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RESEARCH ARTICLE

Kinetic and docking studies of cytosolic/tumor-associated carbonic anhydrase isozymes I, II and IX with some hydroxylic compounds

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Abstract

A series of hydroxylic compounds (**1–10**, **NK-154** and **NK-168**) have been assayed for the inhibition of three physiologically relevant carbonic anhydrase isozymes, the cytosolic isozymes I, II and tumor-associated isozyme IX. The investigated compounds showed inhibition constants in the range of 0.068–4003, 0.012–9.9 and 0.025–115 μm at the hCA I, hCA II and hCA IX enzymes, respectively. In order to investigate the binding mechanisms of these inhibitors, *in silico* studies were also applied. Molecular docking scores of the studied compounds are calculated using scoring algorithms, namely Glide/induced fit docking. The inhibitory potencies of the novel compounds were analyzed at the human isoforms hCA I, hCA II and hCA IX as targets and the K_I values were calculated.

Introduction

Phenol (**1**) is the specific name for hydroxybenzene. It is an important industrial commodity as a precursor to many materials and useful compounds. Phenol and its chemical derivatives are key for building polycarbonates, detergents, herbicides and numerous pharmaceutical drugs[1]. Catechol (**2**) is a natural organic compound with the formula 1,2-dihydroxybenzene. Resorcinol (**3**) and pyrogallol (**4**) are organic compounds. They also have antiseptic properties. Salicylic acid (**5**) is a beta-hydroxybenzoic acid. Salicylic acid and its derivatives are widely used for the treatment of various diseases. *o*-coumaric acid (**6**) is a 2-hydroxycinnamic acid, and it can be found in vinegar[2,3]. Dobutamine (**7**) is used to treat acute but potentially reversible heart failure, such as cardiac surgery or cases of septic or cardiogenic shock, on the basis of its positive inotropic action[4]. Curcumin (**8**), the yellow pigment isolated from the root of the plant *Curcuma longa* L., has several biological effects, the most important being its anticarcinogenic effect. Curcumin is a nutraceutical compound reported to possess therapeutic properties against a variety of diseases ranging from cancer to cystic fibrosis[5,6].

The carbonic anhydrases (CAs, EC 4.2.1.1) are zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide in a two-step reaction, to yield bicarbonate and protons[7]. Among the 16 isoenzymes described up to now in mammalian organisms, CA I and CA II are present at high concentrations in the cytosol of erythrocytes and the gastrointestinal tract[7–11]. CA IX is found in tumor cells and absent (or are present in very limited amount) in normal tissues[12–15]. CA isoenzymes constitute interesting targets for the design of pharmacological agents that are useful in the treatment or prevention of a variety of disorders such as glaucoma, epilepsy, as diuretics or antitumor agents/diagnostic tools[7,14].

Many hydroxy compound derivatives have been widely used as prodrugs or drugs. For instance, paracetamol is a widely used over-the-counter analgesic and antipyretic. Another hydroxy group containing molecule, amoxicillin is antibiotic. Our group recently investigated the interaction of CA isozymes I, II with salicylic acid derivatives, antioxidant phenolic compounds, hydroxy group containing compounds, etc.[2,3,6–11,16] We wanted to extend these earlier investigations to some hydroxy and phenolic compounds in order to discover novel powerful CA inhibitors (CAIs) which might have implications in medicine[6–11,16].

In this work, a series of hydroxylic compounds (**1–10**, **NK-154** and **NK-168**) have been assayed for the inhibition of CA I, II and IX isozymes. Inhibition is reported as K_I (μm) and the results are the average of at least three independent experiments. Since X-ray structures of these small molecules with CA targets are not yet available, *in silico* studies are performed for the investigation of their binding mechanisms.

