Quinazolinones as Competitive Inhibitors of Carbonic Anhydrase-II (Human and Bovine): Synthesis, in-vitro, in-silico, Selectivity, and Kinetics Studies

Ajmal Khan¹, Majid Khan¹, Sobia Ahsan Halim⁴, Zulfiqar Ali Khan³, Zahid Shafiq⁴* and Ahmed Al-Harrasi*¹

¹ Natural and Medical Sciences Research Center, University of Nizwa, Nizwa, Oman, ² International Center for Chemical and Biological Sciences, H. E. J. Research Institute of Chemistry, University of Karachi, Karachi, Pakistan, ³ Department of Chemistry, Government College University, Faisalabad, Pakistan, ⁴ Institute of Chemical Sciences, Bahauddin Zakariya University, Multan, Pakistan

Carbonic anhydrase-II (CA-II) is associated with glaucoma, malignant brain tumors, and renal, gastric, and pancreatic carcinomas and is mainly involved in the regulation of the bicarbonate concentration in the eyes. CA-II inhibitors can be used to reduce the intraocular pressure usually associated with glaucoma. In search of potent CA-II inhibitors, a series of quinazolinones derivatives (4a-p) were synthesized and characterized by IR and NMR spectroscopy. The inhibitory potential of all the compounds was evaluated against bovine carbonic anhydrase-II (bCA-II) and human carbonic anhydrase-II (hCA-II), and compounds displayed moderate to significant inhibition with IC₅₀ values of 8.9–67.3 and 14.0–59.6 µM, respectively. A preliminary structure-activity relationship suggested that the presence of a nitro group on the phenyl ring at R position contributes significantly to the overall activity. Kinetics studies of the most active inhibitor, 4d, against both bCA-II and hCA-II were performed to investigate the mode of inhibition and to determine the inhibition constants (Ki). According to the kinetics results, 4d is a competitive inhibitor of bCA-II and hCA-II with Ki values of 13.0 ± 0.013 and 14.25 ± 0.017 µM, respectively. However, the selectivity index reflects that the compounds 4g and 4o are more selective for hCA-II. The binding mode of these compounds within the active sites of bCA-II and hCA-II was investigated by structure-based molecular docking. The docking results are in complete agreement with the experimental findings.

Keywords: quinazolinones, bovine carbonic anhydrase-II, human carbonic anhydrase-II, structure-activity relationship, kinetics, molecular docking

INTRODUCTION

Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc-containing metallo-enzymes, found in animals, plants, algae, archaea, and eubacteria. CAs are encoded by three gene families, α-CA, β-CA, and γ-CA, that are evolutionarily unrelated (Hewett-Emmett, 2000; Jakubowski et al., 2018). These metallo-enzymes use zinc as a cofactor for the reversible inter-conversion of carbon dioxide and bicarbonate, while α-CAs possess high versatility, being able to catalyze other hydrolytic
processes (Hewett-Emmett, 2000). Carbonic anhydrases are a class of hydrolase enzymes (Pocker and Meany, 1967; Arslan, 2001). In humans, more than 16 isoforms of carbonic anhydrase (hCA) are present (Shank et al., 2005; Shaik et al., 2019). CAs are involved in different physiological and pathological processes (Lindskog, 1997; Aggarwal et al., 2013; Ozensoy Guler et al., 2016). Consequently, these enzymes are interesting therapeutic targets for the treatment of pathological disorders (Chegwidden et al., 2000; Krishnamurthy et al., 2008; Supuran, 2008). CA-II is mainly involved in the regulation of the bicarbonate concentration in the eyes. CA-II inhibitors can be used to reduce the intraocular pressure usually associated with glaucoma (Supuran and Scozzafava, 2007; Pastorekova and Supuran, 2014; Ruusuvuori and Kaila, 2014; Zaraei et al., 2019). Moreover, CA-II is also expressed in malignant brain tumors (Parkkila et al., 1995) and renal, gastric, and pancreatic carcinomas (Frazier et al., 1990; Pastorekova et al., 1997; Parkkila et al., 2000). The inhibitors of CA-II have also been considered as an adjunct in cancer chemotherapy (Zaraei et al., 2019).

These highly abundant proteins are involved in crucial physiological processes related with respiration. These enzymes are mainly involved in pH/CO₂ homeostasis, secretion of electrolytes in tissues/organs, and transportation of CO₂ and bicarbonate between the lungs and metabolizing tissues. Other than that these enzymes are also involved in many other physiological or pathological processes, such as bone resorption, gluconeogenesis, calcification, lipogenesis and ureagenesis, and tumorigenicy (Hewett-Emmett, 2000). CA-II has also been involved in glaucoma, epilepsy, leukemia, and cystic fibrosis (Achal and Pan, 2011; Senturk et al., 2011).

