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Ligand-based and structure-based virtual screening to identify carbonic anhydrase IX inhibitors

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ABSTRACT

A three-dimensional pharmacophore model of CA IX inhibitors was generated and used to screen the ZINC database of commercially available compounds. The hits were docked in a CA IX homology model. By visualizing the binding mode and score of these compounds, six derivatives were selected and evaluated for their inhibitory potency against CA IX. A highly active CA IX inhibitor was identified which may be used as a lead to design novel such derivatives.

1. Introduction

Tumor hypoxia is known to be a crucial feature of the tumor microenvironment, being the result of uncontrolled tumor growth outpacing the rate of vascular proliferation and of architecturally defective microcirculation. This process compromises oxygen diffusion to tumor cells adjacent to neovessels.[1] Hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor activated by hypoxia, triggers the expression of several proteins such as among others carbonic anhydrase IX (CA IX) which is involved in the acid–base regulation of the microenvironment.[2,3] The CA IX expression is dramatically increased in a variety of human tumor, whilst it is low in normal tissues.[4,5] Expression of CA IX in tumors is associated with poor prognosis, tumor progression and aggressiveness suggesting that CA IX is an attractive therapeutic target.[2–4,6]

However, further research is warranted to better understand the exact role of CA IX in cancer.[7] The main goal in the development of CA IX inhibitors is reaching a high selectivity in order to avoid any side effects by inhibiting the other CA isozymes that play physiological roles.[3] Indeed, in human 15 different isozymes have been identified and some of them are involved in a variety of physiological functions such a pH regulation, CO_2 and HCO_3^- transport, production of biological fluids, bone resorption, ureagenesis, gluconeogenesis and lipogenesis.[6]

High-throughput screening of chemical libraries is a well-established method to discover new lead compounds.[8] However, the available databases become larger and their experimental testing is very expensive. Therefore, a small subset of the database which should include promising compounds that likely bind the target should be preferred for experimental screening. This selection can be performed by virtual screening through databases search for molecules fitting a known pharmacophore and/or a three-dimensional structure of the target.

In order to identify new potential leads for CA IX inhibition, we screened the ZINC database which contains commercially available compounds.[9] During this screening, we used several filters such as a pharmacophore model for CA IX inhibitors and the docking of each molecule in the homology model of CA IX previously described.[10] As far as we know, this is the first report on the pharmacophore modeling even the first reported virtual screening study of CA IX inhibitors.

2. Results and discussion

A three-dimensional pharmacophore model of CA IX inhibitors was generated using the software MOE.[11] To form a training set, we selected from literature 10 compounds structurally different and known to strongly and selectively inhibit the CA IX.[12] The

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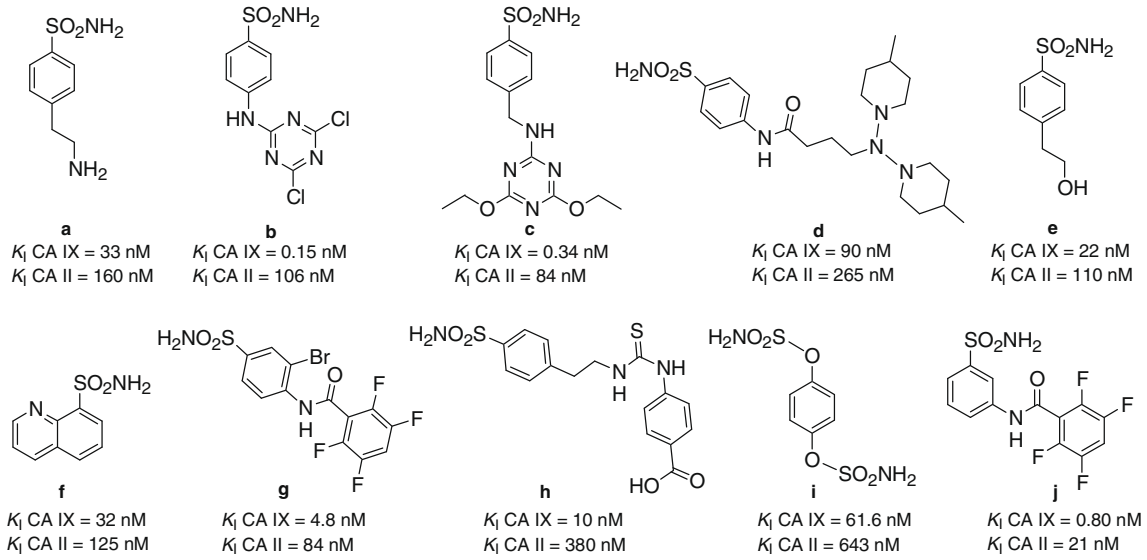

