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Research Article

Synthesis and Evaluation of New Phthalazine Urea and Thiourea Derivatives as Carbonic Anhydrase Inhibitors

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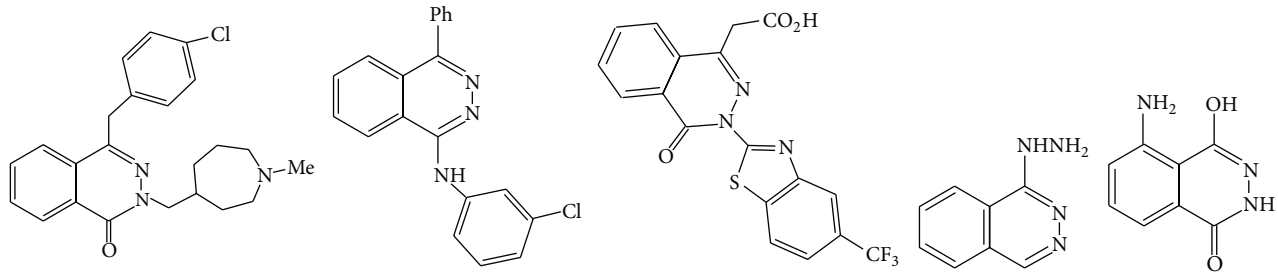
A new series of phthalazine substituted urea and thiourea derivatives were synthesized, and their inhibitory effects on the activity of purified human carbonic anhydrases (hCAs I and II) were evaluated. 2H-Indazolo[2,1-b]phthalazine-trione derivative **(1)** was prepared with 4-nitrobenzaldehyde, dimedone, and phthalhydrazide in the presence of TFA in DMF, and nitro group was reduced to amine derivative **(2)** with $SnCl_2{\cdot}2H_2O$. The compound was reacted with isocyanates and isothiocyanates to get the final products **(3a–p)**. The results showed that all the synthesized compounds inhibited the CA isoenzymes activity. **3a** (IC_{50} = 6.40 μM for hCA I and 6.13 μM for hCA II) has the most inhibitory effect. The synthesized compounds are very bulky to be able to bind near the zinc ion, and they much more probably bind as the coumarin derivatives.

1. Introduction

The carbonic anhydrases (CAs, EC 4.2.1.1.) are commonly characterized as zinc metalloenzymes whose primary physiological function is to rapidly catalyze the reversible hydration of carbon dioxide to form bicarbonate and a proton [1]. CAs are ubiquitous enzymes present in prokaryotes and eukaryotes which are encoded by five known CA structural families, the structurally characterized α-, β-, and γ-classes and the more recently discovered δ- and ζ-classes [2]. The α-CAs are found in vertebrates, algae, eubacteria, and cytoplasm of green plants whereas the γ-CAs are present mainly in Archaea and few eubacteria. The β-CAs are predominantly available in chloroplasts of mono- and dicotyledonous plants along with some algae and eubacteria. The δ-CAs are primarily found in marine diatoms. In humans, 16 isoforms of α-CAs have been reported, of which three are CARP or CA-related proteins [3]. There are sixteen isozymes which are characterized, and many of them are involved in critical physiological processes [4]. CAs are found in a variety of tissues such as kidneys, lungs, eyes, skins, the nervous systems, and the gastrointestinal tract in humans [5]. Biological activities of this metalloenzyme family have several medicinal applications which are commonly used as diuretics for the treatment of symptoms of hypertension [6], as antiglaucoma drugs [7], and for the treatment of high altitude sickness, gastric and duodenal ulcers, epilepsy, and osteoporosis [8]. More recently CA inhibitors have been shown to have potential as antiobesity drugs [9].

Majorities of the drugs used in human medicine are heterocyclic compounds. Common drugs such as Morphine, Lipitor, Penicillin, and nonsteroidal anti-inflammatory agents contain at least one heteroatom in their structure [10]. Heterocyclic compounds containing nitrogen group have large area in nature, and their utilization is becoming progressively important as biologically active pharmaceuticals, agrochemicals, and functional materials [11]. In particular, hydrazine containing heterocyclic compounds have been considered of great importance on account of pharmacological properties and clinical applications [12]. Moreover, these of combined phthalazines have biological properties such as inhibition of p38 MAP kinase [13] for selective binding of GABA receptor [14], antianxiety drug [15], antitumor agent [16], and high-affinity ligand to the a2d-1 subunit of calcium channel [17].

Phthalazine derivatives have been greatly used as therapeutic agents owing to their anticonvulsant, cardiotonic,



Scheme 1: Some commercially used phthalazine derivatives.

vasorelaxant, anti-inflammatory properties [18–23], and antimicrobial activity [24]. Like azelastine, the phthalazine derivatives have antihistaminic effects in the treatment of allergic rhinitis [25], and hydralazine is used as antihypertensive agent in the treatment of pulmonary hypertension [26–28]. Some commercially used phthalazine derivatives are shown in Scheme 1.

