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ORIGINAL PAPER

Facile, highly efficient, and clean one-pot synthesis of acridine sulfonamide derivatives at room temperature and their inhibition of human carbonic anhydrase isoenzymes

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Abstract Reaction of dimedone, 4-amino-*N*-(diaminomethylene)benzenesulfonamide, and aromatic aldehydes was successfully realized using sulfuric acid as a cheap catalyst. Synthesis of novel acridine sulfonamide compounds was performed providing high yields in water as the solvent at room temperature. This method has several advantages such as use of a green solvent, high yields, and efficient one-pot procedure. In addition, human carbonic anhydrase isoenzymes (hCA I and hCA II) were purified from erythrocyte cells by affinity chromatography. The inhibitory effects of acetazolamide and the newly synthesized acridine sulfonamides on hydratase and esterase activities of these isoenzymes was studied in vitro. The esterase IC_{50} values of the new compounds are 47.2–230.1 μM for hCA I and 50.1–275.0 μM for hCA II.

Keywords Green chemistry · Cyclization · Three-component reaction · Aldol reactions · Enzymes · Michael additions

Introduction

Carbonic anhydrases (EC 4.2.1.1) are Zn(II) ion-containing metalloenzymes that catalyze simple but important physiological reactions, like the conversion of CO_2 to HCO_3^- and H^+, and they participate in many crucial physiological processes concerned with pH homeostasis, electrolyte secretion, respiration, CO_2 and ion transport, some biosynthesis reactions (gluconeogenesis, lipogenesis etc.), tumorigenicity, and so on [1–3]. There are five distinct genetic families known to date, the α-, β-, γ-, δ-, and ζ-carbonic anhydrases [4]. The α-carbonic anhydrases are present in mammals. Sixteen different α-carbonic anhydrase isoenzymes have been described in humans [5]. Among these isozymes hCA I and hCA II are cytosolic, and hCA IV is membrane bound. The hCA II and hCA IV isoenzymes are present in human eyes [6, 7]. Glaucoma is a group of diseases characterized by gradual loss of visual field due to an elevation in intraocular pressure (IOP) [8, 9]. Carbonic anhydrase inhibitors reduce intraocular pressure by lowering aqueous humor formation. So carbonic anhydrase isoenzymes (hCA I, hCA II, and hCA IV) are therapeutic targets for the treatment of glaucoma [10, 11].

In this study, novel acridine derivatives were synthesized, characterized, and tested with respect to inhibition of hCA isoenzymes that were purified from human erythrocytes.

Results and discussion

Chemistry

The general synthesis method shown in Scheme 1 was employed to prepare the acridine compounds **4–17**. The syntheses of these compounds were realized in water in a single process through three successive reactions (aldol condensation, Michael addition, and cyclization) using sulfuric acid. Acridine compounds were prepared by means of a one-pot reaction producing high yields and providing a

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Scheme 1

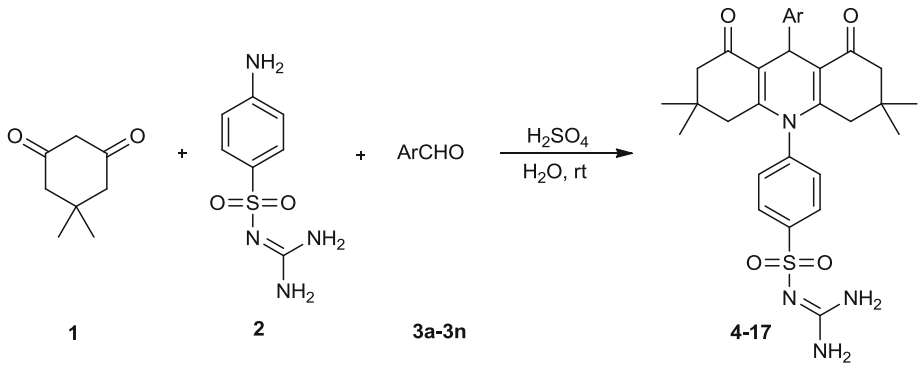


Table 1 Human carbonic anhydrase isozymes (hCA I and hCA II) inhibition data with acridine sulfonamide derivatives **4–17** determined by an esterase assay with 4-nitrophenylacetate as substrate

Product	Ar	Esterase $IC_{50}^{a,b}$/μM		Selectivity ratio (hCA II/hCA I)
		hCA I	hCA II	
AAZ	–	3.02 ± 0.02	2.08 ± 0.02	0.69
4	4-CNC_6H_4	207.0 ± 1.1	198.4 ± 3.0	0.96
5	4-$NO_2C_6H_4$	72.4 ± 2.8	59.0 ± 1.9	0.81
6	3-$NO_2C_6H_4$	230.1 ± 3.2	224.2 ± 3.0	0.97
7	4-FC_6H_4	160.2 ± 1.3	275.0 ± 1.7	1.72
8	4-BrC_6H_4	No inhibition	No inhibition	–
9	2,4-$Cl_2C_6H_3$	145.6 ± 5.2	122.3 ± 4.3	0.84
10	5-Br-2-OHC_6H_3	182.4 ± 3.9	210.5 ± 4.4	1.15
11	4-OHC_6H_4	66.3 ± 4.4	66.0 ± 5.0	0.99
12	4-$CH_3C_6H_4$	47.2 ± 1.7	50.1 ± 1.0	1.06
13	4-$C_2H_5C_6H_4$	103.3 ± 2.4	101.2 ± 2.1	0.98
14	4-$CH_3SC_6H_4$	No inhibition	No inhibition	–
15	4-$CH_3OC_6H_4$	No inhibition	No inhibition	–
16	2,4-$(CH_3O)_2C_6H_3$	No inhibition	No inhibition	–
17	3,4-$(CH_3O)_2C_6H_3$	No inhibition	No inhibition	–

AAZ (acetazolamide) was used as reference compound

[a] Mean ± standard error, from three different assays

[b] $P < 0.005$ for all analysis

simple work-up procedure. Dimedone (**1**) was condensed with sulfaguanidine **2** and aldehydes **3** in the molar ratio of 2:1:1 at room temperature, leading to the acridine sulfonamide-containing compounds **4–17**. By changing the nature of the substituents present in the aldehyde components, a rather large chemical diversity can be incorporated in the acridine sulfonamide reported (Scheme 1; Table 1). These various substituents comprised cyano, nitro, halogen (F, Cl, Br), methoxy, hydroxyl, methylthio, methyl, and ethyl moieties (Table 1).