Quinazolinones are N-containing heterocyclic compounds that are widely distributed in nature, including in plants and microorganisms (He et al., 2017). Quinazolinone emerged as a privileged class of heterocyclic compounds with an increasing number of drug candidates and is regularly used in medicinal chemistry (Khan et al., 2014, 2015, 2016). The 2,3-Disubstituted quinazolinones retain anticancer (Al-Suwaidan et al., 2013), anticonvulsant (Gawad et al., 2011), anti-microbial (Al-Amiery et al., 2014), and anti-inflammatory activities (Alaa et al., 2016). Several derivatives of 3-(4-Aminosulfonyl)phenyl-2-mercapto-substituted-4(3H)-quinazolinones (A) are reported for hCA-I (KIs = 135–282 nM), hCA-II (KIs = 0.25–10.8 nM), hCA-IX (KIs of 3.7–50.4 nM), and CA-XII (KIs of 0.60–52.9 nM) as potent inhibitors (Alafeefy et al., 2016). Several other 2-[(3-substituted-4(3H)-quinazolinon-2-yl)thio]-N-(4-sulfamoylphenyl)acetamides (B) derivatives also showed potent inhibition of α-CA, from Vibrio cholerae (VchCA) and human “hCA-I” and “hCA-II,” at a nanomolar level (Alafeefy et al., 2014) (Figure 1). Recently, 2-[(3-Benzyl-6,7-dimethoxy-4-(3H)-quinazolinon-2-yl)thio]-N-(4-sulfamoylphenyl)propanamide was also reported for hCA-II (KI, 3.30 nM) inhibition (El-Azab et al., 2019).

In this study, we report the synthesis and in-vitro bovine carbonic anhydrase-II (bCA-II) and human carbonic anhydrase-II (hCA-II) inhibitory activities of a series of quinazolinone analogs. Furthermore, the mode of inhibition was further explored by kinetic studies of the active analogs. Additionally, molecular docking studies were carried out to predict the main structural features responsible for the anti-CA-II activities of the compounds to predict the structure-activity relationships.

**EXPERIMENTAL**

**Chemistry**

The chemicals used in this study were purchased from Sigma and Aldrich in extra purified form. Melting points for all compounds were recorded by Büchi 434 melting point apparatus. FT-IR spectra (KBr disks) were measured on a Bruker FT-IR IFS 48 spectrophotometer. NMR spectra were recorded on Bruker Avance 400 spectrometers in DMSO-d6. The chromatograms were visualized under UV light irradiation. The CHN analysis was performed on a Carlo Erba Strumentazione-Mod-1106.

**General Procedure for Synthesis of 2-substituted-4H-benzo[d][1,3]oxazin-4-ones (3a-p)** (Bari et al., 2020)

To an 100 mL round bottom flask, anthranilic acid 1 (0.01 mol) and dry pyridine (30 mL) were added at room temperature with stirring. The solution was cooled to 0°C, followed by dropwise addition of the corresponding aromatic acid chloride 2a-p (0.02 mol) in 10 mL of dry pyridine with constant stirring. After this addition, the reaction mixture was stirred for a further half an hour at room temperature and set aside for 1 h. The product, obtained as a pasty mass, was diluted with ice cold water (50 mL) and treated with aqueous sodium bicarbonate solution.

![FIGURE 1](image-url)
When the effervescence ceased, the precipitate obtained was filtered and washed with water. The crude benzoxazine obtained was dried and recrystallized from aqueous ethanol.

**General Procedure for the Synthesis of Quinazolinone (4a-p) (Panicker et al., 2010)**

To a 50 mL round bottom flask fitted with reflux condenser were added 2-substituted-4H-benzo[d][1,3]oxazin-4-one (0.01 mol) and hydrazine hydrate (0.06 mol) in excess. The reaction mixture was heated under reflux in an oil bath for 10–16 h. The course of the reaction was monitored by thin layer chromatography (TLC). After completion of the reaction as checked by TLC, the reaction mixture was cooled and separated solids were collected by filtration, washed with water/hexane, dried, and purified by column chromatography (methanol/chloroform/water, 10:2:1) to afford the corresponding quinazolinones 4a-p.