Ureidosubstituted benzenesulfonamides show very interesting profile for the inhibition of several human carbonic anhydrases (hCAs) such as hCAs I and II (cytosolic isoforms) and hCAs IX and XII (transmembrane, tumor-associated enzymes). It is mentioned that the compounds have excellent inhibitory effects for all these isoforms due to the urea moiety [29]. On the other hand, it has been reported that some urea derivatives have CA inhibitor activities [30, 31]. Therefore, the investigation of clinically useful ureas/thioureas is a growing field of interest. In this study, a new series of phthalazine substituted urea and thiourea derivatives were synthesized, and their inhibitory effects on the activity of purified human carbonic anhydrases (hCAs I and II) were evaluated.

2. Experimental Procedures

2.1. General. All starting materials and reagents were purchased from commercial suppliers. Reactions were monitored by TLC and TLC plates visualized with short wave UV fluorescence ($k = 254$ nm). Melting points were taken on a Yanagimoto micromelting point apparatus and were uncorrected. IR spectra were measured on a SHIMADZU Prestige-21 (200 VCE) spectrometer. ^{1}H and ^{13}C NMR spectra were measured on spectrometer at VARIAN Infinity Plus 300 and at 75 MHz, respectively. ^{1}H and ^{13}C chemical shifts are referenced to the internal deuterated solvent. The mass spectrometry analysis was carried out with a ABSCIEX-4000 QTRAP LC-MS-MS instrument.

*2.2. Synthesis of 2H-Indazolo[2,1-b]phthalazine-trione Derivative **(1)**.* A mixture of TFA (0.5 mL), 4-nitrobenzaldehyde (1.0 mmol), dimedone (1.0 mmol), and phthalhydrazide (1.0 mmol) in DMF (20 mL) was stirred and refluxed at 90°C for 16 hours. After completion of the reaction, the mixture was allowed to be cooled to room temperature and poured on cold water (50 mL). The precipitate was filtered, washed with water, and dried. The product (2H-indazolo[2,1-b] phthalazine-trione) was purified washing with hot ether.

*2.2.1. 3,3-Dimethyl-13-(4-nitrophenyl)-3,4-dihydro-2H-indazolo[1,2-b]phthalazine-1,6,11(13H)-trione **(1)**.* Yield 80%, m.p. 350–352°C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ, ppm): 1.19 (3H, s), 1.22 (3H, s), 2.34 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.51 (1H, s), 7.59–7.62 (2H, d, *J*: 8.7), 7.88–7.91 (2H, m), 8.19–8.22 (2H, d, *J*: 8.7), 8.23–8.27 (1H, m), 8.37–8.40 (1H, m). ^{13}C NMR ($CDCl_3$, 75 MHz, δ, ppm): 192.1, 155.9, 154.5, 151.6, 147.8, 143.4, 134.8, 133.9, 128.9, 128.6, 128.2, 128.1, 128.0, 127.7, 124.1, 124.0, 117.3, 64.1, 50.7, 38.0, 34.7, 28.6, 28.3. IR (KBr, υ, cm^{-1}): 3078, 2972, 2958, 1647, 1521, 1311.

*2.3. Reduction of Nitro Group **(2)**.* Compound **1** (1 mmol) and $SnCl_2{\cdot}2H_2O$ (5 mmol) in THF-DMF (4 : 2 v/v) were stirred at room temperature for 12 h. After completion of the reaction, the solvent was evaporated under reduced pressure and the mixture was stirred with water, and extracted with chloroform. The organic phase was dried over $MgSO_4$ and filtered and then purified by crystallisation from hexane.

*2.3.1. 13-(4-Aminophenyl)-3,3-dimethyl-3,4-dihydro-2H-indazolo[1,2-b]phthalazine-1,6,11(13H)-trione **(2)**.* Yield 40%, m.p. 263–265°C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ, ppm): 1.20 (3H, s), 1.22 (3H, s), 2.34 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.51 (1H, s), 6.59–6.62 (2H, d, *J*: 8.54), 7.17–7.20 (2H, d, *J*: 8.52), 7.26 (2H, s), 7.81–7.84 (2H, m), 8.25–8.28 (1H, m), 8.31–8.34 (1H, m). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 192.2, 156.0, 154.2, 150.6, 146.8, 134.4, 133.3, 129.2, 128.9, 128.4, 127.9, 127.8, 127.6, 125.9, 118.7, 115.1, 115.0, 64.7, 51.0, 38.0, 34.6, 28.6, 28.5. IR (KBr, υ, cm^{-1}): 3477, 3367, 3028, 2953, 2958, 1651, 1517, 1357. Anal. Calcd. For $C_{23}H_{21}N_3O_3$ *m*/*z*: 387.4; found (M + 1): 388.0.