The reaction of dimedone, 4-amino-*N*-[amino(imino)methyl]benzenesulfonamide (**2**) and 4-cyanobenzaldehyde (**3a**) in the presence of an acid catalyst tested in water was selected as a model reaction. However, the catalysts we tested are outlined in Table 2. The reaction in the presence of *p*-toluenesulfonic acid (TsOH) alone afforded the desired product in a very low yield (entry 6).

Table 2 Comparison of the efficiency of various acid catalysts used in the synthesis of acridine sulfonamide derivatives in water

Entry	Catalyst	Time/h	Yield/%
1	H_2SO_4	48	79.8
2	DBSA	48	78.2
3	HI	48	73.6
4	HCl	48	73.1
5	HBr	48	72.9
6	*p*-TsOH	48	67.2

H_2SO_4 was found to be a good catalyst for this reaction (entry 1), whereas *p*-dodecylbenzenesulfonic acid (DBSA) (entry 2), hydroiodic acid (HI) (entry 3), hydrochloric acid (HCl) (entry 4), and hydrobromic acid (HBr) (entry 5) were much less effective than H_2SO_4, suggesting that the strong acidity of H_2SO_4 is essential for the catalysis.

Table 3 Optimization of conditions for preparation of acridine sulfonamide derivatives using sulfuric acid as a catalyst and in water at room temperature

Entry	1	2	3	4
Concentration/%	0.5	1	2	10
Yield/%	77.6	78.3	79.8	79.8

Table 4 Comparison of the efficiency of various solvents used in the synthesis of acridine sulfonamide derivatives

Entry	Solvent	Yield/%
1	Water	79.8
2	Ethanol	71.3
3	Methanol	72.2
4	Chloroform	70.4
5	Isopropyl alcohol	69.1

The reactions were performed within 48 h

The catalyst plays a crucial role in the success of the reactions in terms of the rate and the yields, and these experiments are summarized in Table 3. For example, 4-cyanobenzaldehyde reacted with dimedone and sulfaguanidine in the presence of 0.5 % H_2SO_4 to give the product **4** in modest yield (77.6 %) in water at room temperature after 48 h of reaction time. Increasing the catalyst to 1, 2, and 10 % resulted in increasing the reaction yields to 78.3, 79.8, and 79.8 %. Use of just 2 % H_2SO_4 in water at room temperature is enough to afford an efficient synthesis. Higher amounts of sulfuric acid did not improve the results significantly.

We also investigated the effects of the solvent on a model reaction, namely, between 4-cyanobenzaldehyde and dimedone catalyzed by H_2SO_4 in various solvents. From the results shown in Table 4, it is obvious that water is the best choice. In addition, water is a green solvent; thus we prefer it for this reaction.

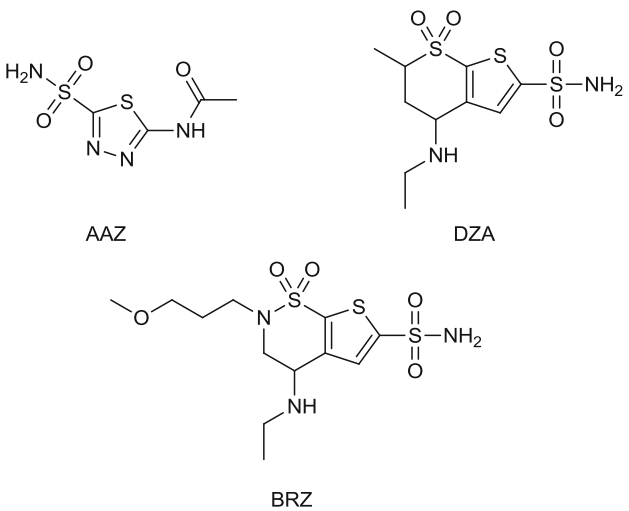
The infrared (IR) spectra of all the acridine compounds showed sharp peaks for the carbonyl groups in the region between 1,616 and 1,643 cm^{-1} [12]. Compounds **4** exhibited a peak at 2,229 cm^{-1} that belongs to a CN group [13]. Besides, in the IR spectra of the compounds, aliphatic C–H stretching bands at 2,928–2,964 cm^{-1} and aromatic C–H stretching bands at 3,101–3,028 cm^{-1} were observed. The NH_2 vibrations of acridine sulfonamide compounds were observed in the region between 3,458 and 3,305 cm^{-1} [14]. The ^{1}H NMR spectra of compounds **4–17** showed singlet peaks that belong to protons of the methyl groups in positions 3 and 6 between 0.71 and 0.91 ppm. The CH_2 group protons of the cyclohexene rings of compounds **4–17** showed doublet peaks at 1.67–2.23 ppm [15]. Compound **12** showed a singlet peak that belongs to protons of the methyl group at 2.23 ppm [15]. The methylthio protons of compound **14** were observed at 2.43 ppm. Compound **13** displayed a triplet peak (3H) at 1.15 ppm and a multiplet (2H) at 1.05 ppm assigned to an ethyl group [15]. The signals for the methoxy group protons for compounds **15**, **16**, and **17** were observed in the range of 3.67–3.69 ppm. The signals for the CH protons were observed at 4.92–5.19 ppm and the signals for the aromatic protons were observed in the range of 6.41–8.15 ppm. Hydroxyl group protons of compound **11** and **10** were observed as a broad signal at 9.09 and 10.61 ppm. The broad peaks between 6.84 and 7.13 ppm were assigned to sulfonamide ($–SO_2NC(NH_2)_2$) group protons of all the compounds **4–17**. The ^{13}C NMR (APT) spectra of compound **4** displayed a signal for the cyano group carbon at 118.95 ppm. All the compounds **4–17** showed carbonyl carbons peaks at 195.08–195.69 ppm. The mass spectra of all the acridines showed molecular ion isotope $[M + 1]^+$ or $[M - 1]^-$ peaks.