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Experimental

All chemicals were obtained commercially from Sigma-Aldrich (Bornem, Belgium). IR spectra were recorded on a PerkinElmer
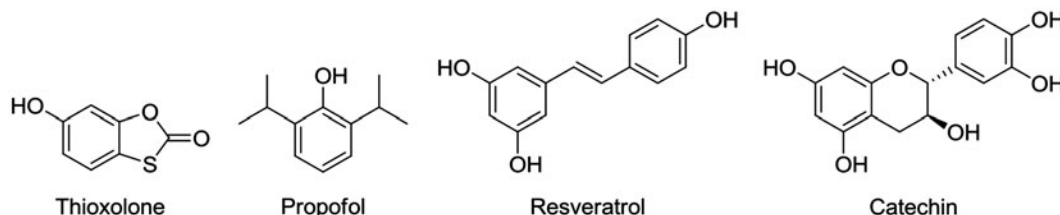
Figure 1. Chemical structures of some carbonic anhydrase inhibitor phenolic compounds.

100 FTIR spectrometer and all ^{1}H-NMR and ^{13}C-NMR spectra were recorded using an Oxford NMR 400 MHz spectrometer using TMS as the internal standard *d* values in ppm at ambient temperature. Melting points were measured on the variable heater.

Synthesis of NK-154 and NK-168

Detailed synthetic procedures for the preparation of N-(1-(2-hydroxyphenylamino)-3-methyl-1-oxobutan-2-ylcarbamothioyl) benzamide (**NK-154**) and N-(1-(2-hydroxyphenylamino)-4-methyl-1-oxopentan-2-ylcarbamothioyl) benzamide (**NK-168**) can be found in 17.

Purification of human carbonic anhydrase isozymes by affinity chromatography

Erythrocytes were purified from fresh human blood obtained from the Blood Center of the Research Hospital at Atatürk University. The blood samples were centrifuged at 1500 rpm for 15 min and the plasma and buffy coat were removed. The red cells were isolated and washed twice with 0.9% NaCl and hemolyzed with 1.5 volumes of ice-cold water. The ghost and intact cells were removed by centrifugation at 20 000 rpm for 30 min at 4 °C. The pH of the hemolysate was adjusted to 8.7 with solid Tris^2. First, benzoyl chloride was stirred for 4 h at room temperature in CH_2Cl_2 cellulose. After the spacer arm cellulose added as a benzyl group and finally diazotized sulfanilamide clamped to the para position of benzyl group as a ligand. The hemolysate was applied to the prepared cellulose-benzyl-sulfanilamide affinity column equilibrated with 25 mm Tris-HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mm Tris-HCl/22 mm Na_2SO_4 (pH 8.7). The human carbonic anhydrase (hCA I and hCA II) isozymes were eluted with 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), respectively. All procedures were performed at 4 °C.

CA IX purification protocol has been used for obtaining high amounts of hCA IX needed in these experiments. The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al.)^{17} was amplified by using PCR and specific primers for the glutathione S-transferase (GST)-Gene Fusion Vector pGEX-3X. The obtained fusion construct was inserted in the pGEX-3X vector and then expressed in *Escherichia coli* BL21 Codon Plus bacterial strain (from Stratagene, San Diego, CA). The bacterial cells were sonicated, then suspended in the lysis buffer (10 mm Tris pH 7.5, 1 mm EDTA pH 8, 150 mm NaCl and 0.2% Triton X-100). After incubation with lysozyme (approx. 0.01 g/l), the protease inhibitors Complete™ were added to a final concentration of 0.2 mm. The obtained supernatant was then applied to a prepacked glutathione sepharose 4B column, extensively washed with buffer and the fusion (GST-CA IX) protein was eluted with a buffer consisting of 5 mm reduced glutathione in 50 mm Tris-HCl, pH 8.0. Finally, the GST part of the fusion protein was cleaved with thrombin. The obtained CA IX was further purified by sulfonamide affinity chromatography.