*2.4. General Procedure **3**: Synthesis of 2H-Indazolo[2,1-b] phthalazine-trione Derivative Substituted Urea and Thiourea Derivatives **(3a–3p)**.* 13-(4-Aminophenyl)-3,3-dimethyl-3,4-dihydro-2H-indazolo[1,2-b]phthalazine-1,6,11(13H)-trione **(2)** (1,3 mmol), Et_3N (1,3 mmol), and isocyanate or isothiocyanate derivatives (3 mmol) in THF (20 mL) were stirred at 60°C for 20 hours. After the reaction was completed, the precipitate was filtered under suction, washed with ether, and dried.

2.4.1. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-phenylthiourea ***(3a)***. Yield 60%, m.p. 362–364°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 1.12 (6H, s), 2.20 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.20 (1H, s), 7.09–7.12 (1H, m), 7.20–7.34 (2H, m), 7.30–7.47 (6H, m), 7.90–7.93 (2H, m), 8.05–8.08 (1H, m), 8.20–8.23 (1H, m), 9.60 (1H, s), 9.73 (1H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.6, 180.0, 156.0, 154.3, 151.9, 140.0, 139.9, 135.2, 134.3, 133.8, 129.6, 129.3, 129.2, 128.3, 128.2, 128.1, 127.4, 125.0, 124.2, 124.1, 124.0, 123.6, 117.9, 117.8, 79.8, 64.6, 50.9, 37.9, 34.9, 28.6. IR (KBr, υ, cm^{-1}): 3286, 3011, 2956, 1656, 1514, 1357. Anal. Calcd. For $C_{30}H_{26}N_4O_3S$ *m*/*z*: 522.6; found (M + 1): 523.1.

2.4.2. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-(3-methoxyphenyl)thiourea ***(3b)***. Yield 70%, m.p. 275–277°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 1.10 (6H, s), 2.24 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 3.69 (3H, s), 6.24 (1H, s), 6.64–6.68 (1H, d, *J*: 7.98), 6.97–7.00 (1H, d, *J*: 7.95), 7.13–7.21 (2H, m), 7.37–7.42 (4H, m), 7.91–7.95 (2H, m), 8.05–8.08 (1H, m), 8.21–8.24 (1H, m), 9.76 (1H, s), 9.83 (1H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.6, 179.8, 159.9, 156.0, 154.3, 151.9, 141.1, 139.9, 135.2, 134.4, 133.9, 129.9, 129.7, 129.3, 128.2, 127.4, 123.7, 117.9, 116.2, 116.0, 110.3, 109.6, 79.8, 64.6, 55.7, 50.9, 37.9, 36.4, 34.9, 31.4, 28.6. IR (KBr, υ, cm^{-1}): 3273, 3015, 2956, 1651, 1514, 1355. Anal. Calcd. For $C_{31}H_{28}N_4O_4S$ *m*/*z*: 552.6; found (M + 1): 553.1.

2.4.3. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-p-tolylthiourea ***(3c)***. Yield 82%, m.p. 348–350°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 1.10 (6H, s), 2.24 (3H, s), 2.28 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.24 (1H, s), 7.08–7.11 (2H, d,: 8.10), 7.21–7.31 (4H, m), 7.37–7.39 (2H, d, *J*: 7.97), 7.92–7.95 (2H, m), 8.06–8.09 (1H, m), 8.22–8.25 (1H, m), 9.67 (1H, s), 9.74 (1H, s).^{13}C NMR (DMSO-d_6, 75 MHz): 192.7, 179.9, 156.0, 154.3, 151.9, 140.0, 138.5, 137.5, 137.3, 135.2, 134.3, 134.2, 133.7, 131.0, 129.7, 129.6, 129.3, 128.2, 127.4, 126.4, 124.5, 124.4, 123.6, 117.9, 79.8, 64.6, 50.9, 37.9, 34.9, 28.7, 28.6. IR (KBr, υ, cm^{-1}): 3277, 3030, 2956, 1656, 1510, 1357. Anal. Calcd. For $C_{31}H_{28}N_4O_3S$ *m*/*z*: 536.6; found (M + 1): 537.1.

2.4.4. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-(4-chlorophenyl)thiourea ***(3d)***. Yield 80%, m.p. 382–384°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 1.12 (6H, s), 2.27 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.27 (1H, s), 7.35–7.38 (2H, d, *J*: 8.75), 7.40 (4H, s), 7.47–7.50 (2H, d, *J*: 8.78), 7.95–7.98 (2H, m), 8.09–8.12 (1H, m), 8.24–8.28 (1H, m), 9.89 (1H, s), 9.94 (1H, s).^{13}C NMR (DMSO-d_6, 75 MHz): 192.7, 180.0, 156.0, 154.4, 152.0, 139.7, 139.1, 135.2, 134.4, 134.0, 129.7, 129.3, 129.2, 129.0, 128.8, 128.3,128.2, 127.4, 125.8, 123.7, 117.9, 80.0, 67.7, 64.6, 50.9, 37.9, 34.9, 28.7, 28.6, 25.8.IR (KBr, υ, cm^{-1}): 3288, 3045, 2958, 1653, 1512, 1357. Anal. Calcd. For $C_{30}H_{25}ClN_4O_3S$ *m*/*z*: 557.0; found (M + 1): 558.5.