Carbonic anhydrase inhibition

Carbonic anhydrase (CA) inhibition has important pharmacologic applications such as antiglaucoma, anticonvulsant, and anticancer agents. It is well known that sulfonamides are the main class of CA inhibitors, which bind to the zinc(II) ion active site of the enzyme [4]. Some sulfonamide drugs, such as acetazolamide (AAZ), dorzolamide (DZA), and brinzolamide (BRZ) are used in glaucoma therapy. These compounds are quite powerful inhibitors but they have some side effects [16]. So syntheses of new CA inhibitors are necessary (Scheme 2).

In the present study, firstly hCA I and hCA II were purified from human erythrocytes. The purification of these isoenzymes was performed using a simple one-step method

Scheme 2



with Sepharose-4B–L-tyrosine-*p*-aminobenzene sulfonamide affinity chromatography. For purity control, SDS-PAGE was performed. Then the newly synthesized acridine compounds **4–17** as well as the reference compound AAZ were tested under in vitro conditions for their enzyme inhibition activities against human CA isoforms hCA I and hCA II. The inhibitor concentration that caused 50 % inhibition (IC_{50}) was determined from activity (%) versus [I] plots [17].

In the in vitro studies all the newly synthesized compounds did not inhibit the hydratase activities of hCA I and hCA II. The effects of the compounds on the esterase activities of hCA I and hCA II are described below. Some of the tested compounds (**8**, **14–17**) have no inhibitory effect on hCA I and hCA II. However, other compounds (**4–13**) have IC_{50} values in the millimolar region. Overall differences between the highest and lowest IC_{50} values are about sixfold (i.e., IC_{50} value of compound **7** for hCA II = 275.0 μM; IC_{50} value of compound **12** for hCA I = 47.2 μM; Table 1).

The slow cytosolic isoform, hCA I, was moderately inhibited by **4–13**. Compounds **14** and **15** have $–OCH_3$ and $–SCH_3$ groups on the *para* position. These compounds did not affect hCA I activity. Compounds **16** and **17** have similar effects on this isozyme. Both of them incorporate two methoxy groups (**16**: 2,4 positions; **17**: 3,4 positions). These functional groups ($–OCH_3$ and $–SCH_3$) are bigger than others investigated in the present study. Therefore steric effects of these groups might block the enzyme–inhibitor interaction. Interestingly, the difference in inhibition is threefold between **5** (IC_{50} value 72.4 μM) and **6** (IC_{50} value 230.1 μM). Both of them have a $–NO_2$ group on the aromatic ring. But the locations of $–NO_2$ groups are different. Compound **12** has a most powerful inhibition effect on hCA I. Compound **12** contains a methyl group, whereas **13** has an ethyl group. Obviously, alkyl group elongation leads to a decrease in inhibition potential of the compounds (IC_{50} value of **12** = 47.2 μM, IC_{50} value of **13** = 103.3 μM). The inhibition effects of the compounds against to hCA I isoform are in the order of **12** > **11** > **5** > **13** > **9** > **7** > **10** > **4** > **6**.

The human hCA II isozyme showed a similar inhibition profile for the tested compounds. No inhibition was observed for **8** or **14–17**. The differences in inhibition potentials of **5** and **6** are similar to those for the hCA I isoform. Compound **12** is the most powerful inhibitor (IC_{50} = 50.1 μM), whereas **7** is the weakest inhibitor (IC_{50} = 275.0 μM) for hCA II. The inhibition effects of the newly synthesized compounds against the hCA II isozyme are in the order of **12** > **5** > **11** > **13** > **9** > **4** > **10** > **6** > **7**. hCA I and hCA II have almost equal selectivity towards the novel compounds (see selectivity ratio in Table 1).

In conclusion, compounds **4–13** have a moderate inhibition effect on human CA isoforms (hCA I and hCA II). However, these newly synthesized CA inhibitors could be evaluated in further studies.

Experimental

The chemicals used in the synthesis of acridine sulfaguanidine derivatives were obtained from Merck and Aldrich Chemical Company, and Sepharose 4B for affinity column and electrophoresis reagents were obtained from Sigma Chem. Co. All chemicals and solvents used for the synthesis were of spectroscopic reagent grade. Melting points were measured on a Bibby Stuart Scientific apparatus. Fourier Transform Infrared (FT–IR) spectra were recorded on a Bruker Optics, ALPHA FT–IR spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were obtained with Bruker DPX-300 and DPX-400 FT-NMR instruments in $CDCl_3$ and DMSO-d_6 as solvents with trimethylsilane as the internal reference. Chemical shifts are expressed in δ units (ppm). The mass analyses were performed on a Waters 2695 Alliance Micromass ZQ instrument LC/MS and Agilent 1100 Series LC/MSD Trap VL&SL. The elemental analyses (C, H, N) were conducted using the Elemental Analyser LECO CHNS-932.