CA inhibition

An applied photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity^{18}. Phenol red (at a concentration of 0.2 mm) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mm Hepes (pH 7.5) as buffer and 20 mm Na_2SO_4 (for maintaining a constant ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mm for the determination of the kinetic parameters and inhibition constants (five different substrate concentrations have been used). For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mm) were prepared in distilled-deionized water and dilutions up to 0.01 nm were done thereafter with distilled-deionized water. Experiments were done using six different inhibitor concentrations, varying from 100 pM to 0.1 nm. Inhibitor and enzyme solutions were preincubated together for 15 min to 24 h at room temperature (15 min) or 4 °C (all other incubation times) prior to assay, in order to allow for the formation of the E-I complex or for the eventual active site-mediated hydrolysis of the inhibitor. Data reported in Table 1 shows the inhibition after 15 min incubation, as there were no differences of inhibitory power when the enzyme and inhibitors were kept for longer periods in incubation^{19,20}. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier^{18,20}, and represent the mean from at least three different determinations. The three CA isozymes used in the experiments were recombinant ones, obtained as reported earlier$^{19–21}$.

In silico docking studies

Glide/SP and Glide/XP docking protocols are applied for the prediction of topologies as well as binding energies (i.e. docking scores) of used compounds at the active sites of CA I, CA II and CA IX enzymes. The active site of the enzyme is defined from the cocrystallized ligands from Protein Data Bank (PDB) files. Crystal structures of hCA I, II and IX are retrieved from PDB server (PDB codes: 2FW4, 5AML and 3IAI), respectively$^{22–24}$. Throughout the docking simulations, both partial flexibility and full flexibility around the active site residues are performed by Glide/SP/XP and induced fit docking (IFD) approaches^{25}. IFD procedure was established in three consequence stages which involve: (i) docking of all ligands into the generated model, (ii) refining of amino acid residues within 4 Å of docked poses and (iii) re-docking of docked ligands against the refined protein.

Results and discussion

Phenolic and polyphenolic compounds have in fact been evaluated as CAIs by our group earlier, being shown that many such derivatives are potent inhibitors of several physiologically

relevant isoforms, such as CA I and II[8–11,16]. However, no X-ray crystallographic characterization of such adducts has been performed yet, the polyphenolic compounds being the first simple phenol (**1**) for which the X-ray crystal structure in complex with hCA II has been reported by Christianson's group[26]. The rationale of investigating phenols as CAIs is due to the fact that the simple phenol (**1**) has been shown to be the only competitive inhibitor with CO_2 as a substrate for the main isoform of CA, i.e. human CA II (hCA II)[26]. Our group investigated the interactions of a few simple phenols (**1–5**), antioxidant phenolic compounds, some natural product and polyphenolic compounds with some mammalian isozymes (Figure 1)[21,27–32]. Results for these molecules were in low micromolar/submicromolar inhibitions and derived molecules may assist to design isozyme selective CAIs. Indeed, the inhibition profile of various isozymes

with this class of agents was diverse, with inhibition constants ranging from the millimolar to the submicromolar for many simple phenols[19].

We report here the first study on the inhibitory effects of compounds **NK-154** and **NK-168** on the CO_2 hydration activity of hCA I, II and IX. These compounds were docked at the binding site of the targets (hCA I, hCA II and hCA IX). Glide SP/XP docking scores of docked inhibitors at hCA I, II and IX targets and corresponding binding interactions were tabulated in Table 1. The previous reports by Oztürk Sarikaya et al.[3] investigated pyrogallol (**4**) by using an esterase assay, for monitoring CA inhibition. Data of Table 1 show the following regarding inhibition of hCA I, II and IX with compounds **1–10**, **NK-154** and **NK-168**, by using a stopped-flow, CO_2 hydration assay for monitoring CA inhibition (Figure 2 and Table 1).

Against the slow cytosolic isozyme hCA I, catechol and resorcinol behave as moderate inhibitors, with K_I values in the range of 795–4003 μm, similarly to the structurally related compounds **1**, **4** and **5** (K_Is of 7.41–10.2 μm). It is interesting to note that the compounds **NK-154** and **NK-168** were much better hCA I inhibitors as compared to the corresponding compounds **6**–**10** from which they were prepared. Kinetic investigations (Lineweaver–Burk plots, data not shown) indicate that similarly to sulfonamides and inorganic anions[33–39], all the investigated natural compounds act as competitive inhibitors with CO_2 as

Table 1. K_I values for the *in vitro* inhibition of hCA I, hCA II and IX with compounds **1–10**, **NK-154**, **NK-168** and **AZA**.