2.4.5. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-(4-iodophenyl)thiourea ***(3e)***. Yield 84%, m.p. 395–397°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 1.09 (6H, s), 2.24 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.24 (1H, s), 7.29–7.26 (2H, d, *J*: 7.30), 7.37 (4H, s), 7.60–7.63 (2H, d, *J*: 7.18), 7.92–7.96 (2H, m), 8.06–8.09 (1H, m), 8.22–8.25 (1H, m), 9.84 (1H, s), 9.88 (1H, s).^{13}C NMR (DMSO-d_6, 75 MHz): 192.7, 179.9, 156.0, 154.4, 152.0, 140.0, 139.9, 139.7, 137.7, 135.2, 134.4, 134.0, 129.7, 129.3, 128.3, 128.2, 127.4, 126.4, 126.2, 126.0, 123.7, 117.9, 89.2, 64.6, 50.9, 37.9, 34.9, 32.6, 28.7, 28.6. IR (KBr, υ, cm^{-1}): 3259, 3037, 2953, 1647, 1529, 1359. Anal. Calcd. For $C_{30}H_{25}IN_4O_3S$ *m*/*z*: 648.5; found (M + 1): 649.0.

2.4.6. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-(4-bromophenyl)thiourea ***(3f)***. Yield 80%, m.p. 373–375°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 1.09 (6H, s), 2.24 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.24 (1H, s), 7.38 (4H, s), 7.39–7.48 (4H, m), 7.92–7.95 (2H, m), 8.06–8.09 (1H, m), 8.22–8.25 (1H, m), 9.86 (1H, s), 9.91 (1H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.7, 180.0, 156.0, 154.4, 151.9, 151.6, 139.7, 139.5, 134.0, 132.5, 132.0, 131.9, 129.7, 129.3, 128.3, 127.4, 126.0, 125.9, 123.7, 118.6, 117.9, 117.8, 117.0, 79.8, 64.6, 50.9, 37.9, 34.9, 28.7, 28.6. IR (KBr, υ, cm^{-1}): 3265, 3060, 2951, 1651, 1537, 1358. Anal. Calcd. For $C_{30}H_{25}BrN_4O_3S$ *m*/*z*: 601.5; found (M + 1): 602.9.

2.4.7. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-(4-florophenyl) thiourea ***(3g)***. Yield 68%, m.p. 220–222°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 1.09 (6H, s), 2.24 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.24 (1H, s), 7.09–7.15 (2H, t, *J*: 8.90), 7.37–7.39 (4H, m), 7.41–7.44 (2H, t, *J*: 7.19), 7.91–7.95 (2H, m), 8.06–8.09 (1H, m), 8.21–8.24 (1H, m), 8.29 (1H, s), 8.30 (1H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.6, 180.4, 161.3, 156.0, 154.4, 151.9, 139.8, 136.3, 136.2, 135.2, 134.4, 133.9, 129.7, 129.3, 128.3, 128.2, 127.4, 126.9, 126.8, 123.7, 117.9, 115.8, 115.5, 79.8, 64.6, 50.9, 37.9, 34.9, 28.7, 28.6. IR (KBr, υ, cm^{-1}): 3278, 3067, 2958, 1654, 1504, 1357. Anal. Calcd. For $C_{30}H_{25}FN_4O_3S$ *m*/*z*: 540.6; found (M + 1): 540.9.

2.4.8. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-phenylurea ***(3h)***. Yield 56%, m.p. 370–372°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 1.20 (3H, s), 1.21 (3H, s), 2.33 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.36 (1H, s), 6.93–6.99 (1H, m), 7.03–7.06 (2H, d, *J*: 8.95), 7.20–7.26 (4H, m), 7.31–7.34 (2H, d, *J*: 9.13), 7.50 (1H, s), 7.86–7.89 (2H, m), 7.94 (1H, s), 8.23–8.26 (1H, m), 8.37–8.40 (1H, m). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.6, 156.0, 154.3, 153.1, 151.7, 140.3, 140.2, 140.1, 135.2, 134.3, 134.2, 131.4, 129.7, 129.4, 128.6, 128.2, 127.4, 122.5, 120.2, 119.5, 118.8, 118.7, 118.6, 118.1, 104.1, 64.7, 51.0, 37.9, 34.9, 28.7. IR (KBr, υ, cm^{-1}): 3477, 3365, 3037, 2953, 1645, 1538, 1307. Anal. Calcd. For $C_{30}H_{26}N_4O_4$ *m*/*z*: 506.5; found (M + 1): 507.3.