*General procedure for preparation of acridine sulfonamide derivatives **4–17***

A mixture of 0.280 g dimedone (**1**, 2 mmol), 0.214 g 4-amino-*N*-(diaminomethylene)benzenesulfonamide (**2**, 1 mmol), 0.131 g 4-cyanobenzaldehyde (**3a**, 1 mmol), and 0.50 g H_2SO_4 (2 %) in 25 cm^3 water was stirred continuously for 48 h at room temperature. The reaction progress was monitored by TLC. The solid product was filtered, washed with 500 cm^3 water, and recrystallized from ethanol/water.

4-[9-(4-Cyanophenyl)-3,3,6,6-tetramethyl-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]-N-(diaminomethylene)benzenesulfonamide
(**4**, $C_{31}H_{33}N_5O_4S$)
Yellow solid; 80 % yield; m.p.: 292–293 °C (EtOH/water); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 0.73 (s, 6H, 2 CH_3), 0.91 (s, 6H, 2 CH_3), 1.77 (d, 2H, J = 16.5 Hz, CH_2), 2.02 (d, 2H, J = 16.5 Hz, CH_2), 2.21 (m, 4H, 2 CH_2), 5.09 (s, 1H, CH), 6.87 (s, 4H, 2 NH_2), 7.51 (d, 2H, J = 8.5 Hz, Ar–H), 7.62 (d, 2H, J = 8.5 Hz, Ar–H), 7.73 (d, 2H, J = 8.5 Hz, Ar–H), 7.97 (d, 2H, J = 8.5 Hz, Ar–H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 26.12 (CH_3), 29.05 (CH_3), 32.05 (C), 32.96 (CH), 40.93 (CH_2), 49.36 (CH_2), 108.65 (CN), 112.14 (C), 118.95 (C), 127.16 (CH), 128.76 (CH), 129.22 (CH), 132.00 (CH), 140.33 (C), 145.35 (C), 150.50 (C), 151.38 (C), 158.20 (C), 195.09 (C)

ppm; FT–IR: $\bar{\nu}$ = 3,453 and 3,343 (NH_2), 2,964 (C–H), 2,229 (C≡N), 1,616 (C=O), 1,557 and 1,494 (C=C), 1,361 and 1,220 (SO_2) cm^{-1}; MS (ESI): $m/z = 572$ ($[M + 1]^+$).

N-(Diaminomethylene)-4-[3,3,6,6-tetramethyl-9-(4-nitrophenyl)-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]benzenesulfonamide
(**5**, $C_{30}H_{33}N_5O_6S$)
Yellow solid; 76 % yield; m.p.: 250 °C (EtOH/water); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.71 (s, 6H, 2 CH_3), 0.89 (s, 6H, 2 CH_3), 1.76 (d, 2H, J = 17.4 Hz, CH_2), 2.01 (d, 2H, J = 16.0 Hz, CH_2), 2.21 (d, 4H, J = 16.6 Hz, 2 CH_2), 5.15 (s, 1H, CH), 6.85 (s, 4H, 2 NH_2), 7.60 (d, 2H, J = 8.8 Hz, Ar–H), 7.64 (d, 2H, J = 8.5 Hz, Ar–H), 7.96 (d, 2H, J = 9.0 Hz, Ar–H), 8.15 (d, 2H, J = 8.8 Hz, Ar–H) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 27.01 (CH_3), 29.55 (CH_3), 32.53 (C), 33.36 (CH), 41.41 (CH_2), 49.85 (CH_2), 112.49 (C), 123.77 (CH), 127.65 (CH), 129.43 (CH), 130.86 (CH), 140.80 (C), 145.88 (C), 146.17 (C), 151.07 (C), 153.95 (C), 158.70 (C), 195.53 (C) ppm; FT–IR: $\bar{\nu}$ = 3,367 and 3,334 (NH_2), 2,954 (C–H), 1,639 (C=O), 1,555 and 1,513 (C=C), 1,336 and 1,218 (SO_2) cm^{-1}; MS (ESI): $m/z = 592$ ($[M + 1]^+$).

N-(Diaminomethylene)-4-[3,3,6,6-tetramethyl-9-(3-nitrophenyl)-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]benzenesulfonamide
(**6**, $C_{30}H_{33}N_5O_6S$)
Yellow solid; 77 % yield; m.p.: 295 °C (EtOH/water); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.71 (s, 6H, 2 CH_3) 0.89 (s, 6H, 2 CH_3), 1.81 (d, 2H, J = 17.4 Hz, CH_2), 2.02 (d, 2H, J = 16.1 Hz, CH_2), 2.22 (d, 4H, J = 17.3 Hz, 2 CH_2), 5.13 (s, 1H, CH), 6.85 (s, 4H, 2 NH_2), 7.61 (d, 2H, J = 7.8 Hz, Ar–H), 7.79 (d, 2H, J = 7.7 Hz, Ar–H), 8.00 (t, 3H, J = 8.5 Hz, Ar–H), 8.13 (s, 1H, Ar–H) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 26.53 (CH_3), 26.56 (CH_3), 32.59 (C), 32.96 (CH), 41.28 (CH_2), 49.82 (CH_2), 112.79 (C), 121.53 (CH), 123.05 (CH), 127.73 (CH), 130.00 (CH), 130.23 (CH), 134.71 (CH), 140.77 (C), 145.91 (C), 147.87 (C), 148.55 (C), 151.13 (C), 158.69 (C), 195.64 (C) ppm; FT–IR: $\bar{\nu}$ = 3,458 and 3,414 (NH_2), 2,959 (C–H), 1,636 (C=O), 1,557 and 1,521 (C=C), 1,360 and 1,221 (SO_2) cm^{-1}; MS (ESI): $m/z = 592$ ($[M + 1]^+$).