**Experimental Data**

### 3-Amino-2-(4-Chlorophenyl) Quinazolin-4(3H)-one (4a)

Yield: 76%; m.p: 224–225°C; FT-IR (υ_max, KBr, cm⁻¹): 3,307, 3,053, 1,670, 1,603, 1,548, 1,488, 1,355, 1,250, 708; 1H NMR (400 MHz, DMSO-d⁶, δ, ppm): 5.42 (2H, s), 7.62–7.68 (3H, m), 8.07 (1H, d, J = 7.8 Hz), 8.2 (1H, d, J = 7.2 Hz), 8.27 (1H, d, J = 7.8 Hz), 8.56 (1H, s); Anal. Calcd for C₁₄H₁₀ClN₂O: C, 70.57; H, 3.57; N, 19.85. Found: C, 70.56; H, 3.58; N, 19.85.

### 3-Amino-2-(4-Nitrophenyl) quinazolin-4(3H)-one (4d)

Yield: 84%; m.p: 206–208°C; FT-IR (υ_max, KBr, cm⁻¹): 3,308, 3,053, 1,675, 1,600; 1H NMR (400 MHz, DMSO-d⁶, δ, ppm): 5.48 (2H, s), 7.62–7.68 (3H, m), 8.07 (1H, d, J = 7.8 Hz), 8.2 (1H, d, J = 7.2 Hz), 8.27 (1H, d, J = 7.8 Hz), 8.56 (1H, s); Anal. Calcd for C₁₄H₁₀N₂O: C, 70.57; H, 3.57; N, 19.85. Found: C, 70.56; H, 3.58; N, 19.85.

### 3-Amino-2-(4-Nitrophenyl)-quinazolin-4(3H)-one (4e) (Panidi and Dodiya, 2013)

Yield: 77%; m.p: 180–182°C; FT-IR (υ_max, KBr, cm⁻¹): 3,309, 3,053, 1,673, 1,603, 1,548, 1,489, 1,354, 1,250, 701; 1H NMR (400 MHz, DMSO-d⁶, δ, ppm): 5.52 (2H, s), 7.51–7.63 (4H, m), 7.91–8.02 (4H, m); Anal. Calcd for C₁₅H₁₃N₃O₂: C, 60.27; H, 3.89; N, 15.65. Found: C, 60.26; H, 3.89; N, 15.65.

### 3-Amino-2-(4-Chlorophenyl)-quinazolin-4(3H)-one (4f)

Yield: 85%; m.p: 184–185°C; FT-IR (υ_max, KBr, cm⁻¹): 3,309, 3,053, 1,676, 1,610, 1,010, 690; 1H NMR (400 MHz, DMSO-d⁶, δ, ppm): 5.26 (2H, s), 7.52 (2H, d, J = 7.45 Hz), 7.61–7.67 (3H, m), 7.74 (2H, d, J = 7.6 Hz), 8.1 (1H, d, J = 7.55 Hz); Anal. Calcd for C₁₄H₁₇BrN₂O: C, 53.19; H, 3.19; N, 13.29. Found: C, 53.17; H, 3.20; N, 13.28.

### 3-Amino-2-(4-Methylphenyl)-quinazolin-4(3H)-one (4h) (Babu et al., 2014)

Yield: 84%; m.p: 169–170°C; FT-IR (υ_max, KBr, cm⁻¹): 3,308, 3,053, 1,681, 1,610, 1,329, 506; 1H NMR (400 MHz, DMSO-d⁶, δ, ppm): 4.98 (2H, s), 8.20 (1H, d, J = 7.2 Hz), 7.77 (1H, bd, J = 8.4 Hz), 7.59–7.67 (4H, m), 7.29–7.36 (2H, m); Anal. Calcd for C₁₅H₁₃N₂O: C, 71.70; H, 5.21; N, 16.72. Found: C, 71.71; H, 5.20; N, 16.73.

### References

Babu et al., 2014

Panidi and Dodiya, 2013

Panicker et al., 2010

Khan et al. Quinazolinones as Competitive Inhibitors of Carbonic Anhydrase-II
3-Amino-2-(3-Chlorophenyl) Quinazolin-4(3H)-one (4m) (Pandit and Dodiya, 2013)
Yield: 78%; m.p: 221–222 °C; FT-IR (υmax, KBr, cm⁻¹): 3.310, 3.053, 2.950, 1.689, 1.610, 1.495; ¹H NMR (400 MHz, DMSO-d⁶, δ, ppm): 3.79 (6H, s), 3.82 (3H, s), 4.88 (2H, s), 6.62 (2H, s), 7.48–7.52 (3H, m), 8.01 (1H, d, J = 7.3 Hz); Anal. Calcd. for C₁₇H₁₇N₃O₄: C, 62.38; H, 5.23; N, 12.84; Found C, 62.38; H, 5.24; N, 12.83.

