4.5-dihydropyrazole derivatives had low nanomolar K_i values in ranging of 12 ± 2–35 ± 4 nM against the cytosolic isoform hCA I [78]. Among this series, the most active compound was bromine atom bearing compound with K_i value of 12 ± 2 nM. The results of two studies are in concordance with each other. It was indicated that hCA I is involved in cerebral oedema and retinal cells. In addition, inhibition of hCA I could be a significant gadget

Table 4 Inhibition profiles of novel 1,3,5-trisubstituted pyrazoline derivatives **2a**–**2n** on human carbonic anhydrase I and II isoenzymes (hCA I and II) and acetylcholinesterase (AChE) enzymes

Compounds	IC_{50}/nM						K_i/nM		
	HCA I	R^2	HCA II	R^2	AChE	R^2	HCA I	HCA II	AChE
2a	4.91	0.9767	3.34	0.9603	54.56	0.9412	4.54 ± 1.25	3.64 ± 1.11	38.85 ± 9.05
2b	5.21	0.9685	4.35	0.9753	44.94	0.9880	4.65 ± 1.18	3.14 ± 0.78	26.44 ± 3.70
2c	5.31	0.9502	3.13	0.9759	38.35	0.9889	6.21 ± 2.07	2.48 ± 0.81	35.47 ± 4.32
2d	4.27	0.9762	3.26	0.9891	61.16	0.9452	4.48 ± 0.86	2.07 ± 0.37	46.33 ± 6.43
2e	6.07	0.9435	5.45	0.9259	37.84	0.9556	5.91 ± 1.50	6.04 ± 2.38	42.46 ± 19.01
2f	4.52	0.9681	3.38	0.9702	42.48	0.9662	5.24 ± 1.76	4.01 ± 1.23	48.42 ± 12.41
2 g	6.13	0.9590	5.41	0.9486	20.87	0.9973	7.20 ± 1.66	8.47 ± 2.38	10.76 ± 2.02
2 h	6.07	0.9673	3.98	0.9522	41.22	0.9850	5.60 ± 1.15	4.96 ± 1.18	26.68 ± 2.71
2i	4.22	0.9573	3.01	0.9804	22.68	0.9789	4.70 ± 2.22	4.15 ± 0.62	9.61 ± 0.72
2j	4.47	0.9776	3.26	0.9852	22.08	0.9870	3.33 ± 0.77	3.94 ± 1.08	11.61 ± 0.76
2 k	5.33	0.9760	4.07	0.9565	51.25	0.9611	5.82 ± 1.14	4.73 ± 0.74	29.36 ± 5.42
2 l	5.87	0.9329	4.58	0.9744	41.52	0.9928	5.63 ± 1.74	5.60 ± 1.22	22.01 ± 7.82
2 m	6.86	0.9766	5.17	0.9630	59.13	0.9843	7.90 ± 1.74	5.74 ± 1.64	36.81 ± 6.95
2n	6.24	0.9905	4.98	0.9812	26.60	0.9891	6.38 ± 1.14	6.31 ± 0.72	15.54 ± 1.35
AZA*	16.91	0.9654	13.58	0.9416	–	–	18.64 ± 5.03	9.85 ± 1.94	–
TAC**	–	–	–	–	181.22	0.9576	–	–	110.30 ± 36.32

*AZA (acetazolamide) was used as positive control for hCA I and II isoenzymes

**TAC (Tacrine) was used as positive control for AChE enzyme

for fighting uneven position. In addition, acetazolamide (AZA) showed K_i values of 18.64 ± 5.03 nM against hCA I that defined as broad-specificity hCA inhibitor owing to its current inhibition of CAs. Among the tested novel hydrazine compounds **2a**–**2n**, the bromo derivative **2j** was found the best hCA I inhibitor with K_i value of 3.33 ± 0.77 nM.

(2) Human CA II recognized as participating in multiple other hydrolytic actions and some catalytic diversity, which likely define nonphysiological substrates [57, 79]. Against, the physiologically predominant isoenzyme hCA II, novel hydrazine compounds **2a**–**2n** demonstrated K_i values from 2.07 ± 0.37 to 8.47 ± 2.38 nM (Table 4). Between which compound **2d** was found the best effective hCA II inhibitor (K_i: 2.07 ± 0.37 nM). On the other hand, AZA that is practically used as positive standard for both CA isoenzymes obtained K_i value of 9.85 ± 1.94 nM towards hCA II. In our recent study, novel carbothioamide substituted-4,5-dihydropyrazole derivatives had similar results and demonstrated K_is varying from 23 ± 6 to 58 ± 14 nM [78].