2.4.9. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-(4-nitrophenyl)urea ***(3i)***. Yield 70%, m.p. 383–385°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 1.09 (3H, s), 1.10 (3H, s), 2.23 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.25 (1H, s), 7.35-7.36 (4H, s), 7.62–7.66 (2H, d, *J*: 9.35), 7.91–7.94 (2H, m), 8.04–8.08 (1H, m), 8.13–8.16 (2H, d, *J*: 9.32), 8.21–8.24 (1H, m), 8.92 (1H, s), 9.41 (1H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.6, 156.0, 154.3, 152.5, 152.3, 151.7, 147.0, 146.3, 142.1, 141.6, 139.3, 132.1, 129.7, 129.3, 128.7, 128.6, 125.8, 125.7, 119.1, 118.6, 118.1, 118.0, 80.0, 64.6, 38.9, 37.9, 34.9, 28.7, 28.5, 25.8. IR (KBr, υ, cm^{-1}): 3369, 3099, 2956, 1664, 1598, 1301. Anal. Calcd. For $C_{30}H_{25}N_5O_6$ *m*/*z*: 551.4; found (M + 1): 552.5.

2.4.10. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-(3-methoxyphenyl)urea ***(3j)***. Yield 70%, m.p. 330–332°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 1.10 (6H, s), 2.24 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 3.68–3.70 (3H, s), 6.20 (1H, s), 6.49–6.54 (2H, m), 6.87–6.91 (2H, m), 7.13-7.14 (2H, m), 7.32 (2H, s), 7.91–7.96 (2H, m), 8.06–8.11 (1H, m), 8.22–8.27 (1H, m), 8.63 (1H, s), 8.64 (1H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.6, 179.8, 159.9, 156.0, 154.3, 151.9, 141.1, 139.9, 135.2, 134.4, 133.9, 129.9, 129.7, 129.3, 128.2, 127.4, 123.7, 117.9, 116.2, 116.0, 110.3, 109.6, 79.8, 64.6, 55.7, 50.9, 37.9, 36.4, 34.9, 31.4, 28.6. IR (KBr, υ, cm^{-1}): 3360, 3067, 1664, 1539, 1355. Anal. Calcd. For $C_{31}H_{28}N_4O_5$ *m*/*z*: 536.5; found (M + 1): 536.9.

2.4.11. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-p-tolylurea ***(3k)***. Yield 66%, m.p. 397-399°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 1.10 (6H, s); 2.20 (3H, s), 2.24 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.20 (1H, s), 7.02–7.05 (2H, m), 7.27–7.32 (4H, m), 7.91–7.94 (2H, m), 8.04–8.08 (1H, m), 8.21–8.25 (1H, m), 8.28–8.29 (2H, m), 8.50 (1H, s), 8.60 (1H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.6, 156.1, 156.0, 154.3, 153.1, 151.7, 140.2, 137.7, 135.2, 134.3, 131.2, 129.8, 129.7, 129.4, 128.6, 128.2, 127.4, 118.9, 118.6, 118.1, 80.0, 79.8, 79.6, 79.2, 64.7, 50.9, 37.9, 34.9, 28.7, 28.5, 21.0. IR (KBr, υ, cm^{-1}): 3383, 3298, 3082, 2953, 1666, 1510, 1309. Anal. Calcd. For $C_{31}H_{28}N_4O_4$ *m*/*z*: 520.5; found (M + 1): 521.3.

2.4.12. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-(4-florophenyl)urea ***(3l)***. Yield 64%, m.p. 375–377°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 1.21 (6H, s), 2.31 (2H, s), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.33 (1H, s), 6.93–6.98 (2H, m), 7.29–7.32 (2H, t, *J*: 1.76), 7.34–7.37 (2H, m), 7.39–7.42 (2H, t, *J*: 1.08), 7.87–7.90 (2H, m), 8.17–8.20 (1H, m), 8.30–8.33 (1H, m), 8.38 (1H, m), 8.42 (1H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.6, 156.4, 156.0, 154.3, 153.2, 151.7, 140.1, 136.6, 135.1, 134.3, 131.4, 129.6, 129.4, 128.6, 128.1, 127.5, 127.4, 120.6, 120.5, 118.7, 118.6, 118.1, 116.0, 115.8, 64.7, 50.9, 37.9, 34.9, 28.7, 28.5. IR (KBr, υ, cm^{-1}): 3367, 3313, 3075, 1664, 1504, 1307. Anal. Calcd. For $C_{30}H_{25}FN_4O_4$ *m*/*z*: 524.5; found (M + 1): 525.6.

2.4.13. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-hexylurea ***(3m)***. Yield 60%, m.p. 265–267°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 0.80–0.84 (3H, t, *J*: 6.26), 1.09 (6H, s), 1.22 (6H, s), 1.32–1.38 (2H, m), 2.23 (2H, s), 2.98–3.02 (2H, t, *J*: 6.06), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.04–6.08 (1H, t, *J*: 5.58), 6.17 (1H, s), 7.24 (4H, s), 7.91–7.94 (2H, m), 8.05–8.08 (1H, m), 8.21–8.24 (1H, m), 8.38 (1H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.6, 156.0, 154.2, 151.7, 141.0, 135.2, 134.3, 130.3, 129.6, 129.4, 128.5, 128.1, 127.3, 118.1, 117.9, 80.0, 79.6, 79.1, 64.7, 50.9, 37.9, 34.9, 31.7, 31.3, 30.3, 28.7, 28.5, 26.7, 22.7, 14.6. IR (KBr, υ, cm^{-1}): 3402, 3354, 3040, 2960, 1662, 1541, 1352. Anal. Calcd. For $C_{30}H_{34}N_4O_4$ *m*/*z*: 514.6; found (M + 1): 515.5.