3-Amino-2-(4-Methoxyphenyl) Quinazolin-4(3H)-one (4o) (Pandit and Dodiya, 2013)
Yield: 88%; m.p: 221–223 °C; FT-IR (υmax, KBr, cm⁻¹): 3.310, 3.053, 2.940, 1.689, 1.610, 1.490; ¹H NMR (400 MHz, DMSO-d⁶, δ, ppm): 4.92 (2H,s), 3.76 (3H, s), 6.96 (2H, d, J = 7.5 Hz); 7.34 (2H, d, J = 7.5 Hz), 7.60–7.68 (3H, m), 8.09 (1H, d, J = 7.3 Hz); Anal. Calcd. for C₁₃H₁₃N₃O₂: C, 76.40; H, 4.90; N, 15.72; Found C, 76.42; H, 4.88; N, 15.71.

In-vitro Assay Protocol
In-vitro bCA-II and hCA-II activities were measured by following the spectrophotometric method described by Pocker and Meany with slight modifications (Pocker and Meany, 1967; Ur Rehman et al., 2020). The spectrophotometric assay was conducted in HEPES-Tris buffer of pH 7.4 (20 mM) at 25 °C. Each inhibitory well-consisted of 140 µL of HEPES-Tris buffer solution, 20 µL of bCA-II enzyme solution (0.1 mg/ml HEPES-Tris buffer), and 20 µL of test compound in HPLC grade DMSO (maintain 10% of the final concentration). The mixture solution was pre-incubated for 15 min at 25 °C. Substrate p-nitrophenyl acetate (p-NPA) (0.7 mM) was prepared in HPLC grade methanol and the reaction was started by adding 20 µL to a well in a 96-well-plate. The amount of product formed was measured for 30 min continuously at 1 min intervals at 400 nm in a 96-well-plate using xMARK microplate spectrophotometer, Bio-Rad (USA). The activity of the controlled compound was taken as 100%. All experiments were carried out in triplicates of each used concentration, and results are represented as a mean of the triplicate.

RESULTS AND DISCUSSION

Chemistry
The target compounds were synthesized according to the route depicted in Scheme 1. Anthranilic acid 1 was treated with corresponding acid chlorides (2a-p) in the presence of pyridine to form precursor heterocycle (3a-p) via literature method (Scheme 1) (Bari et al., 2020). The corresponding benzoazinones were refluxed in excess of hydratine hydrate to furnish the 3-Amino-2-aryl quinazolin-4(3H)-ones (4a-p) in

![Scheme 1: Synthesis of quinazolinone derivatives.](image-url)
The structures of compounds (4a-p) were established using microanalysis (CHN) and spectral data, i.e., IR and $^1$H NMR. The C=N band in FTIR appeared in the range of 1,489–1,521 cm$^{-1}$. Moreover, the carbonyl group of compounds (4a-p) appeared at 1,660–1,689 cm$^{-1}$. The amino moiety (-NH$_2$) of compounds (4a-p) was verified by the characteristic peak at 3,300–3,319 cm$^{-1}$ in FT-IR spectra. The amino-moiety of compounds (4a-p) was verified by $^1$H NMR spectra which appeared as a singlet for amino protons in a range from δ 4.92–5.52 ppm. The spectral data of other aromatic and aliphatic protons was also in accordance with the structures of anticipated compounds. CHN analysis also supported the anticipated structures (4a-p) and the observed values were in good agreement with the values found.

### Biology

**In-vitro bCA-II and hCA-II inhibitions**

All quinazolinone analogs (4a-p) were evaluated against bovine carbonic anhydrase-II (bCA-II) and human carbonic anhydrate-II (hCA-II) for their ability to act as an inhibitor of CA-II. All the assays were carried out at a micromolar level using acetazolamide as a standard inhibitor. After initial screening, all the analogs demonstrated significant inhibitory activity against bCA-II with IC$_{50}$ values in the range of 8.9 ± 0.31 – 67.3 ± 0.42 µM, except compounds 4i and 4o, which were found to be inactive (Table 2). Moreover, compounds 4c, 4e, 4f, 4l, and 4m showed more superior activity than the standard drug ‘acetazolamide’ (IC$_{50}$ = 18.2 ± 0.43 µM). Compound 4m was the most active inhibitor (IC$_{50}$ = 8.9 ± 0.31 µM), followed by 4e (IC$_{50}$ = 9.1 ± 0.21 µM), 4l (IC$_{50}$ = 10.7 ± 0.82 µM), 4c (IC$_{50}$ = 11.7 ± 0.36 µM), and 4f (IC$_{50}$ = 17.9 ± 0.56 µM). However, compounds 4d, 4k, 4n, and 4p depicted biological activity comparable to the standard acetazolamide with IC$_{50}$ values in range of 18.3 ± 0.51–28.2 ± 0.01 µM, while compounds 4a, 4b, 4g, 4h, and 4j were found