Chart 1. Chemical structures of representative CA IX inhibitors selected for the pharmacophore modeling.

chemical structures as well as experimental K_I values of the training set are given in Chart 1. It may be observed that all inhibitors incorporate a sulfonamide or sulfamate moiety which is essential for the inhibition of CAs.[6]

In order to highlight the common structural features of the training set, a flexible alignment of these compounds was performed following the method implemented in MOE.[11] A score in given to each alignment and allows an evaluation of the alignment quality.

The sulfonamide moiety is known to bind the zinc ion present in the active site and therefore constitutes an important pharmacophore element. We thus imposed during the flexible alignment procedure the alignment of the sulfonamide moiety. The pharmacophore was generated on the first aligned solution which was characterized by the best score and by low internal strain energy. In MOE, pharmacophoric structure features are represented by a point encased in a sphere.[11] The spheres depicts the tolerance of location allowed during database searching. A six-point pharmacophore of CA IX inhibitors was obtained (Fig. 1). The first three points correspond to the zinc binding function which is typically a sulfonamide moiety in many CA inhibitors (sphere #1 radius: 0.52 Å, sphere #2 and #3 radius: 1 Å).[6] Point #4 is characterized by an aromatic ring (radius: 1.3 Å). Points #5 (radius: 1 Å) and #6 (radius: 1 Å) cover various features such as hydrophobic region, H-bond donor or acceptor.

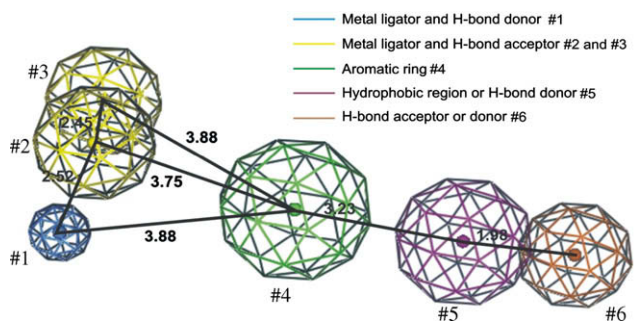

Figure 1. Pharmacophore model obtained by the alignment of selective CA IX inhibitors (distance between spheres are expressed in Å).

The pharmacophore model was used as in silico filter to screen the ZINC database of commercially available compounds. This can be performed by the MOE software. Nevertheless, the size of the database should be restricted as much as possible. A first selection of drugable compounds within the ZINC database was thus conducted (Fig. 2). To the parameters used for the query, the unsubstituted sulfonamide moiety was added as the first three point of the pharmacophore model is dedicated to a metal ligator. 1176 compounds emerged and were loaded in the MOE environment where the Conformation Import methodology was applied to generate low-energy conformations. The conformers of each compound were then filtered by the pharmacophore model. To be considered as a hit the compound has to fit all the features of the pharmacophore.

500 hits were obtained and were docked with the help of the automated GOLD program[13] in the active site of the previously described CA IX model.[10] Finally, we selected a total of 6 compounds from the ranked top 100 compounds, which was based on a visual look of their binding mode as well as on our experience. The selected compounds (Fig. 3) have been shifted to in vitro studies for their inhibitory potency evaluation. Selectivity was assessed by determining the inhibitory activity against the ubiquitous CA I and II isoforms. These CA isozymes are physiologically important and play a role in respiration, electrolyte secretion, acid–base homeostasis, biosynthetic reaction.[6] The inhibition activities together with the K_I ratios are presented in Table 1.