Inhibitor	K_I (μM)*		
	hCA I	hCA II	hCA IX
Phenol (**1**)[a]	10.2	5.50	8.80
Catechol (**2**)[a]	4003	9.9	115
Resorcinol (**3**)[a]	795	7.7	69.7
Pyrogallol (**4**)[b]	7.41	0.54	–
Salicylic acid (**5**)[a]	9.9	7.1	78.7
o-Coumaric acid (**6**)[c]	3.1	9.2	>1000
Dobutamine (**7**)[c]	1.92	0.48	9.82
Curcumin (**8**)[c]	2.41	0.38	4.05
NK-154	0.074	>1000	0.481
NK-168	0.068	>1000	0.490
9[d]	8.03	3.83	7.15
10[d]	3.34	350	21
AZA[a]	0.25	0.012	0.025

*Mean from at least three determinations. Errors in the range of ~2% of the reported value (data not shown).
[a]From ref. 19.
[b]From ref. 3.
[c]From ref. 21.
[d]From ref. 20.

Table 2. Docking score values of selected ligands against hCA I, hCA II and hCA IX.

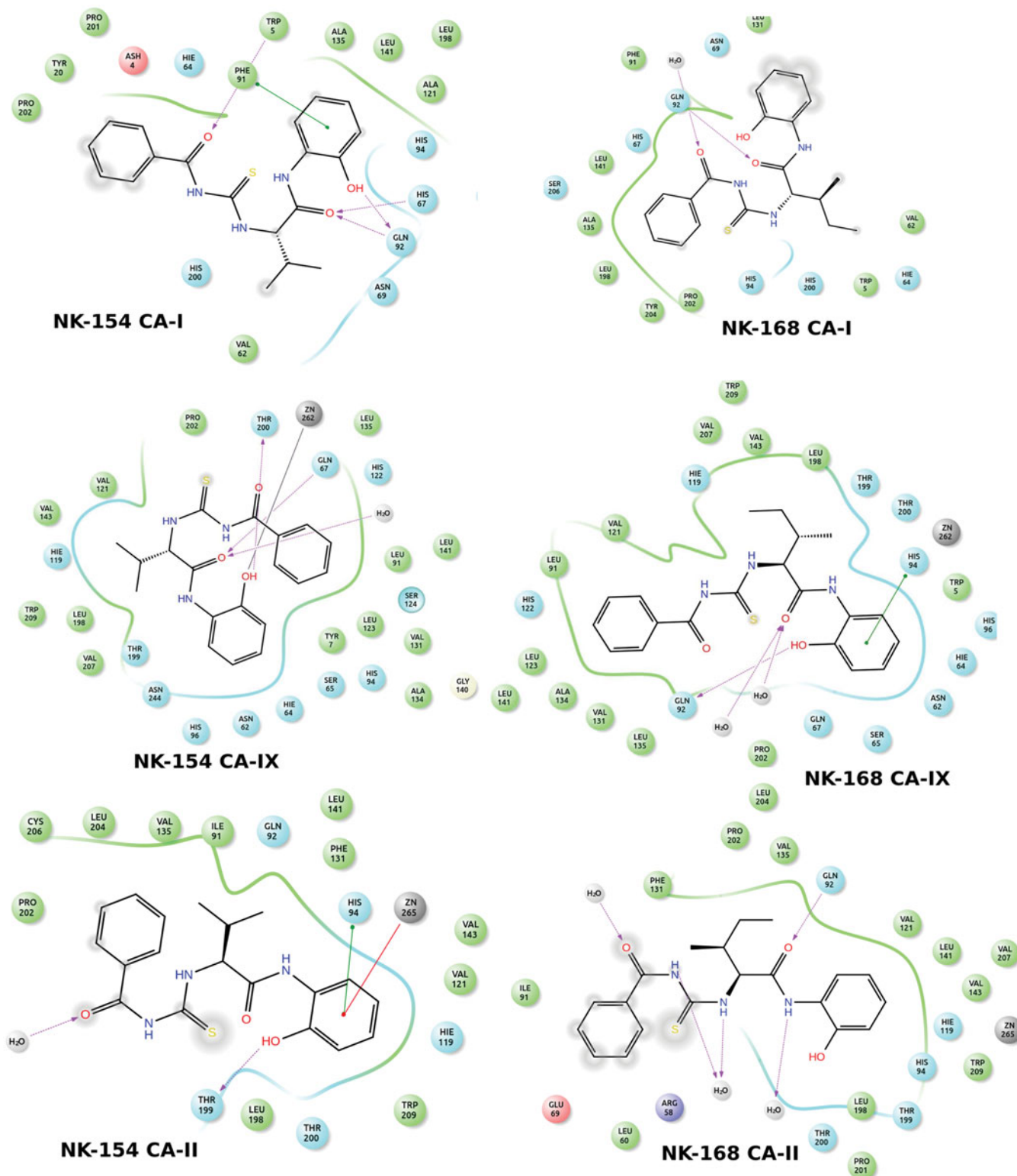| Compound	hCA I IFD results (kcal/mole)	hCA II IFD results (kcal/mole)	hCA IX IFD results (kcal/mole)
Phenol (**1**)	−5.36	−5.72	−5.62
Catechol (**2**)	−6.04	−6.28	−5.78
Resorcinol (**3**)	−6.01	−6.74	−7.24
NK-154	−7.87	−8.69	−9.87
NK-168	−7.19	−9.02	−9.82
AZA	−5.82	−7.85	−7.39