### Table 1 | Different analogs, % yield, and reaction time of quinazolinones.

| Entry | Compounds | R     | % Yields | Reaction time (h) |
|-------|-----------|-------|----------|------------------|
| 1     | 4a        | Phenyl| 82       | 12               |
| 2     | 4b        | 1- Naphthyl| 80   | 10               |
| 3     | 4c        | 2- Nitrophenyl| 77  | 16               |
| 4     | 4d        | 3- Nitrophenyl| 84  | 20               |
| 5     | 4e        | 4- Nitrophenyl| 76  | 20               |
| 6     | 4f        | 2- Bromophenyl| 79  | 11               |
| 7     | 4g        | 4- Bromophenyl| 81  | 12               |
| 8     | 4h        | 4- Methylphenyl| 85  | 8                |
| 9     | 4i        | 2- Fluorophenyl| 84  | 12               |
| 10    | 4j        | 3- Fluorophenyl| 78  | 14               |
| 11    | 4k        | 4- Fluorophenyl| 81  | 10               |
| 12    | 4l        | 2- Chlorophenyl| 85  | 10               |
| 13    | 4m        | 3- Chlorophenyl| 79  | 12               |
| 14    | 4n        | 4- Chlorophenyl| 86  | 10               |
| 15    | 4o        | 4- Methoxyphenyl| 88  | 15               |
| 16    | 4p        | 3,4,5-Trimethoxyphenyl| 78  | 13               |

### Table 2 | In-vitro inhibition results of compounds (4a-p) against bCA-II and hCA-II.

| Compounds | % Inhibition (0.5 mM) | IC$_{50}$ ± SEM (µM) | % Inhibition (0.5 mM) | IC$_{50}$ ± SEM (µM) |
|-----------|-----------------------|----------------------|-----------------------|----------------------|
| 4a        | 75.2                  | 53.6 ± 0.85          | 75.2                  | 59.6 ± 1.03          |
| 4b        | 83.6                  | 67.3 ± 0.42          | 38.1                  | NA                   |
| 4c        | 80.5                  | 11.7 ± 0.36          | 88.5                  | 21.1 ± 1.36          |
| 4d        | 92.9                  | 19.7 ± 1.02          | 94.2                  | 21.5 ± 0.52          |
| 4e        | 80.7                  | 9.1 ± 0.21           | 92.7                  | 39.9 ± 2.21          |
| 4f        | 88.9                  | 17.9 ± 0.56          | 98.9                  | 43.5 ± 1.51          |
| 4g        | 85.3                  | 33.6 ± 0.22          | 85.3                  | 14.0 ± 0.60          |
| 4h        | 92.4                  | 38.0 ± 0.62          | 92.4                  | 53.0 ± 2.12          |
| 4i        | 17.6                  | NA                   | 27.8                  | NA                   |
| 4j        | 78.6                  | 46.6 ± 0.39          | 28.1                  | NA                   |
| 4k        | 76.3                  | 18.3 ± 0.51          | 36.2                  | NA                   |
| 4l        | 78.2                  | 10.7 ± 0.82          | 28.7                  | NA                   |
| 4m        | 60.0                  | 8.9 ± 0.31           | 30.5                  | NA                   |
| 4n        | 54.2                  | 28.2 ± 0.01          | 24.3                  | NA                   |
| 4o        | 19.9                  | NA                   | 89.4                  | 22.0 ± 0.40          |
| 4p        | 87.3                  | 26.8 ± 0.23          | 27.4                  | NA                   |
| Acetazolamide | 86.4               | 18.2 ± 0.43          | 83.2                  | 19.6 ± 1.23          |