CA IX inhibition activities obtained are ranging from 0.29 to 2.75 μM (Table 1). Three compounds (**2**, **3** and **5**) exhibit similar inhibition potency around 0.30 μM. However, they are characterized by different selectivity profile. The K_I ratios of CA I over CA IX highlight that the six compounds are CA IX selective vs. CA I while the K_I ratios of CA II over CA IX reveal unfortunately no CA IX versus CA II selective compound. Regarding the structure of the six compounds, we observed that compounds **3** and **4** share a common scaffold. However, they exhibit different CA IX inhibition profiles with K_I of 0.29 and 1.07 μM, respectively. This can be explained through the analysis of their different binding mode inside CA IX (Fig. 4). On one hand, **3** directs its side chain towards the specific residues of CA IX active site contained in the hydrophobic pocket (Pro202, Ala204, Leu135, Val131). Its amide link interacts by H-bond with Thr200 (O···NH, distance 2.98 Å). On the other hand, **4** adopts an extended conformation within the CA IX active site with its side chain pointing towards

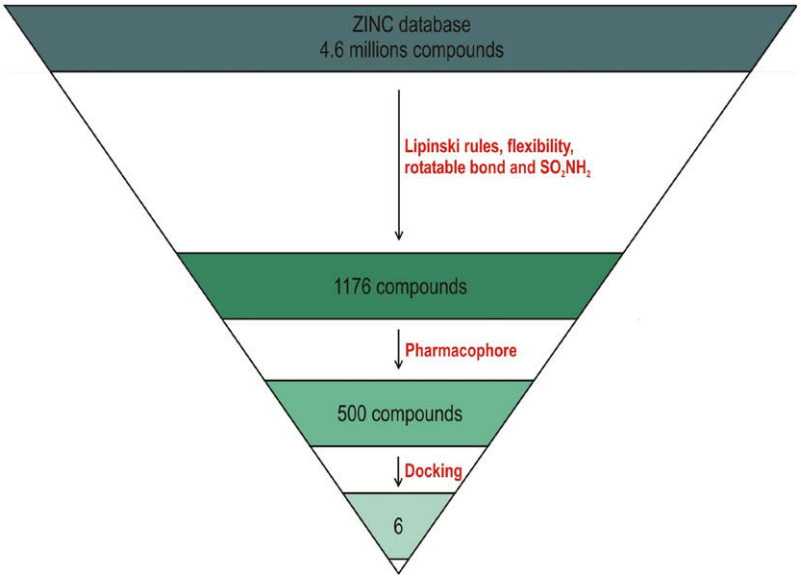

Figure 2. Virtual screening strategy.

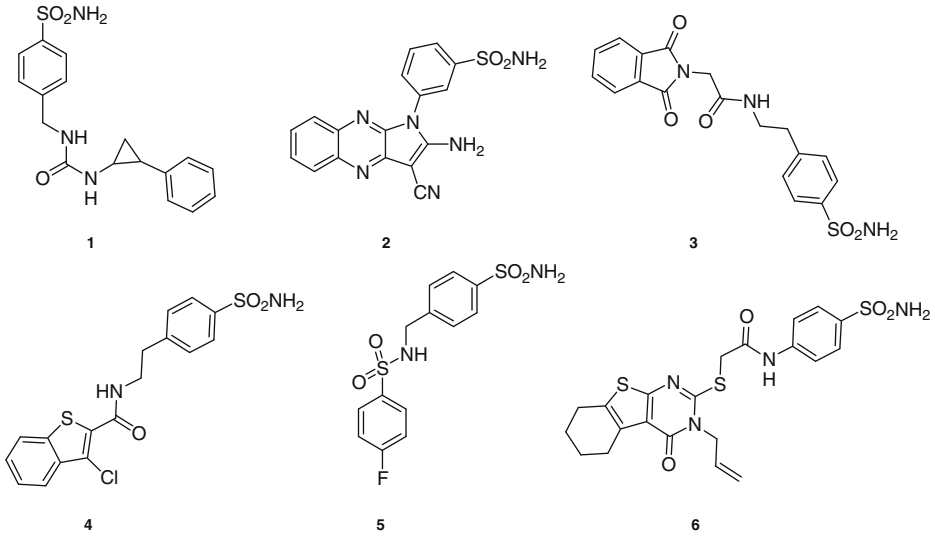

Figure 3. Compounds selected from the virtual screening strategy.