N-(Diaminomethylene)-4-[9-(4-fluorophenyl)-3,3,6,6-tetramethyl-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]benzenesulfonamide (**7**, $C_{30}H_{33}FN_4O_4S$)
Yellow solid; 75 % yield; m.p.: 290–291 °C (EtOH/water); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 0.73 (s, 6H, 2 CH_3), 0.89 (s, 6H, 2 CH_3), 1.75 (d, 2H, J = 16.0 Hz, CH_2), 2.02 (d, 2H, J = 16.0 Hz, CH_2), 2.20 (d, 4H, J = 16.0 Hz, 2 CH_2), 5.05 (s, 1H, CH), 6.88 (s, 4H, 2 NH_2), 7.07 (t, 2H, J = 8.0 Hz, Ar–H), 7.35-7.45 (m, 2H, Ar–H), 7.60 (d, 2H, J = 8.0 Hz, Ar–H), 7.97 (d, 2H, J = 8.0 Hz, Ar–H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 26.06 (CH_3), 29.14 (CH_3), 31.39 (CH), 32.04 (C), 40.90 (CH_2), 49.46 (CH_2), 112.99 (C), 114.44 (CH), 114.65 (CH), 127.17 (CH), 129.25 (CH), 140.50 (C), 142.30 (C), 145.26 (C), 149.91 (C), 158.19 (C), 162.96 (C), 195.11 (C) ppm; FT–IR: $\bar{\nu}$ = 3,442 and 3,367 (NH_2), 2,958 (C–H), 1,641 (C=O), 1,541 (C=C), 1,362 and 1,221 (SO_2) cm^{-1}; MS (ESI): $m/z = 565$ ($[M + 1]^+$).

4-[9-(4-Bromophenyl)-3,3,6,6-tetramethyl-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]-N-(diaminomethylene)benzenesulfonamide
(**8**, $C_{30}H_{33}BrN_4O_4S$)
Yellow solid; 76 % yield; m.p.: 240 °C (EtOH/water); ^{1}H NMR (400 MHz, DMSO-d_6): δ = 0.72 (s, 6H, 2 CH_3), 0.88 (s, 6H, 2 CH_3), 1.77 (d, 2H, J = 16.0 Hz, CH_2), 2.02 (d, 2H, J = 16.0 Hz, CH_2), 2.21 (d, 4H, J = 16.0 Hz, 2 CH_2), 5.02 (s, 1H, CH), 7.13 (s, 4H, 2 NH_2), 7.28 (d, 2H, J = 4.0 Hz, Ar–H), 7.45 (d, 2H, J = 8.0 Hz, Ar–H), 7.58 (d, 2H, J = 4.0 Hz, Ar–H), 8.15 (d, 2H, J = 8.0 Hz, Ar–H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): δ = 26.11 (CH_3), 29.13 (CH_3), 31.81 (C), 32.04 (CH), 40.89 (CH_2), 49.44 (CH_2), 112.65 (C), 113.87 (C), 118.82 (C), 127.15 (CH), 129.87 (CH), 130.33 (CH), 130.81 (CH), 140.44 (C), 145.29 (C), 150.08 (C), 158.19 (C), 195.09 (C) ppm; FT–IR: $\bar{\nu}$ = 3,446 and 3,371 (NH_2), 2,957 (C–H), 1,637 (C=O), 1,540 (C=C), 1,360 and 1,221 (SO_2) cm^{-1}; MS (ESI): $m/z = 625$ ($[M + 1]^+$).

N-(Diaminomethylene)-4-[9-(2,4-dichlorophenyl)-3,3,6,6-tetramethyl-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]benzenesulfonamide (**9**, $C_{30}H_{32}Cl_2N_4O_4S$)
Yellow solid; 75 % yield; m.p.: 250 °C (EtOH/water); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.74 (s, 6H, 2 CH_3), 0.86 (s, 6H, 2 CH_3), 1.71 (d, 2H, J = 17.3 Hz, CH_2), 1.94 (d, 2H, J = 16.0 Hz, CH_2), 2.11 (m, 4H, 2 CH_2), 5.19 (s, 1H, CH), 6.84 (s, 4H, 2 NH_2), 7.32 (d, 2H, J = 10.3 Hz, Ar–H), 7.40 (s, 1H, Ar–H), 7.51 (d, 1H, J = 8.4 Hz, Ar–H), 7.58 (d, 2H, J = 8.1 Hz, Ar–H), 7.95 (d, 1H, J = 8.1 Hz, Ar–H) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 26.49 (CH_3), 29.63 (CH_3), 32.34 (C), 33.37 (CH), 41.63 (CH_2), 49.87 (CH_2), 111.99 (C), 126.99 (CH), 127.20 (CH), 127.68 (CH), 131.05 (CH), 131.39 (C), 133.96 (C), 134.46 (CH), 141.02 (C), 142.49 (C), 145.79 (C), 151.09 (C), 158.69 (C), 195.40 (C) ppm; FT–IR: $\bar{\nu}$ = 3,451 and 3,334 (NH_2), 2,960 (C–H), 1,626 (C=O), 1,541 (C=C), 1,360 and 1,223 (SO_2) cm^{-1}; MS (ESI): $m/z = 615$ ($[M + 1]^+$).