SEM, Standard error mean; NA, Not active.
to be the least active hits of the series. For hCA-II, compounds 4a, 4c-4h, and 4o were found to be active with IC$_{50}$ values in the range of 14.0 ± 0.60–59.6 ± 1.03 µM, as compared to acetazolamide (IC$_{50}$ = 19.6 ± 1.23 µM) (Table 2). Among all the compounds, 4g (IC$_{50}$ = 14.0 ± 0.60 µM) exhibited better activity than the standard drug, while compounds 4c (IC$_{50}$ = 21.1 ± 1.36 µM), 4d (IC$_{50}$ = 21.5 ± 0.52 µM), and 4o (IC$_{50}$ = 22.0 ± 0.40 µM) demonstrated activities comparable to acetazolamide. Compounds 4e (IC$_{50}$ = 39.9 ± 2.21 µM), 4f (IC$_{50}$ = 43.5 ± 1.51 µM), 4h (IC$_{50}$ = 53.0 ± 2.12 µM), and 4a (IC$_{50}$ = 59.6 ± 1.03 µM) were shown to be the least active hits in this series. The in-vitro results indicated that compounds 4g and 4o are more selective inhibitors for hCA-II. The biological activities of all the compounds are tabulated in Table 2.

**Kinetics Studies**

To investigate the mechanism of action of these compounds, kinetics studies were performed. The kinetics studies were used to discover the type of inhibition and dissociation constant (Ki). Kinetics studies of the most active compound, 4d, against both bCA-II and hCA-II were performed, using different substrate concentrations on one side with different concentrations of 4d on the other side. Compound 4d inhibited both the bCA-II and hCA-II enzymes in a concentration-dependent manner with Ki values of 13.0 ± 0.013 and 14.25 ± 0.017 µM, respectively. From the kinetics studies, it was deduced that the compound 4d is a competitive inhibitor for both bCA-II and hCA-II. The Lineweaver-Burk plots were used for determination of the type of inhibition, in which the reciprocal of substrate concentrations was plotted against the reciprocal of the rate of the reaction to monitor the effect of the inhibitor on both K$_m$ and V$_{max}$. The Lineweaver-Burk plots of 4d against both bCA-II and hCA-II clearly showed that the mode of inhibition of 4d is competitive (Figures 2A, 3A). In competitive inhibition, the K$_m$ of enzyme increased, while V$_{max}$ are not affected. The Lineweaver-Burk plots of compounds 4d (Figures 2A, 3A) showed that in the presence of compounds 4d, the K$_m$ of both enzymes bCA-II and hCA-II increased significantly on applying compounds 4d, while the V$_{max}$ remained unchanged, which described the competitive behavior of compounds 4d and its interaction in the active site of the enzyme.

The K$_i$ values of compounds 4d for both enzymes bCA-II and hCA-II were deduced by secondary replots of Lineweaver-Burk plots by plotting the slope of each line in the Lineweaver-Burk plots.
plots against different concentrations of compound 4d (Figures 2B, 3B). The $K_i$ values were confirmed by Dixon plot after plotting the reciprocal of the rate of reaction against different concentrations of compound 4d (Figures 2C, 3C).

**Computational Studies**

Computational medicinal chemistry has expedited the pace of drug design and discovery over the last four decades (Lin et al., 2020). Docking is a computational method which is widely used to study and understand the interaction between two macro-molecules (for e.g., protein-protein or protein-DNA) or between a macromolecule and ligand (e.g., for a drug-receptor, drug-DNA, or drug-RNA) and effectively predict the inhibitory mechanism of drugs. Therefore, we predicted the mode of interactions of all the active compounds through molecular docking. The reference inhibitor (acetazolamide) and quinazolinones were docked into the catalytic active pocket of hCA-II and bCA-II. The binding modes and receptor-ligand interactions in the binding site of bCA-II and hCA-II in the three-dimensional and two-dimensional form were carefully examined by visual analysis through MOE. The results are presented in Tables 3, 4. Molecular docking revealed that all the active quinazolinones derivatives were exactly fitted into the active catalytic pocket of both enzymes (hCA-II and bCA-II). The docked orientation of compounds revealed direct interactions of ligands with the zinc ion present in the active site. Moreover, interaction of compounds with the active site residues and water molecule stabilize the compounds in the active site.

The docking process was first validated by re-docking the standard inhibitor acetazolamide in the active site of enzyme and is presented in Figures 4D, 6D.