Table 1
Inhibition activities of the reference CA inhibitor acetazolamide (**AZA**) and of the compounds selected by virtual screening

Compounds	K_I (μM)[a]			K_I ratios	
	hCA I	hCA II	hCA IX	K_I hCA I/K_I hCA IX	K_I hCA II/K_I hCA IX
AZA	0.90	0.012	0.025	36	0.48
1	12.0	2.61	1.84	6.5	1.4
2	8.35	0.005	0.30	28	0.018
3	4.23	0.66	0.29	15	2.3
4	8.10	0.051	1.07	7.5	0.048
5	0.87	0.10	0.29	3.0	0.36
6	15.0	0.33	2.75	5.4	0.11

[a] Errors in the range of 5–10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[13]

the hydrophilic pocket (Gln67, Gln92). These two binding modes can explain the differences in the inhibition activities observed. Moreover, Tuccinardi et al. showed that both lipophilic and electrostatic interactions play fundamental role in determining the ligand affinity.[7] In our case, we observed a better inhibition activity for compound **3** that interacts with CA IX by means H-bond whereas for compound **4** no particular interaction were observed and its inhibition activity was found weaker.

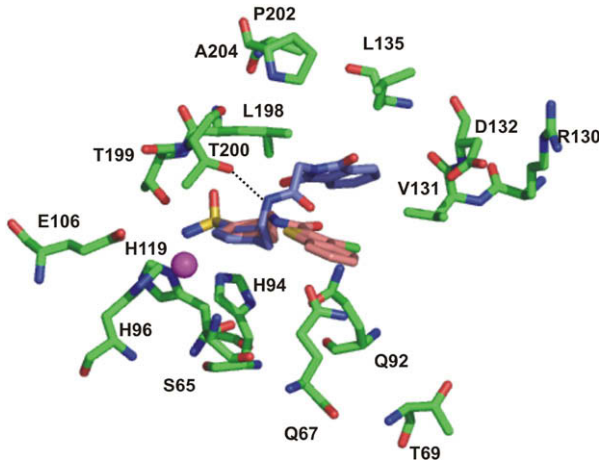

Figure 4. Binding model of **3** (blue) and **4** (pink) in CA IX. H-bond is indicated by dotted lines.[14]

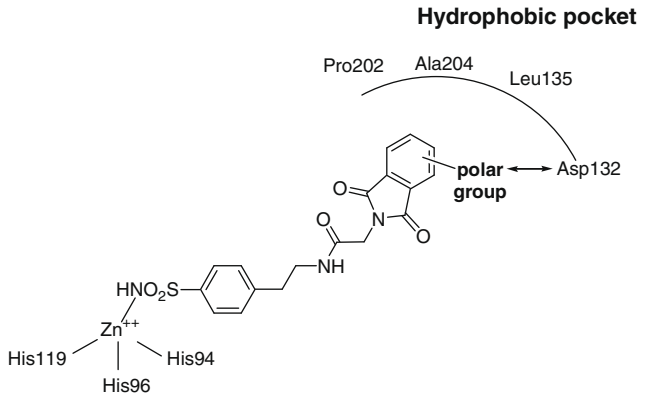

Figure 5. Pharmacomodulation suggestion on compound **3** to enhance the CA IX selectivity by targeting Asp132.

3. Conclusions

We report here an indirect approach of drug design conducted to highlight new lead compounds that might be used for the design of CA IX inhibitors. The ZINC database was screened through drug-like filters, a six-point pharmacophore of CA IX inhibitors and by docking compounds inside the active site of a CA IX model. From this study, we identified compound **3** as being an active CA IX inhibitor (K_I CA I = 4.2 μM, K_I CA II 0.6 μM and K_I CA IX = 0.3 μM). CA IX selectivity versus CA I is reached while CA IX selectivity versus CA II is not obtained. The isoindole dione moiety of compound **3** is directed towards the specific residue Asp132 only present in CA IX. We therefore suggest substituting a polar or positively charged group on this moiety to interact favorably with Asp132 in order to enhance the CA IX selectivity (Fig. 5). The synthesis and the results of the CA inhibition of such new compounds will be reported in the near future.

4. Experimental

4.1. Molecular modeling

4.1.1. Pharmacophore modeling

The flexible alignment available in MOE is a stochastic procedure that simultaneously searches the conformation space of a collection of molecules and the space of alignments of those molecules. The forcefield used was MMFF94x, the iteration limit and the failure limit was set up to 20 and the energy cutoff to 10 kcal mol^{-1}. Each alignment generates a score that quantifies the quality of the alignment in terms of both internal strain and overlap of molecular features. To generate the pharmacophore, we used the PCH (polarity-charge-hydrophobicity) scheme. Tolerance parameter, defined by the neighborhood distance threshold, was set up to 1.21 Å and the threshold, which represents the percentage of molecules which have groups of the same feature type, to 89% for points #1 to #4 of the pharmacophore and to 59% for #5 and #6.