2.4.14. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-propylurea ***(3n)***. Yield 74%, m.p. 353–355°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 0.82–0.84 (3H, t, *J*: 6.26), 1.08 (6H, s), 1.32–1.39 (2H, s), 2.23 (2H, s), 2.98–3.02 (2H, t), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.04–6.07 (1H, t, *J*: 5.58), 6.12 (1H, s), 7.22 (4H, s), 7.90–7.93 (2H, m), 8.05–8.09 (1H, m), 8.20–8.23 (1H, m), 8.37 (1H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.6, 156.0, 154.2, 151.7, 141.0, 135.2, 134.3, 130.3, 129.6, 129.4, 128.5, 128.1, 127.3, 118.1, 117.9, 79.6, 79.1, 64.7, 50.9, 37.9, 34.9, 31.3, 28.7, 28.5, 26.7, 22.7, 14.6. IR (KBr, υ, cm^{-1}): 3394, 3354, 3042, 2958, 2932, 1649, 1537, 1354. Anal. Calcd. For $C_{27}H_{28}N_4O_4$ *m*/*z*: 486.5; found (M + 1): 487.2.

2.4.15. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-isopropylurea ***(3o)***. Yield 50%, m.p. 218-219°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 0.82–0.87 (3H, t, *J*: 6.26), 1.08 (6H, s), 1.22–1.39 (3H, s), 2.20 (2H, s), 2.98–3.04 (1H, t, *J*: 6.06), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.02–6.06 (1H, t, *J*: 5.58), 6.11 (1H, s), 7.25 (4H, s), 7.90–7.93 (2H, m), 8.09-8.10 (1H, m), 8.19–8.21 (1H, m), 8.39 (1H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.6, 156.0, 154.2, 151.7, 141.0, 135.2, 134.3, 130.3, 129.6, 129.4, 128.5, 128.1, 127.3, 118.1, 117.9, 79.6, 79.1, 64.7, 50.9, 37.9, 34.9, 31.3, 28.7, 28.5, 26.7, 22.7, 14.6. IR (KBr, υ, cm^{-1}): 3392, 3354, 3045, 2958, 2932, 1649, 1537, 1355. Anal. Calcd. For $C_{27}H_{28}N_4O_4$ *m*/*z*: 472.5; found (M + 1): 473.7.

2.4.16. 1-(4-(3,3-Dimethyl-1,6,11-trioxo-2,3,4,6,11,13-hexahydro-1H-indazolo[1,2-b]phthalazin-13-yl)phenyl)-3-ethylurea ***(3p)***. Yield 48%, m.p. 360–362°C; ^{1}H NMR (DMSO-d_6, 300 MHz): 0.81–0.86 (3H, t, *J*: 6.26), 1.07 (6H, s), 2.19 (2H, s), 2.97–3.04 (2H, t, *J*: 6.06), 3.18–3.44 (2H, AB system, *J*: 19.21), 6.02–6.06 (1H, t, *J*: 5.58), 6.12 (1H, s), 7.23 (4H, s), 7.90–7.93 (2H, m), 8.09-8.10 (1H, m), 8.19–8.21 (1H, m), 8.39 (1H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): 192.6, 156.0, 155.7, 154.2, 151.7, 141.0, 135.2, 134.3, 130.4, 129.7, 129.5, 129.4, 128.5, 128.1, 127.4, 118.2, 118.1, 118.0, 64.7, 50.9, 37.9, 34.9, 34.5, 28.7, 28.5, 16.1. IR (KBr, υ, cm^{-1}): 3406, 3367, 3045, 2976, 2960, 1651, 1533, 1355. Anal. Calcd. For $C_{26}H_{26}N_4O_4$ *m*/*z*: 458.5; found (M + 1): 459.0.

2.5. CA Enzyme Assay. Blood samples (25 mL) were taken from healthy human volunteers. They were anticoagulated with acid-citrate-dextrose and centrifuged at 2000 g for 20 min at 4°C, and the supernatant was removed. The packed

erythrocytes were washed three times with 0.9% NaCl and then haemolysed in cold water. The ghosts and any intact cells were removed by centrifugation at 2000 g for 25 min at 4°C, and the pH of the haemolysate was adjusted to pH 8.5 with solid tris-base. The 25 mL haemolysate was applied to an affinity column containing L-tyrosine-sulfonamide-Sepharose-4B [32] equilibrated with 25 mM tris-HCl/0.1 M Na_2SO_4 (pH 8.5). The affinity gel was washed with 50 mL of 25 mM tris-HCl/22 mM Na_2SO_4 (pH 8.5). The human CA (hCA) isozymes were then eluted with 0.1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), which recovered hCA I and hCA II, respectively. Fractions of 3 mL were collected, and their absorbance was measured at 280 nm. CA activity was measured by the Maren method which is based on determination of the time required for the pH to decrease from 10.0 to 7.4 due to CO_2 hydration [33]. CO_2-hydratase activity (enzyme units (EU)) was calculated by using the equation $t_0 - t_c/t_c$ where t_0 and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