4-[9-(5-Bromo-2-hydroxyphenyl)-3,3,6,6-tetramethyl-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]-N-(diaminomethylene)benzenesulfonamide
(**10**, $C_{30}H_{33}BrN_4O_5S$)
Yellow solid; 74 % yield; m.p.: 312 °C (EtOH/water); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.73 (s, 6H, 2 CH_3),

0.88 (s, 6H, 2 CH_3), 1.67 (d, 2H, $J = 17.4$ Hz, CH_2), 1.98 (d, 2H, $J = 16.0$ Hz, CH_2), 2.23 (m, 4H, 2 CH_2), 4.92 (s, 1H, CH), 6.85 (s, 4H, 2 NH_2), 6.94 (d, 2H, $J = 8.0$ Hz, Ar–H), 7.03 (s, 1H, Ar–H), 7.10 (d, 1H, $J = 8.0$ Hz, Ar–H), 7.30 (d, 2H, $J = 8.0$ Hz, Ar–H), 7.95 (s, 1H, $J = 8.0$ Hz, Ar–H), 10.61 (s, 1H, OH) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): $\delta = 29.74$ (CH_3), 31.43 (CH_3), 32.08 (CH), 32.27 (C), 32.46 (CH_2), 41.47 (CH_2), 110.90 (C), 118.22 (CH), 127.58 (CH), 128.74 (C), 129.97 (CH), 131.02 (CH), 133.45 (C), 134.24 (CH), 141.53 (C), 145.69 (C), 151.63 (C), 155.70 (C), 158.68 (C), 196.15 (C) ppm; FT–IR: $\bar{\nu} =$ 3,448 and 3,346 (NH_2), 2,958 (C–H), 1,617 (C=O), 1,542 (C=C), 1,370 and 1,234 (SO_2) cm^{-1}; MS (ESI): $m/z = 641$ ($[M + 1]^+$).

N-(Diaminomethylene)-4-[9-(4-hydroxyphenyl)-3,3,6,6-tetramethyl-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]benzenesulfonamide (**11**, $C_{30}H_{34}N_4O_5S$)
Yellow solid; 76 % yield; m.p.: 225 °C (EtOH/water); ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 0.73$ (s, 6H, 2 CH_3), 0.88 (s, 6H, 2 CH_3), 1.71 (d, 2H, $J = 17.3$ Hz, CH_2), 2.00 (d, 2H, $J = 16.0$ Hz, CH_2), 2.18 (d, 4H, $J = 16.7$ Hz, 2 CH_2), 4.92 (s, 1H, CH), 6.84 (s, 4H, 2 NH_2), 6.61 (d, 2H, $J = 8.5$ Hz, Ar–H), 7.08 (d, 2H, $J = 8.5$ Hz, Ar–H), 7.54 (d, 2H, $J = 8.5$ Hz, Ar–H), 7.94 (d, 2H, $J = 8.5$ Hz, Ar–H), 9.09 (s, 1H, OH) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): $\delta = 26.56$ (CH_3), 29.70 (CH_3), 31.24 (CH), 32.31 (C), 41.40 (CH_2), 50.08 (CH_2), 114.05 (C), 115.13 (CH), 127.65 (CH), 128.88 (CH), 130.80 (CH), 137.27 (C), 141.17 (C), 145.67 (C), 149.89 (C), 155.78 (C), 158.67 (C), 195.60 (C) ppm; FT–IR: $\bar{\nu} =$ 3,435 and 3,321 (NH_2), 2,955 (C–H), 1,622 (C=O), 1,527 (C=C), 1,365 and 1,223 (SO_2) cm^{-1}; MS (ESI): $m/z = 563$ ($[M + 1]^+$).

N-(Diaminomethylene)-4-[3,3,6,6-tetramethyl-1,8-dioxo-9-(p-tolyl)-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]-benzenesulfonamide (**12**, $C_{31}H_{36}N_4O_4S$)
Yellow solid; 76 % yield; m.p.: 191 °C (EtOH/water); ^{1}H NMR (400 MHz, DMSO-d_6): $\delta = 0.74$ (s, 6H, 2 CH_3), 0.89 (s, 6H, 2 CH_3), 1.74 (d, 2H, $J = 16.0$ Hz, CH_2), 2.01 (d, 2H, $J = 16.0$ Hz, CH_2), 2.19 (d, 4H, $J = 20.0$ Hz, 2 CH_2), 2.23 (s, 3H, CH_3), 5.02 (s, 1H, CH), 6.85 (s, 4H, 2 NH_2), 7.05 (d, 2H, $J = 8.0$ Hz, Ar–H), 7.21 (d, 2H, $J = 8.0$ Hz, Ar–H), 7.57 (d, 2H, $J = 8.0$ Hz, Ar–H), 7.97 (d, 2H, $J = 8.0$ Hz, Ar–H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 20.06$ (CH_3), 26.07 (CH_3), 29.21 (CH), 31.41 (CH_3), 32.03 (C), 40.91 (CH_2), 49.54 (CH_2), 113.25 (C), 127.20 (CH), 127.44 (CH), 128.51 (CH), 130.32 (CH), 134.65 (C), 140.62 (C), 143.19 (C), 145.21 (C), 149.68 (C), 158.17 (C), 195.08 (C) ppm; FT–IR: $\bar{\nu} =$ 3,455 and 3,375 (NH_2), 2,928 (C–H), 1,638 (C=O), 1,164 (C=C), 1,361 and 1,222 (SO_2) cm^{-1}; MS (ESI): $m/z = 561$ ($[M + 1]^+$).