Figure 2. Chemical structures of tested compounds.
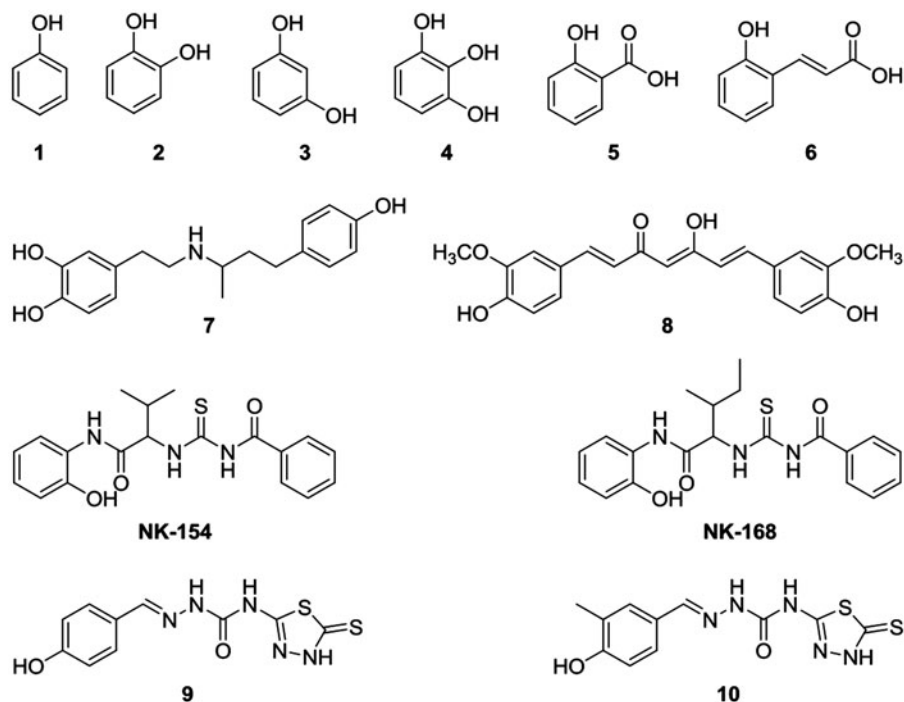
Figure 3. 2D docking poses of **NK-154** and **NK-168** into the hCAI, hCA II and hCAIX receptors. Polar, hydrophobic and charged domains were highlighted by different colors as shown in diagram legend.

substrate (i.e. they bind in different regions of the active site cavity as compared to the substrate).