2.6. In Vitro Inhibition Studies. For the *in vitro* inhibition studies of urea and thiourea derivatives, different concentrations of these compounds were added to the enzyme. Activity percentage values of CA for different concentrations of each compound were determined by regression analysis using Microsoft Office 2000 Excel. CA enzyme activity without a synthesized compounds solution was accepted as 100% activity.

3. Results and Discussion

For evaluation of the physiologically relevant human CA isozymes (hCAs I and II) inhibitory activity, several new urea and thiourea compounds were subjected to CA inhibition assay with CO_2 as a substrate. The results showed that phthalazine substituted urea and thiourea derivatives **(3a–p)** inhibited the CA enzyme activity. The inhibition constants of the synthesized compounds against CAs were given in Table 1. We have determined the IC_{50} values of 6.40–20.38 μM and 6.13–23.63 μM for hCA I and hCA II, respectively, and they are all competitive inhibitors.

2H-Indazolo[2,1-b]phthalazine-trione compound **(1)** was prepared with 4-nitrobenzaldehyde, dimedone, and phthalhydrazide in the presence of TFA in DMF [34]. The nitro containing phthalhydrazide was reduced with tin (II) chloride in ethanol [35]. The amino phthalhydrazide was reacted with isocyanates or thioisocyanates to get the final products **(3a–p)** (Scheme 2) at moderate yields [36].

All new compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR, and MS. In the infrared spectra of compounds **(3a–p)**, it was possible to observe the absorptions between 3250 and 3410 cm^{-1} relating to NH stretch and absorptions in 1645–1670 cm^{-1} from urea carbonyl moiety stretching. The ^{1}H NMR spectra for all the synthesized urea and thiourea compounds show signals as singlet between 6.10 and 10.00 ppm relating to hydrogens attached to the nitrogen. The signals for aromatic hydrogens are between 6.50 and 8.50 ppm. The signals around 3.18 ppm are seen as AB system with the coupling constant 19.21 Hz. which is the germinal-germinal interaction. Through the ^{13}C NMR data, a sign can be seen about 150.0–160.0 ppm for urea carbonyl and 175.0–180.0 ppm for thiourea carbonyl.

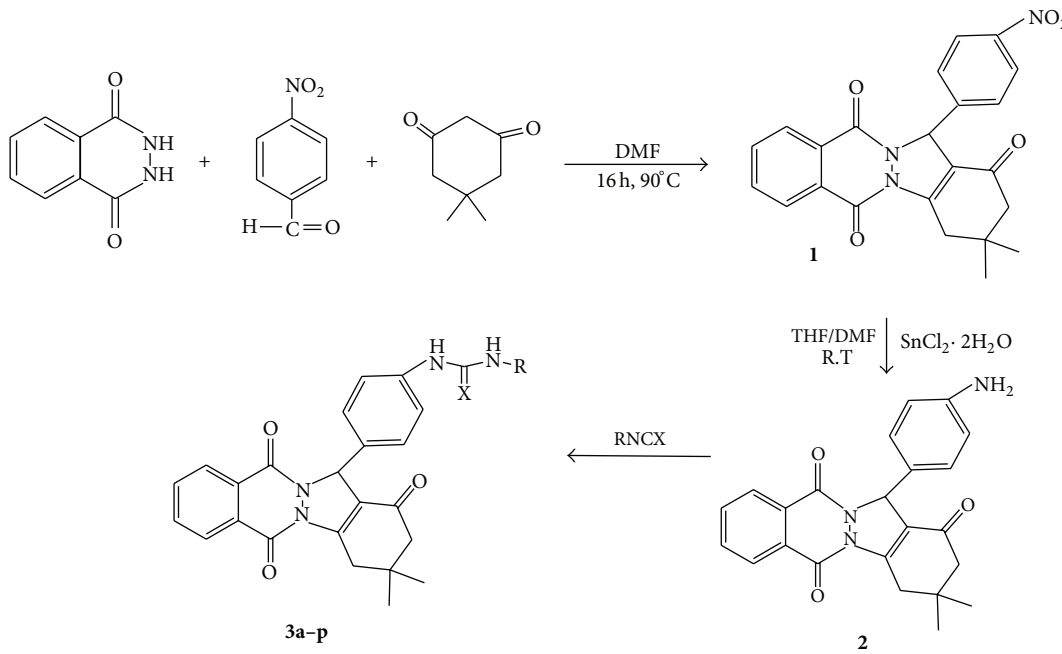
Table 1: IC_{50} (μM) values of the phthalazine substituted urea and thiourea derivatives.