(3) AD therapy has been realized by the use of AChE inhibitors [54, 57]. In the investigation for novel compounds with the potential effect of inhibiting the enzyme AChE are significantly shown that multitude heterocyclics have recorded important inhibitory activity on AChE which currently including three of the four AD drugs on the market [54]. All of new 1,3,5-trisubstituted pyrazolines **2a**–**2n** had substantially higher AChE inhibitory activity than tacrine (K_i: 110.30 ± 36.32 nM), which used as anti-cholinesterase. Indeed, the K_i values of standard compound (tacrine) and new 1,3,5-trisubstituted pyrazolines **2a**–**2n** are shown in Table 4. In this study, as can be seen in the results recorded from Table 4, AChE was significantly higher inhibited by new 1,3,5-trisubstituted pyrazolines **2a**–**2n**, with K_i values in the range of 9.61 ± 0.72 to 48.42 ± 12.41 nM. The most active one is **2i** and showed a K_i value of 9.61 ± 0.72 nM. However, all new 1,3,5-trisubstituted pyrazolines **2a**–**2n** obtained higher AChE inhibition activity than that of tacrine (K_i: 110.30 ± 36.32 nM). Recently, we found that AChE enzyme was significantly inhibited by novel carbothioamide substituted-4,5-dihydropyrazole derivatives, with K_i values in the range of 57 ± 20 to 256 ± 82 nM [78]. Among this series, the most active compound was methoxy group containing compound with K_i value of 57 ± 20 nM. These results showed that *N*-acetyl substituted pyrazolines **2a**–**2n** had higher affinity against AChE than that of carbothioamide substituted pyrazolines [78]. This high affinity of *N*-acetylpyrazolines **2a**–**2n** can be attributed to the acetyl group.

Conclusions

In the present study, a series of new 1,3,5-trisubstituted pyrazoline derivatives **2a**–**2n** were synthesized, characterized, and investigated their anticancer activity against C6 rat glio-carcinoma, antimicrobial activity against some human pathogen microorganism and evaluated inhibition effects against hCA I, hCA II, and AChE enzymes. The compounds **2c**, **2d**, **2e**, **2f**, **2g**, **2j**, and **2k** showed very high activity with the IC_{50} values range of 33.46–95.04 μM compared to 5-FU (IC_{50}: 116.80 μM) at all concentrations. All compounds **2a**–**2n** were evaluated for their AChE, hCA I, and II inhibitory properties that obtained at the nanomolar levels. The results clearly showed that all new synthesized 1,3,5-trisubstituted pyrazoline derivatives **2a**–**2n** can be selective inhibitor of AChE and both cytosolic CA isoenzymes. In addition, these compounds **2a**–**2n** recognized in this work which can be good candidates for the therapy of disparate other memory impairments and mild-to-moderate AD.

Experimental

Melting points were measured on Electrothermal 9100 apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker-rAvance DPX-400 instrument. As internal standards served TMS ($\delta = 0.00$) for ^{1}H NMR and $CDCl_3$ ($\delta = 77.0$) for ^{13}C NMR spectroscopy. *J* values are given in Hz. The multiplicities of the signals in the ^{1}H NMR spectra are abbreviated by s (singlet), d (doublet), t (triplet), q (quarted), m (multiplet), br (broad), and combinations thereof. Elemental analyses were obtained from a LECO CHNS 932 Elemental Analyser.