A better inhibitory activity has been observed with compounds dobutamine and curcumin investigated here for the inhibition of the rapid cytosolic isozyme hCA II (Table 1). Structure-activity relationship is thus quite sharp for this polyphenolic series of hydroxylic compounds: the N-(1-(2-hydroxyphenylamino) containing compounds **NK-154** and **NK-168** are ineffective leads, with monophenol ring-containing compounds **1–3**, **5** and **6** is

already a submicromolar hCA II inhibitor. The best hCA II inhibitor in this compounds was referans sulfonamide compound **AZA**, which with a K_I of 0.012 μm.

In addition to partial flexible docking simulation, flexible receptor docking at the active site was also carried out to enhance the induce fitting treatment of amino acid residues within 4 Å of the ligand. The IFD results were tabulated in Table 2 for CAI, II and IX receptors (top docking scores were considered). Revealed binding energies from IFD simulations are lower (i.e. higher
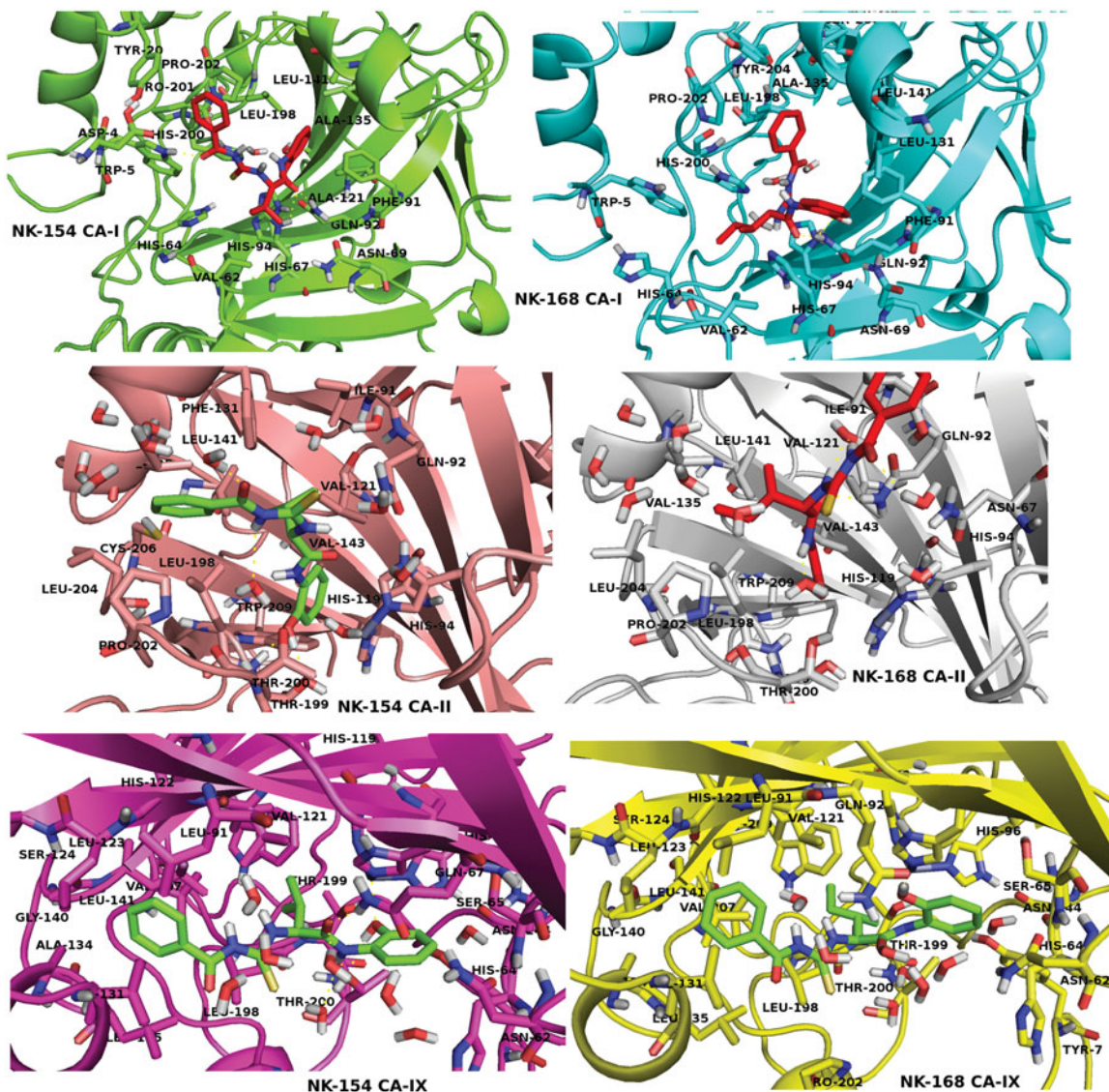
Figure 4. 3D docking poses of **NK-154** and **NK-168** into the hCAI, hCA II and hCA IX receptors. Key amino acid residues were determined in the 3D positions in active sites.