	hCA I	hCA II		hCA I	hCA II
1	12,08	11,89	**3h**	14,04	17,78
2	10,18	10,25	**3i**	6,76	6,75
3a	6,40	6,13	**3j**	20,38	22,73
3b	13,97	15,35	**3k**	16,38	16,34
3c	18,20	23,63	**3l**	12,62	12,34
3d	19,59	18,21	**3m**	7,63	9,67
3e	11,02	8,50	**3n**	8,20	8,74
3f	12,20	19,12	**3o**	7,45	10,03
3g	8,72	8,25	**3p**	11,72	10,04

CA inhibitors decrease intraocular pressure by reducing bicarbonate formation in the ciliary process, so lowering Na^+ transport and flow of aqueous humour: this is the basis for their use in glaucoma treatment. Unfortunately, systemic therapy with parenteral sulphonamides leads to significant side effects, many of them being probably due to inhibition of CA isoforms in other tissues. Acetazolamide which is 20 times less active against hCA I than against hCA II in erythrocytes is the most widely used inhibitor. But the inhibition of various CA isoforms which are present in tissues other than eye leads to an entire range of side effects, the most prominent being numbness and tingling of extremities, metallic taste, depression, fatigue, malaise, weight loss, decreased libido, gastrointestinal irritation, metabolic acidosis, renal calculi, and transient myopia [7, 36, 37].

Sulfonamide compounds are coordinated to the zinc (II) ion within the hCAs active site, whereas its organic scaffold fills the entire enzyme cavity, making an extensive series of van der Waals and polar interactions with amino acid residues both at the bottom, middle, and entrance of the active site cavity [38]. Coumarins derivatives may possess various tautomeric forms which may bind within the CA active site similarly to phenols, that is, by anchoring to the zinc-bound water molecule/hydroxide ion [39]. Coumarins cannot bind enzyme effectively in the restricted space near Zn^{2+} ion because they have bulky group and exhibit unusual binding mode not interacting with the metal ion of the enzyme [40]. We assume that the synthesized compounds are very big pendant group to be able to bind near the zinc ion. Hence, they much more probably bind as the coumarin derivatives.

The results showed that all the compounds **(3a–p)** inhibited the enzyme activity. The inhibition constants of the synthesized compounds against CAs were given in Table 1. The following structure-activity relationship (SAR) observations can be drawn from the data. The slow cytosolic isoform hCA I and the second off-target isoform hCA II were inhibited by the synthesized compounds with inhibition values in the



	3a	3b	3c	3d	3e	3f	3g	3h
X	S	S	S	S	S	S	S	O
R	Ph–	3-MeO–Ph–	4-CH_3–Ph–	4-Cl–Ph–	4-I–Ph–	4-Br–Ph–	4-F–Ph–	Ph–

	3i	3j	3k	3l	3m	3n	3o	3p
X	O	O	O	O	O	O	O	O
R	4-NO_2–Ph–	3-MeO–Ph–	4-CH_3–Ph–	4-F–Ph–	$CH_3(CH_2)_5$–	$CH_3(CH_2)_2$–	$(CH_3)_2CH$–	CH_3CH_2–

Scheme 2: Synthesis of phthalazine substituted urea and thiourea derivatives.

range of 6.00–24.00 μM. The best hCA I and hCA II inhibitors among the newly synthesized and investigated compounds were **3a** and **3i**. For urea derivatives of aryl-phthalazine substituted compounds, electron withdrawing groups (nitro and fluorine) bonded on phenyl ring (**3i** and **3l**) increased the hCAs I and II inhibitory activity. In contrast, electron donating groups (methoxy, methyl) on phenyl ring (**3j** and **3k**) have moderate inhibitory activity for the hCAIs and II. For the aryl-aryl thiourea derivatives, electron donating groups as mesomeric or inductive (methoxy, methyl, and halogens) on the phenyl ring (from **3b** to **3f**) have moderate inhibitory activity, but the compound **(3g)** with fluorine atom has good inhibition effect on hCAs I and II. Flouro substituted urea derivatives (**3l** and **3g**) showed more inhibitory effect than methoxy, methyl, chloro, and bromo substituted ureas. Fluorophenyl sulfamate adducts were reported where the sulfamates possess a rather variable binding pattern within the hCA II active site [41, 42]. Alkyl-phthalazine substituted ureas have different inhibition effects. When alkyl chain increases, inhibition effect increases with alkyl chain length due to their steric effect. It is obviously clear that bulky phthalazine group affects inhibition for the compounds.

In summary, enzyme inhibition is a more important issue for drug design and biochemical applications [43–46]. The results showed that new phthalazine substituted urea and thiourea derivatives inhibited the hCAs I and II enzyme activity. Therefore, our results suggested that the compounds are likely to be adopted as candidates to treat glaucoma and may be taken for further evaluation in *in vivo* studies.

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