**Binding Interactions of Active Compounds and Their Predictive SAR Against bCA-II**

From the docked poses of the all active compounds, it was confirmed that the compounds directly interact with Zn$^{2+}$ ion through their quinazolinone-carbonyl moiety. Compounds 4c, 4d, 4e, 4f, 4k, 4l, and 4m were found to be potent inhibitors of bCA-II with IC$_{50}$ values in the range of 8.9–19.7 $\mu$M. Compound 4m was the most potent inhibitor (IC$_{50}$ = 8.9 ± 0.31$\mu$M), followed by 4e > 4l > 4c > 4f > 4k > 4d, while compounds 4p > 4n displayed moderate active, and 4g > 4h > 4j >
4a > 4b were retrieved as the least active, as compared to standard acetazolamide. The docked pose of 4m indicates that the quinazolinone-carbonyl moiety of 4m formed a metallic bond (3.44 Å) with the Zn$^{2+}$ ion, while the quinazolinone-nitrogen formed hydrogen bonds with the side chain of Thr198 and Gln91 at a distance of 2.28 and 3.41 Å, respectively. Similarly, carbonyl oxygen and the nitro groups of 4e mediated a metallic bond with Zn$^{2+}$ ion (3.58 Å), and H-bonds with the side chains of Thr198 (2.80 Å) and Gln91 (3.09 Å). Additionally, the quinazolinone substituted amino group of 4e mediated a H-bond with a water molecule in the active site (3.17 Å). The third most active compounds, 4l and 4c, also followed a similar type of interaction, however, 4c was also stabilized by the side chains of Asn66 and Gln92 through hydrogen bonding. The carbonyl oxygen of 4f and 4k interacted with the Zn$^{2+}$ ion, however 4f lost the interactions with Thr198 and Gln91, instead mediating H-bonding with two water molecules (WAT493 and WAT279), however 4k retained H-bonds with Thr198 and Gln91. Compound 4d showed a metallic and a H-bond with the Zn$^{2+}$ ion and Thr199, respectively, however it lost the interactions with Gln91 and water molecules. The quinazolinone moiety of the moderate active compounds (4p and 4n) interacted with the Zn$^{2+}$ ion, while the quinazolinone moiety of both the compounds mediated hydrogen bonding with the side chains of Gln91 and Thr198.
TABLE 4 | Computational analysis of all active compounds against hCA-II.

| Code | Docking score | Ligand atoms | receptor Atoms | interaction type | Distance (Å) |
|------|----------------|--------------|----------------|-----------------|--------------|
| 4g   | −4.51          | N18          | HOH270         | HBD             | 2.45         |
|      |                | N18          | ND2-ASN62      | HBA             | 3.34         |
|      |                | N18          | OG1-THR200     | HBA             | 2.99         |
| 4c   | −4.96          | O20          | NE2-GLN92      | HBA             | 3.42         |
|      |                | N18          | HOH270         | HBA             | 2.78         |
| 4d   | −5.36          | O21          | ZN             | Metallic        | 2.73         |
|      |                | N11          | OG1-THR200     | HBA             | 2.68         |
| 4o   | −5.31          | O20          | ZN             | Metallic        | 2.71         |
|      |                | N7           | NE2-GLN92      | HBA             | 2.79         |
|      |                | N18          | OG1-THR200     | HBD             | 3.38         |
| 4e   | −5.20          | O20          | ZN             | Metallic        | 2.54         |
|      |                | N7           | NE2-GLN92      | HBA             | 2.65         |
| 4f   | −4.43          | N18          | OG1-THR200     | HBD             | 3.10         |
|      |                | O19          | HOH270         | HBA             | 2.89         |
| 4h   | −4.73          | N18          | OG1-THR200     | HBA             | 3.33         |
|      |                | O19          | HOH270         | HBA             | 2.38         |
| 4a   | −4.44          | O19          | ND2-ASN62      | HBD             | 1.89         |
|      |                | N18          | OD1-ASN67      | HBA             | 2.74         |
| Standard (Acetazolamide) | −4.85 | O10          | ZN             | Metallic        | 2.73         |
|      |                | O11          | OG1-THR199     | HBA             | 3.12         |
|      |                | O11          | OG1-THR-200    | HBD             | 3.60         |

HBA, Hydrogen bond acceptor; HBD, Hydrogen bond donor.

FIGURE 4 | The 3D interaction of the (A) most active compound (4m, shown in gray stick model), (B) moderate active compound (4n, green stick model), (C) least active compound (4a, pink stick model), and (D) standard drug (acetazolamide, shown in cyan sticks) in the active site of bCA-II. The active site residues are depicted in pink stick model. The hydrogen bonds are presented in black lines.