General procedure for the synthesis of 2a–2n

A solution of chalcone derivatives **1a**–**1n** (1 mmol) and hydrazine hydrate (2 mmol) in 10 cm^3 acetic acid was refluxed for 4 h. After the reaction was complete, the mixture was poured into ice-water and neutralized with ammonia by controlling with pH paper. The mixture was allowed to stand at room temperature overnight, and the formed precipitate was filtered off and dried. The pure products were obtained by crystallization from ethanol.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(3-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2a, $C_{27}H_{25}N_3O_4$) Yield 83%; m.p.: 125–128 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.80$ (d, $J = 8.4$ Hz, 2H), 7.27–7.23 (m, 3H), 6.81–6.75 (m, 3H), 6.29 (bs, 2H), 5.58 (dd, $J = 11.8$ Hz, 4.8 Hz, 1H), 3.79–3.67 (m, 4H), 3.54 (bs, 2H), 3.47 (t, $J = 1.6$ Hz, 2H), 3.14 (dd, $J = 17.6$ Hz, 4.8 Hz, 1H), 2.44 (bs, 3H), 1.82 (d, $J = 8.8$ Hz, 1H), 1.65 (d, $J = 8.8$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 176.5$ (2C), 168.7, 160.0, 152.8, 143.3, 134.6 (2C), 133.3, 131.5, 130.0, 127.1 (2C), 126.8 (2C), 117.6, 112.9, 111.3, 60.0, 55.2, 52.3, 45.8 (2C), 45.5 (2C), 42.2, 21.9 ppm; IR (KBr): $\overline{V} = 1708$, 1664, 1600, 1378, 1326, 1288, 1257, 1178, 1049 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2b, $C_{27}H_{25}N_3O_4$) Yield 87%; m.p.: 117–121 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.81$ (d, $J = 8.4$ Hz, 2H, H12,16), 7.27 (d, $J = 8.4$ Hz, 2H, H13,15), 7.16 (d, $J = 8.8$ Hz, 2H, H28,32), 6.85 (d, $J = 8.8$ Hz, 2H, H29,31), 6.29 (bs, 2H, H1,5), 5.56 (dd, $J = 11.6$ Hz, 4.4 Hz, 1H, H19), 3.79 (bs, 3H, $-OCH_3$), 3.72 (dd, $J = 17.6$ Hz, 12.0 Hz, 1H, H18), 3.54 (brs, 2H, H3,4), 3.47 (t, $J = 1.4$ Hz, 2H, H2,6), 3.15 (dd, $J = 17.6$ Hz, 4.4 Hz, 1H, H18), 2.41 (bs, 3H, $-CH_3$), 1.82 (d, $J = 8.8$ Hz, 1H, H7), 1.65 (d, $J = 8.8$ Hz, 1H, H7) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 176.5$ (2C), 168.9, 159.0, 152.8, 134.6 (2C), 133.9, 133.2, 131.6, 127.1 (2C), 126.9 (2C), 126.8 (2C), 114.2 (2C), 59.6, 55.2, 52.3, 45.8 (2C), 45.5 (2C), 42.1, 21.9 ppm; IR (KBr): $\overline{V} = 2360$, 1708, 1660, 1610, 1513, 1444, 1378, 1326, 1290, 1247, 1178 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(*o*-tolyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2c, $C_{27}H_{25}N_3O_3$) Yield 78%; m.p.: 201–203 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.79$ (d, $J = 8.4$ Hz, 2H), 7.23 (d, $J = 8.4$ Hz, 2H), 7.19–7.14 (m, 3H), 6.98–6.96 (m, 1H), 6.28 (bs, 2H), 5.77 (dd, $J = 12.0$ Hz, 4.8 Hz, 1H), 3.75 (dd, $J = 17.2$ Hz, 12.0 Hz, 1H), 3.54 (bs, 2H), 3.48 (t, $J = 1.6$ Hz, 2H), 3.00 (dd, $J = 17.2$ Hz, 4.8 Hz, 1H), 2.47 (s, 6H), 1.82 (d, $J = 8.8$ Hz,1H), 1.64 (d, $J = 8.8$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 176.5$ (2C), 168.8, 152.7, 139.6, 134.6 (2C), 134.0, 133.2, 131.6, 130.7, 127.5, 127.1 (2C), 126.8 (2C), 126.7, 123.9, 57.2, 52.3, 45.8 (2C), 45.7 (2C), 41.5, 21.9, 19.4 ppm; IR (KBr): $\overline{V} = 1708$, 1656, 1446, 1376, 1330, 1178 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(*m*-tolyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2d, $C_{27}H_{25}N_3O_3$) Yield 69%; m.p.: 205–208 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.81$ (d, $J = 8.4$ Hz, 2H), 7.24 (d, $J = 8.4$ Hz, 2H), 7.20 (d, $J = 7.6$ Hz, 1H), 7.07 (d, $J = 7.6$ Hz, 1H), 7.02 (d, $J = 7.6$ Hz, 2H), 6.29 (bs, 2H), 5.57 (dd, $J = 11.6$ Hz, 4.8 Hz, 1H), 3.73 (dd, $J = 17.6$ Hz, 11.6 Hz, 1H), 3.54 (bs, 2H), 3.48 (t, $J = 1.6$ Hz, 2H), 3.14 (dd, $J = 17.6$ Hz, 4.8 Hz, 1H), 2.43 (bs, 3H), 2.33 (bs, 3H), 1.82 (d, $J = 8.8$ Hz, 1H), 1.64 (d, $J = 8.8$ Hz, 1H) ppm; ^{13}C NMR