N-(Diaminomethylene)-4-[9-(4-ethylphenyl)-3,3,6,6-tetramethyl-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]benzenesulfonamide (**13**, $C_{32}H_{38}N_4O_4S$)
Yellow solid; 75 % yield; m.p.: 180 °C (EtOH/water); ^{1}H NMR (400 MHz, DMSO-d_6): $\delta = 0.74$ (s, 6H, 2 CH_3), 0.89 (s, 6H, 2 CH_3), 1.02–1.05 (m, 2H, CH_2), 1.15 (t, 3H, $J = 8.0$ Hz, CH_3), 1.75 (d, 2H, $J = 20.0$ Hz, CH_2), 2.02 (d, 2H, $J = 16.0$ Hz, CH_2), 2.20 (d, 4H, $J = 20.0$ Hz, 2 CH_2), 5.02 (s, 1H, CH), 6.87 (s, 4H, 2 NH_2), 7.09 (d, 2H, $J = 8.0$ Hz, Ar–H), 7.23 (d, 2H, $J = 8.0$ Hz, Ar–H), 7.57 (d, 2H, $J = 8.0$ Hz, Ar–H), 7.97 (d, 2H, $J = 8.0$ Hz, Ar–H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 15.45$ (CH_3), 26.14 (CH_3), 27.70 (CH_2), 29.17 (CH), 31.42 (CH_3), 32.04 (C), 40.90 (CH_2), 49.55 (CH_2), 113.27 (C), 127.28 (CH), 127.95 (CH), 128.57 (CH), 129.70 (CH), 140.61 (C), 143.44 (C), 145.22 (C), 149.70 (C), 158.18 (C), 162.77 (C), 195.10 (C) ppm; FT–IR: $\bar{\nu} =$ 3,427 and 3,337 (NH_2), 2,957 (C–H), 1,623 (C=O), 1,537 (C=C), 1,361 and 1,221 (SO_2) cm^{-1}; MS (ESI): $m/z = 575$ ($[M + 1]^+$).

N-(Diaminomethylene)-4-[3,3,6,6-tetramethyl-9-[4-(methylthio)phenyl]-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]benzenesulfonamide (**14**, $C_{31}H_{36}N_4O_4S_2$)
Yellow solid; 76 % yield; m.p.: 229–230 °C (EtOH/water); ^{1}H NMR (400 MHz, DMSO-d_6): $\delta = 0.74$ (s, 6H, 2 CH_3), 0.89 (s, 6H, 2 CH_3), 1.74 (d, 2H, $J = 17.0$ Hz, CH_2), 2.02 (d, 2H, $J = 16.0$ Hz, CH_2), 2.21 (d, 4H, $J = 19.0$ Hz, 2 CH_2), 2.43 (s, 3H, -SCH_3), 5.00 (s, 1H, CH), 6.89 (s, 4H, 2 NH_2), 7.13 (t, 2H, $J = 10.0$ Hz, Ar–H), 7.27 (d, 2H, $J = 8.0$ Hz, Ar–H), 7.59-7.70 (m, 2H, Ar–H), 7.97 (d, 2H, $J = 8.0$ Hz, Ar–H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6): $\delta = 14.66$ (CH_3), 26.13 (CH_3), 29.17 (CH_3), 31.50 (CH), 32.04 (C), 40.91 (CH_2), 49.50 (CH_2), 113.01 (C), 123.53 (CH), 125.57 (CH), 127.18 (CH), 128.22 (CH), 135.01 (C), 140.56 (C), 142.93 (C), 145.24 (C), 149.82 (C), 158.18 (C), 195.11 (C) ppm; FT–IR: $\bar{\nu} =$ 3,435 and 3,336 (NH_2), 2,958 (C–H), 1,624 (C=O), 1,489 (C=C), 1,363 and 1,223 (SO_2) cm^{-1}; MS (ESI): $m/z = 593$ ($[M + 1]^+$).

N-(Diaminomethylene)-4-[9-(4-methoxyphenyl)-3,3,6,6-tetramethyl-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]benzenesulfonamide (**15**, $C_{31}H_{36}N_4O_5S$)
Yellow solid; 75 % yield; m.p.: 203 °C (EtOH/water); ^{1}H NMR (300 MHz, DMSO-d_6): $\delta = 0.72$ (s, 6H, 2 CH_3), 0.87 (s, 6H, 2 CH_3), 1.71 (d, 2H, $J = 16.1$ Hz, CH_2), 1.99 (d, 2H, $J = 16.0$ Hz, CH_2), 2.17 (d, 4H, $J = 17.4$ Hz, 2 CH_2), 3.67 (s, 3H, OCH_3), 4.98 (s, 1H, CH), 6.85 (s, 4H, 2 NH_2), 6.79 (d, 2H, $J = 8.5$ Hz), 7.20 (d, 2H, $J = 8.5$ Hz, Ar–H), 7.54 (d, 2H, $J = 7.8$ Hz, Ar–H), 7.94 (d, 2H, $J = 8.5$ Hz, Ar–H) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): $\delta = 26.59$ (CH_3), 29.15 (CH_3), 30.75 (CH), 32.51 (C), 41.40 (CH_2), 50.05 (CH_2), 55.31 (OCH_3), 113.72 (CH),

113.89 (C), 127.65 (CH), 128.98 (CH), 130.79 (CH), 138.89 (C), 141.12 (C), 145.72 (C), 150.02 (C), 157.77 (C), 158.68 (C), 195.56 (C) ppm; FT–IR: $\bar{\nu}$ = 3,415 and 3,305 (NH_2), 2,956 (C–H), 1,635 (C=O), 1,542 (C=C), 1,362 and 1,229 (SO_2) cm^{-1}; MS (ESI): m/z = 577 ($[M + 1]^+$).