The docked view of the least active compounds (4g, 4h, 4j, 4a, and 4b) showed that quinazolinone carbonyl of 4g interacted with the side chain of Thr198 via H-bond (2.81 Å) and the side chain of Asn66 offered a hydrophobic interaction to the compound (3.50 Å). Whereas, quinazolinone-amide group of 4h mediated a H-bond with the side chain of Thr198...
FIGURE 5 | (A) The docked view of compound 4d is shown in the active site of bCA-II in 3D-form. (B) The binding interactions of 4d in two-dimensional form is demonstrated. (C) The binding modes of all the active compounds are shown. The hydrogen bonds are shown in black lines.

FIGURE 6 | The 3D docked view of the (A) most active compound (4g, showed as lime green stick model), (B) moderate active compound (4c, shown as green stick model) (C) least active compound (4a, shown as sky blue stick model) and (D) standard (acetazolamide shown as yellow stick model) against hCA-II. The active site residues are depicted in cyan stick model. The hydrogen bonds are presented in black lines.
(2.99 Å), and the amide group of 4j interacted with a water molecule (WAT340) present in the active site. Similarly, the carbonyl oxygen and amide nitrogen of compounds 4a and 4b mediated H-bonds with the side chain of Thr197 and Gln91, respectively. Additionally, 4b formed a bond with a water molecule (WAT435). The binding interactions and docking scores of each docked compound are tabulated in Table 3. Figure 4 shows the binding interactions of the most active (4m), moderate (4n), and the least active (4a) compounds, however, a docked view of the most potent compound (4d) and all the compounds are presented in Figure 5.

**Binding Interactions of Active Compounds and Their Predictive SAR Against hCA-II**

Docking analysis deduced that the quinazolinone moieties of these compounds are responsible for the formation of metallic interactions with the Zn\(^{2+}\) atom in the catalytic cavity. Compound 4g (IC\(_{50}\) = 14.0 ± 0.60 μM) was the most potent and selective inhibitor of hCA-II, as compared to standard acetazolamide (IC\(_{50}\) = 19.6 ± 1.23 μM), followed by compounds 4c > 4d > 4o and least active 4e > 4f > 4h > 4a. The quinazolinone moiety of 4g interacted with the side chain of Asn62, Thr200, and Wat270 with bond lengths of 3.34, 2.99, and 2.45 Å, respectively. Similarly, 4c interacted with the side chain of Gln92 and Wat270, whereas 4d was linked with Zn\(^{2+}\) atom through its nitro-oxygen group. The nitro-oxygen and aminooquinoline moiety of 4o interacted with Zn\(^{2+}\) atom and the side chains of Gln92 and Thr200 via hydrogen bonds. However, the quinazolinone moiety of 4e interacted with the Zn\(^{2+}\) ion and Gln92. On the other hand, the least active compounds, 4a, 4f, and 4h, interacted with the side chains of Asn62, Asn67, Thr200, and water molecules within the active site. The detailed binding interactions of active compounds and their docking scores are tabulated in Table 4. The binding interactions of most active (4g), moderate (4c), and least active (4a) compounds in the active site of hCA-II are presented in Figure 6. The docked orientation of all active compounds and the binding interaction of compound 4d are shown in Figure 7.

**CONCLUSION**

Quinazolinone derivatives (4a-p) were synthesized in search of new therapeutic agents for glaucoma and other diseases associated with hyperactivity of CA-II. The in-vitro results showed that these skeletons displayed moderate to significant inhibition of both the enzymes (bCA-II and hCA-II).
Compounds 4c, 4e, 4f, 4l, and 4m showed superior activity against bCA-II, while compounds 4g, 4c, 4d, and 4o were found to be significantly active against hCA-II. The structure-activity relationship reflected that the nitro group on phenyl ring at R position plays an important role in the overall inhibitory activities of compounds. Among the tested compounds, 4g and 4o are more selective for hCA-II. Moreover, kinetics studies showed the competitive behavior of inhibition of this series. Additionally, molecular docking predicted that the compounds bind efficiently with Zinc ion and several residues within the active site, therefore, through appropriate fitting and binding, these compounds effectively inhibit both bCA-II and hCA-II enzymes.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article-supplementary material, further inquiries can be directed to the corresponding author/s.

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**AUTHOR CONTRIBUTIONS**

AA-H and ZS conceived and designed the study. ZK synthesized all compounds. AK and MK performed *in-vitro* assay. MK and SH performed the computational studies and analyzed the data. AK wrote the manuscript with input and comments from MK, SH, ZK, ZS, and AA-H. All authors have read and approved the final version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with one of the authors with the authors AK, SH, and AA-H.

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