(100 MHz, $CDCl_3$): $\delta = 176.5$ (2C), 168.9, 152.8, 141.7, 138.6, 134.6 (2C), 133.3, 131.5, 128.8, 128.5, 127.2 (2C), 126.8 (2C), 126.0, 122.5, 60.1, 52.3, 45.8 (2C), 45.5 (2C), 42.3, 22.0, 21.4 ppm; IR (KBr): $\overline{V} = 1710$, 1664, 1606, 1519, 1444, 1378, 1326, 1178 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(*p*-tolyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2e, $C_{27}H_{25}N_3O_3$) Yield 60%; m.p.: 120–124 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.80$ (d, $J = 8.4$ Hz, 2H), 7.24 (d, $J = 8.4$ Hz, 2H), 7.15–7.10 (m, 4H), 6.29 (brs, 2H), 5.57 (dd, $J = 12.0$ Hz, 4.4 Hz, 1H), 3.74 (dd, $J = 17.8$ Hz, 12.0 Hz, 1H), 3.54 (bs, 2H), 3.48 (t, $J = 1.6$ Hz, 2H), 3.14 (dd, $J = 17.6$ Hz, 4.8 Hz, 1H), 2.42 (bs, 3H), 2.32 (bs, 3H), 1.82 (d, $J = 8.8$ Hz, 1H), 1.65 (d, $J = 8.8$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 176.5$ (2C), 168.9, 152.8, 138.8, 137.4, 134.6 (2C), 133.2, 131.6, 129.5 (2C), 127.1 (2C), 126.8 (2C), 125.5 (2C), 59.9, 52.3, 45.8 (2C), 45.5 (2C), 42.2, 21.9, 21.1 ppm; IR (KBr): $\overline{V} = 2360$, 1710, 1662, 1517, 1376, 1326, 1178 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(2-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2f, $C_{26}H_{22}ClN_3O_3$) Yield 86%; m.p.: 226–229 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.79$ (d, $J = 8.0$ Hz, 2H), 7.42–7.40 (m, 1H), 7.24–7.21 (m, 4H), 7.06–7.04 (m, 1H), 6.28 (bs, 2H), 5.94 (dd, $J = 11.6$ Hz, 4.8 Hz, 1H), 3.84 (dd, $J = 17.8$ Hz, 11.8 Hz, 1H), 3.54 (bs, 2H), 3.47 (bs, 2H), 3.05 (dd, $J = 17.6$ Hz, 4 Hz, 1H), 2.49 (bs, 3H), 1.82 (d, $J = 8.8$ Hz, 1H), 1.64 (d, $J = 8.8$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 176.5$ (2C), 168.9, 153.1, 140.5, 138.3, 134.6 (2C), 133.4, 131.6, 131.3, 130.0, 128.8, 127.3, 127.1 (2C), 126.8 (2C), 57.9, 52.3, 45.8 (2C), 45.5 (2C), 41.3, 21.9 ppm; IR (KBr): $\overline{V} = 1770$, 1708, 1656, 1519, 1448, 1378, 1330, 1170, 1039 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(3-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2g, $C_{26}H_{22}ClN_3O_3$) Yield 50%; m.p.: 150–154 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.81$ (d, $J = 8.0$ Hz, 2H), 7.27–7.25 (m, 4H), 7.20 (bs, 1 H), 7.11 (d, $J = 6.8$ Hz, 1H), 6.29 (bs, 2H), 5.57 (dd, $J = 11.8$ Hz, 4.6 Hz, 1H), 3.76 (dd, $J = 17.8$ Hz, 11.8 Hz, 1H), 3.54 (bs, 2H), 3.48 (bs, 2H), 3.14 (dd, $J = 17.4$ Hz, 4.6 Hz, 1H), 2.44 (bs, 3H), 1.82 (d, $J = 8.8$ Hz, 1H), 1.65 (d, $J = 8.8$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 176.5$ (2C), 169.0, 152.7, 143.6, 134.8, 134.6 (2C), 133.4, 131.2, 130.2, 128.0, 127.2 (2C), 126.8 (2C), 125.7, 123.7, 59.6, 52.3, 45.9 (2C), 45.5 (2C), 42.1, 21.9 ppm; IR (KBr): $\overline{V} = 2360$, 1708, 1664, 1421, 1407, 1376, 1326, 1178 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(4-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2h, $C_{26}H_{22}ClN_3O_3$) Yield 91%; m.p.: 124–128 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.80$ (d, $J = 8.4$ Hz, 2H), 7.30 (d, $J = 8.4$ Hz, 2H), 7.25 (d, $J = 8.8$ Hz, 2H), 7.16 (d, $J = 8.4$ Hz, 2H), 6.28 (bs, 2H), 5.56 (dd, $J = 11.8$ Hz, 4.6 Hz, 1H), 3.74 (dd, $J = 17.8$ Hz, 11.8 Hz, 1H), 3.53 (bs, 2H), 3.47 (t, $J = 1.6$ Hz, 2H), 3.12 (dd, $J = 17.8$ Hz, 4.6 Hz, 1H), 2.42 (bs, 3H), 1.81 (d, $J = 8.8$ Hz, 1H), 1.64 (d, $J = 8.8$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 176.5$ (2C), 168.9, 152.7, 140.2, 134.6 (2C), 133.5, 133.4, 131.3, 129.1 (2C), 127.1 (2C), 127.0 (2C), 126.8 (2C), 59.5, 52.3, 45.8 (2C), 45.5 (2C), 42.1, 21.9 ppm; IR (KBr): $\overline{V} = 2360$, 1708, 1662, 1376, 1324, 1292, 1176, 1089 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(2-bromophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2i, $C_{26}H_{22}BrN_3O_3$) Yield 93%; m.p.: 198–202 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.79$ (d, $J = 8.4$ Hz, 2H), 7.60 (d, $J = 8.0$ Hz, 1H), 7.28–7.27 (m, 1H), 7.23 (d, $J = 8.4$ Hz, 2H), 7.15 (t, $J = 7.6$ Hz, 1H), 7.02 (d, $J = 7.6$ Hz, 1H), 6.28 (bs, 2H), 5.92 (dd, $J = 11.4$ Hz, 4.8 Hz, 1H), 3.86 (dd, $J = 17.8$ Hz, 12.0 Hz, 1H), 3.54 (bs, 2H), 3.47 (bs, 2H), 3.03 (dd, $J = 17.8$ Hz, 4.8 Hz, 1H), 2.49 (bs, 3H), 1.82 (d, $J = 8.8$ Hz, 1H), 1.64 (d, $J = 8.8$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 176.5$ (2C), 168.9, 153.0, 139.9, 134.6 (2C), 133.4, 133.2, 131.3, 129.1, 127.9, 127.1 (2C), 126.8 (2C), 125.8, 121.5, 60.0, 52.3, 45.8 (2C), 45.5 (2C), 41.5, 21.9 ppm; IR (KBr): $\overline{V} = 1708$, 1658, 1409, 1378, 1328, 1178,1025 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(3-bromophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2j, $C_{26}H_{22}BrN_3O_3$) Yield 83%; m.p.: 124–127 °C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.80$ (d, $J = 8.8$ Hz, 2H), 7.40 (d, $J = 8.0$ Hz, 1H), 7.27 (d, $J = 8.8$ Hz, 2H), 7.19 (d, $J = 8.0$ Hz, 2H), 7.15 (d, $J = 8.0$ Hz, 1H), 6.29 (bs, 2H), 5.56 (dd, $J = 11.8$ Hz, 4.8 Hz, 1H), 3.75 (dd, $J = 17.6$ Hz, 12.0 Hz, 1H), 3.54 (bs, 2H), 3.48 (t, $J = 1.6$ Hz, 2H), 3.13 (dd, $J = 17.6$ Hz, 4.8 Hz, 1H), 2.44 (bs, 3H), 1.82 (d, $J = 9.2$ Hz, 1H), 1.65 (d, $J = 8.8$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 176.5$ (2C), 168.9, 152.6, 143.9, 134.6 (2C), 133.4, 131.2, 130.9, 130.5, 128.6, 127.2 (2C), 126.8 (2C), 124.2, 123.0, 59.5, 52.3, 45.8 (2C), 45.5 (2C), 42.1, 21.9 ppm; IR (KBr): $\overline{V} = 2360$, 1708, 1664, 1594, 1569, 1517, 1376, 1324, 1176 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(4-bromophenyl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2k, $C_{26}H_{22}BrN_3O_3$) Yield 95%; m.p.: 128–132 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.80 (d, J = 8.4 Hz, 2H), 7.45 (d, J = 8.4 Hz, 2H), 7.25 (d, J = 8.4 Hz, 2H), 7.11 (d, J = 8.4 Hz, 2H), 6.29 (bs, 2H), 5.56 (dd, J = 11.8 Hz, 4.8 Hz, 1H), 3.75 (dd, J = 17.6 Hz, 12 Hz, 1H), 3.54 (bs, 2H), 3.47 (t, J = 1.6 Hz, 2H), 3.12 (dd, J = 17.6 Hz, 4.8 Hz, 1H), 2.42 (s, 3H), 1.82 (d, J = 8.8 Hz, 1H), 1.65 (d, J = 8.8 Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 176.5 (2C), 169.0, 152.7, 140.7, 134.6 (2C), 133.4, 132.0 (2C), 131.2, 127.4 (2C), 127.2 (2C), 126.8 (2C), 121.6, 59.6, 52.3, 45.9 (2C), 45.5 (2C), 42.0, 21.9 ppm; IR (KBr): $\overline{V}$ = 2358, 1708, 1662, 1407, 1376, 1326, 1176 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(furan-2-yl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2l, $C_{24}H_{21}N_3O_4$) Yield 75%; m.p.: 246–248 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.82 (d, J = 8.4 Hz, 2H), 7.31–730 (m, 1H), 7.25 (d, J = 8.4 Hz, 2H), 6.35–6.32 (m, 2H), 6.30–6.29 (m, 2H), 5.72 (dd, J = 11.6 Hz, 4.8 Hz, 1H), 3.62–3.40 (m, 6H), 2.40 (s, 3H), 1.82 (d, J = 8.8 Hz, 1H), 1.65 (d, J = 8.8 Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 176.6 (2C), 169.1, 153.1, 151.9, 142.0, 134.7 (2C), 133.4, 131.5, 127.2 (2C), 126.8 (2C), 110.6, 107.7, 53.5, 52.3, 45.9 (2C), 45.6 (2C), 38.1, 21.9 ppm; IR (KBr): $\overline{V}$ = 3459, 2977, 2944, 2873, 1770, 1704, 1656, 1608, 1519, 1506, 1382, 1332, 1184, 1122 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2m, $C_{24}H_{21}N_3O_3S$) Yield 90%; m.p.: 248–250 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.82 (d, J = 8.8 Hz, 2H), 7.26 (d, J = 8.4 Hz, 2H), 7.20 (d, J = 5.2 Hz, 1H), 7.03 (d, J = 3.2 Hz, 1H), 6.93 (dd, J = 5.2 Hz, 3.2 Hz, 1H), 6.29 (bs, 2H), 5.93 (dd, J = 11.2 Hz, 4.0 Hz, 1H), 3.73 (dd, J = 17.6 Hz, 11.2 Hz, 1H), 3.55 (bs, 2H), 3.48 (t, J = 1.6 Hz, 2H), 3.34 (dd, J = 17.6 Hz, 4.0 Hz, 1H), 2.41 (s, 3H), 1.83 (d, J = 8.8 Hz, 1H), 1.65 (m, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 176.6 (2C), 169.0, 152.8, 144.1, 134.7 (2C), 133.4, 131.4, 127.2 (2C), 126.9, 126.8, 124.9 (2C), 124.7, 55.4, 52.4, 45.9 (2C), 45.6 (2C), 41.9, 22.0 ppm; IR (KBr): $\overline{V}$ = 3457, 3428, 2360, 1702, 1654, 1380, 1332, 1182, 1174 cm^{-1}.