N-(Diaminomethylene)-4-[9-(2,4-dimethoxyphenyl)-3,3,6,6-tetramethyl-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]benzenesulfonamide
(**16**, $C_{32}H_{38}N_4O_6S$)
Yellow solid; 74 % yield; m.p.: 223 °C (EtOH/water); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.71 (s, 6H, 2 CH_3), 0.86 (s, 6H, 2 CH_3), 1.69 (d, 2H, J = 17.1 Hz, CH_2), 1.91 (d, 2H, J = 16.2 Hz, CH_2), 2.06 (d, 4H, J = 12.0 Hz, 2 CH_2), 3.69 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 5.06 (s, 1H, CH), 6.85 (s, 4H, 2 NH_2), 6.41 (t, 2H, J = 9.7 Hz, Ar–H), 7.21 (d, 1H, J = 8.5 Hz, Ar–H), 7.54 (d, 2H, J = 8.5 Hz, Ar–H), 7.96 (d, 2H, J = 8.5 Hz, Ar–H) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 26.04 (CH_3), 29.86 (CH_3), 31.16 (CH), 32.44 (C), 41.79 (CH_2), 50.12 (CH_2), 55.34 (OCH_3), 56.02 (OCH_3), 98.66 (CH), 104.32 (CH), 112.27 (C), 125.51 (CH), 127.58 (CH), 130.90 (CH), 133.08 (C), 141.63 (C), 145.45 (C), 150.17 (C), 151.38 (C), 158.67 (C), 159.15 (C), 195.36 (C) ppm; FT–IR: $\bar{\nu}$ = 3,401 and 3,322 (NH_2), 2,955 (C–H), 1,632 (C=O), 1,497 (C=C), 1,363 and 1,228 (SO_2) cm^{-1}; MS (ESI): m/z = 605 ($[M - 1]^-$).

N-(Diaminomethylene)-4-[9-(3,4-dimethoxyphenyl)-3,3,6,6-tetramethyl-1,8-dioxo-1,2,3,4,5,6,7,8-octahydroacridin-10(9H)-yl]benzenesulfonamide
(**17**, $C_{32}H_{38}N_4O_6S$)
Yellow solid; 74 % yield; m.p.: 215 °C (EtOH/water); ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.78 (s, 6H, 2 CH_3), 0.91 (s, 6H, 2 CH_3), 1.73 (d, 2H, J = 17.3 Hz, CH_2), 2.02 (d, 2H, J = 16.1 Hz, CH_2), 2.20 (d, 4H, J = 18.0 Hz, 2 CH_2), 3.69 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 5.00 (s, 1H, CH), 6.85 (s, 4H, 2 NH_2), 6.83 (d, 3H, J = 6.5 Hz, Ar–H), 7.51 (d, 2H, J = 7.6 Hz, Ar–H), 7.96 (d, 2H, J = 8.7 Hz, Ar–H) ppm; ^{13}C NMR (75 MHz, DMSO-d_6): δ = 26.52 (CH_3), 29.71 (CH_3), 31.52 (CH), 32.52 (C), 41.42 (CH_2), 50.04 (CH_2), 55.74 (OCH_3), 55.83 (OCH_3), 111.69 (CH), 111.87 (CH), 113.75 (C), 119.85 (CH), 127.73 (CH), 130.69 (CH), 139.17 (C), 141.06 (C), 145.70 (C), 147.39 (C), 148.60 (C), 150.15 (C), 158.66 (C), 195.69 (C) ppm; FT–IR: $\bar{\nu}$ = 3,443 and 3,344 (NH_2), 2,954 (C–H), 1,643 (C=O), 1,464 (C=C), 1,363 and 1,263 (SO_2) cm^{-1}; MS (ESI): m/z = 605 ($[M - 1]^-$).

CA inhibition assay

Purification of carbonic anhydrase I and II isoenzymes from human erythrocytes
Erythrocytes were purified from human blood. The blood samples were centrifuged at 1,500 rpm for 20 min and plasma was removed. Later, red cells were washed with NaCl (0.9 %), and the erythrocytes were hemolyzed with 1.5 volumes of ice-cold water. Cell membranes were removed by centrifugation at 4 °C, 20,000 rpm for 30 min. The pH of hemolysate was adjusted to 8.7 with solid TRIS [tris(hydroxymethyl)aminomethane]. The hemolysate was applied to an affinity column (Sepharose-4B–L-tyrosine-*p*-aminobenzene sulfonamide) and equilibrated with 25 μM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with solution of 25 μM Tris–HCl/22 mM Na_2SO_4 (pH 8.7). The hCA I and hCA II isoenzymes were diluted with a solution of 1 M NaCl/25 μM Na_2HPO_4 (pH 6.3) and 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6) [18]. For protein content estimation, the Bradford method was used with bovine serum albumin as a standard [19]. SDS–polyacrylamide gel electrophoresis was performed after the purification of the enzyme [20].

Determination of hydratase and esterase activities of hCA I and hCA II
The CO_2 hydratase activity of the enzyme was determined at 0 °C in a veronal buffer (pH 8.15) with pH–stat method as indicator and saturated carbon dioxide solution as substrate in a final volume of 4.2 cm^3. The time (in seconds) taken for the solution to change from pH 8.15 to pH 6.5 was measured by the pH meter. The enzyme unit (EU) is the amount of enzyme that reduces the non-enzymatic reaction time by 50 %. The activity of an enzyme unit was calculated by using the equation ($t_0 - t_c/t_c$), where t_0 and t_c are times for pH change of the non-enzymatic and enzymatic reactions, respectively [21].

Esterase activities of hCA I and hCA II isoenzymes were determined by hydrolysis of *p*-nitrophenyl acetate. The change of absorbance was determined at 348 nm after 3 min [22].

Determination of IC_{50} values of the compounds
The IC_{50} values of inhibitors were determined by monitoring the hydrolysis of *p*-nitrophenyl acetate in the presence of the CA isoenzymes and various concentrations of inhibitors via the change in absorbance at 348 nm after 3 min [22]. Regression analysis graphs were drawn by plotting inhibitor concentrations versus enzyme activity and the IC_{50} values were determined.

Statistical analysis

All the presented data were confirmed in at least three independent experiments and are expressed as the mean ± standard deviation (SD). Data were analyzed by using a one-way analysis of variance for multiple comparisons. $P < 0.005$ was considered to be statistically significant.

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