docking scores) than the results of Glide/SP and XP results. In the case of compounds **NK-154** and **NK-168**, lowest binding energies against CAI, II and IX receptors were connected to these ligands. 2D and 3D ligand binding interaction diagrams of docking poses of both novel inhibitors into the ligand binding site of CAI, II and IX receptors were represented in Figures 3 and 4. These ligand diagrams clearly identify key amino acid residues which participate in ligand-binding interactions for both receptors. Hydrophobicity and polarity of binding pockets were identified for each complex on diagrams. H-bonding and π–π stacking interactions were displayed by red and blue lines, respectively. Zinc metal ion in all complexes was located in the active site and the neighborhood of inhibitor. The His94 amino acid residue was constrained in all complexes, which plays a critical role in CAI and II inhibitory processes. In the case of CA-I, Gln92 amino acid residue forms an H-bond with the hydroxyl group of compounds **7** and **8**. In the case of CA-II, Thr200 has participated in ligand-receptor interaction and hydroxyl groups of compounds **7** and **8** form H bonds. Figures 3 and 4 display 3D docking poses of ligands **7** and **8** at the binding pockets of CAI and II, respectively. In order to determine active sites and orientations of ligands, positions of the docked ligand were zoomed graphically for each complex and the corresponded residues around 4 Å of ligands

were labeled. According to the superimposition data of the complexes, in individual CAI, II and IX receptors, both inhibitors convulsively have shared the same positions in ligand-binding pockets.

In a recent study, it was reported that catechol (**13**) and resorcinol (**14**)[18] as simple compounds which they not involve sulfonamide, sulfamate or related functional groups and these compounds are found as CAIs, and could represent the starting point for a new class of inhibitors that may have advantages for patients with sulfonamide allergies[18,28]. The sulfonamide zinc-binding group is thus superior to the thiol one (from the thioxolone hydrolysis product) for generating CAIs with a varied and sometimes isozyme-selective inhibition profile against the mammalian enzymes. However, it is critically important to explore further classes of potent CAIs in order to detect compounds with a different inhibition profile as compared to the sulfonamides and their bioisosteres and to find novel applications for the inhibitors of these widespread enzymes.

Conclusion

Compounds **NK-154** and **NK-168** used in this study affect the activity of CA isozymes due to the presence of the different

functional groups (OH, phenyl, benzyl and methyl) present in their aromatic and non-aromatic scaffold. Our findings here indicate thus another class of possible CAIs of interest, in addition to the well-known sulfonamides/sulfamates/sulfamides. Indeed, some hydroxylic compounds investigated here showed effective hCA I and hCA IX inhibitory activity, in the low micromolar range, by the CO_2 hydrase method which usually gives K_I-s. These findings point out that substituted hydroxylic compounds may be used as leads for generating potent CAIs eventually targeting other isoforms which have not been assayed yet for their interactions with such agents.

Declaration of interest

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