(3a*R*,4*S*,7*R*,7a*S*)-2-[4-[1-Acetyl-5-(pyridin-4-yl)-4,5-dihydro-1*H*-pyrazol-3-yl]phenyl]-3a,4,7,7a-tetrahydro-1*H*-4,7-methanoisoindole-1,3(2*H*)-dione (2n, $C_{25}H_{22}N_4O_3$) Yield 55%; m.p.: 270–272 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.58 (d, J = 8.4 Hz, 2H), 7.79 (d, J = 8.8 Hz, 2H), 7.26 (d, J = 8.4 Hz, 2H), 7.14 (d, J = 8.8, 2H), 6.28 (bs, 2H), 5.58 (dd, J = 12.0 Hz, 4.8 Hz, 1H), 3.79 (dd, J = 17.6 Hz, 12.0 Hz, 1H), 3.54 (bs, 2H), 3.47 (t, J = 1.6 Hz, 2H), 3.10 (dd, J = 17.6 Hz, 4.8 Hz, 1H), 2.45 (s, 3H), 1.82 (d, J = 8.8 Hz, 1H), 1.65 (d, J = 8.8 Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 176.4 (2C), 169.0, 152.7, 150.4 (2C), 150.0, 134.6 (2C), 133.6, 130.9, 127.2 (2C), 126.9 (2C), 120.5 (2C), 59.1, 52.3, 45.9 (2C), 45.5 (2C), 41.7, 21.8 ppm; IR (KBr): $\overline{V}$ = 3455, 3004, 2871, 1766, 1706, 1658, 1600, 1650, 1519, 1438, 1413, 1375, 1332, 1174 cm^{-1}.