4.1.2. Virtual screening

Parameters used for the query of the ZINC database compounds are: $-2 < \log P < +4$; rotatable bonds < 12; H-bond donor < 5, H-bond acceptor < 10; polar surface area < 140; molecular weight < 500; SMARTS code: [ND1]S(=O)(=O).

4.1.3. Docking studies

GOLD is based on a genetic algorithm. It performs docking of flexible ligands into protein with partial flexibility in the neighborhood of the active site. The torsion angles of Ser, Thr and Tyr hydroxyl groups as well as Lys NH_3^+ moieties are optimized during the run so that hydrogen bond formation is favored. The interaction sphere was centered in the active site and delimited by a 12 Å radius. A tetrahedral geometry was imposed on the zinc binding site. The speed-up was set up to three. Five solutions were generated and were ranked by GOLD score. We allowed an early termination of the job if three best solutions were found within a root mean square deviation of 1.5 Å.

4.2. Enzymatic evaluation

An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 5–30 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. 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 with 10–20% of DMSO and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least squares methods using PRISM 3 and represent the mean from at least three different determinations.

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References and notes

1. (a) Dang, C. V.; Semenza, G. L. *Trends Biochem. Sci.* **1999**, *24*, 68; (b) Korkolopoulou, P.; Perdiki, M.; Thymara, I.; Boviatsis, E.; Agrogiannis, G.; Kotsiakis, X.; Angelidakis, D.; Rologis, D.; Diamantopoulou, K.; Thomas-Tsagli, E.; Kaklamanis, L.; Gatter, K.; Patsouris, E. *Hum. Pathol.* **2007**, *38*, 629.

2. Thiry, A.; Dogné, J. M.; Masereel, B.; Supuran, C. T. *Trends Pharmacol. Sci.* **2006**, *27*, 566.
3. Thiry, A.; Supuran, C. T.; Masereel, B.; Dogné, J. M. *J. Med. Chem.* **2008**, *51*, 3051.
4. Pastorekova, S.; Kopacek, J.; Pastorek, J. *Curr. Top. Med. Chem.* **2007**, *7*, 865.
5. Pastorekova, S.; Parkkila, S.; Zavada, J. *Adv. Clin. Chem.* **2006**, *42*, 167.
6. Supuran, C. T. *Nat. Rev. Drug Discovery* **2008**, *7*, 168.
7. Tuccinardi, T.; Ortore, G.; Rossello, A.; Supuran, C. T.; Martinelli, A. *J. Chem. Inf. Model.* **2007**, *47*, 2253.
8. Höltje, H.-D.; Sippl, W.; Rognan, D.; Folkers, G. In *Molecular Modeling: basic principles and applications*. In *Wiley-VCH*; Weinheim, 2003.
9. Irwin, J. J.; Shoichet, B. K. *J. Chem. Inf. Model.* **2005**, *45*, 177.
10. Thiry, A.; Ledecq, M.; Cecchi, A.; Dogné, J. M.; Wouters, J.; Supuran, C. T.; Masereel, B. *J. Med. Chem.* **2006**, *49*, 2743.
11. ChemicalComputingGroup *MOE: Molecular Operating Environment*, versions 2005.06 ed.
12. (a) Garaj, V.; Puccetti, L.; Fasolis, G.; Winum, J. Y.; Montero, J. L.; Scozzafava, A.; Vullo, D.; Innocenti, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5427; (b) Turkmen, H.; Durgun, M.; Yilmaztekin, S.; Emul, M.; Innocenti, A.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 367; (c) Vullo, D.; Franchi, M.; Gallori, E.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1005; (d) Vullo, D.; Scozzafava, A.; Pastoreková, S.; Pastorek, J.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2351; (e) Vullo, D.; Steffansen, B.; Brodin, B.; Supuran, C. T.; Scozzafava, A.; Nielsen, C. U. *Bioorg. Med. Chem. Lett.* **2006**, *14*, 2418; (f) Winum, J. Y.; Innocenti, A.; Nasr, J.; Montero, J. L.; Scozzafava, A.; Vullo, D.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2353.
13. Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. *J. Mol. Biol.* **1997**, *267*, 727.
14. DeLano, W.L. In *The PyMOL Molecular Graphics System*; DeLano Scientific: Palo Alto, CA, USA.