Anticancer studies

The anticancer activity of compounds **2a**–**2n** was investigated against C6 cells using the BrdU Cell Proliferation Assay (BCPA) according to literature methods [62].

Antimicrobial activity

All newly synthesized compounds were screened for their in vitro antimicrobial activities by disc-diffusion method using Mueller–Hilton agar medium against *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC-6633, which are Gram-positive bacteria, *Proteus vulgaris* KUEN 1329, *Pseudomonas aureginosa* ATCC 9027, *Escherichia coli* AÜ tıp which are Gram-negative bacteria, and *Candida albicans* ATCC 1213 which are fungus. In these tests, SCF (sulbactam/cefoperazone) were used as standard [62].

Enzyme studies

Carbonic anhydrase I and II isoforms purification and inhibition studies

Both CA isoforms (hCA I and II) in this study by Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography were purified in a single purification step [80]. In addition, pH of the buffer was adjusted to 8.7 using by solid Tris. In addition, supernatant was transferred to the affinity column, which previously prepared [81]. Then, the proteins from the column were spectrophotometrically measured at 280 nm [82]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was tested for registration of both isoforms purity [83]. Human CA isoforms activity designation was perceived spectrophotometric procedure of Verpoorte et al. [84] as described previously [85]. In this test, changes in absorbance were obtained during 3 min at 25 °C. *p*-Nitrophenylacetate (PNA) compound was used as substrate and transformed by both isoforms to *p*-nitrophenolate ion [86]. For definition of protein amount, Bradford procedure was used during the purification stages [52, 55]. The bovine serum albumin was used as standard protein for this designation, which was done at 595 nm [87]. For designating

the inhibition results of each isoform, novel hydrazine compounds **2a**–**2n** and an activity (%) [hydrazine ethers] graph was drawn. To calculate K_i values, three disparate novel hydrazine **2a**–**2n** concentrations were used [88].

AChE activity determination

The inhibitory effect of novel hydrazines **2a**–**2n** on AChE activity was tested according to spectrophotometric procedure of Ellman et al. [76]. Acetylthiocholine iodide (AChI) was used as substrate for this test. For the measurement of the AChE activity, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, D8130-1G, Sigma-Aldrich, Sternheim, Germany) was applied [89]. Briefly, 50 mm^3 of Tris/HCl solution (1.0 M, pH 8.0) and 10 mm^3 of sample solution dissolved in distilled water at disparate concentrations and 50 mm^3 AChE (5.32×10^{-3} U) solution were mixed and incubated for 12 min at 26 °C [90].

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