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Discovery of New Potential Anti-Infective Compounds Based on Carbonic Anhydrase Inhibitors by Rational Target-Focused Repurposing Approaches

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Discovery of New, Potential Anti-infective Compounds based on Carbonic Anhydrase Inhibitors by Rational-target-focus Repurposing Approaches

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Abstract:	In academia compound-recycling represents an alternative drug discovery strategy to identify new pharmaceutical targets for a library of chemical compounds available in-house. Herein, we report the application of a rational target-based drug repurposing approach to find diverse applications for our in-house collection of compounds. The Carbonic Anhydrase (CA, EC 4.2.1.1) metalloenzyme superfamily was identified

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Discovery of New, Potential Anti-infective Compounds based on Carbonic Anhydrase Inhibitors by Rational-target-focus Repurposing Approaches

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- **Abstract**

In academia compound-recycling represents an alternative drug discovery strategy to identify new pharmaceutical targets for a library of chemical compounds available in-house. Herein, we report the application of a rational target-based drug repurposing approach to find diverse applications for our in-house collection of compounds.

The Carbonic Anhydrase (CA, EC 4.2.1.1) metalloenzyme superfamily was identified as a potential target of our compounds. The combination of a thoroughly validated docking screening protocol, together with *in vitro assays* against different CA families and isoforms, allowed us to identify two unprecedented chemotypes as CA inhibitors (CAIs). The identified compounds have the ability to preferentially bind pathogenic (bacterial/protozoan) CAs over the human ones, representing excellent hits to be further optimized in hits-to-lead campaigns.

- **Introduction**

Drug-repurposing or repositioning (DR) denotes an ensemble of tasks aimed at the identification of new drug indications for existing drugs,[1] and is an emerging alternative strategy in drug discovery program, in both pharma and academia fields.

In academia, DR can be also translated into compound-recycling (CR) that is the repurposing of compound library collections already available in-house.[1–3] Indeed, small-molecules already synthetized, that resulted inactive against a target of interest, can be tested on other targets, leading to a new-purpose for an old molecule. Potential new targets for unemployed compounds can be identified through the cross analysis of the fragments composition of the whole compound library with the data available in literature for a specific class of protein (target focus approach).[1,4–6] Indeed, in academia CR approaches have found great application, leading to the development of several successful repurposing strategies.[2,3]

In this *scenario* we embarked in a project aimed at the repurposing of the compound libraries available in-house, looking for new potential applications for our compounds.

Therefore, we proceeded to the analysis of the fragments and chemotypes present in our libraries by applying the Maximum Common Substructure (MCS) decomposition approach.[7,8] The analysis of the data available in literature, for similar classes of chemical structures, allowed us to identify the Carbonic Anhydrase (CA, EC 4.2.1.1) metalloenzyme family as a potential target of some of our compound series. Prompted by these results, we set up and thoroughly validated a docking protocol against all the CA classes and isoforms so far crystallized. Such a method allowed us to identify eleven compounds as potential CA inhibitors (CAIs). The compounds were, therefore, tested *in*

vitro for their ability to inhibits different classes and isoforms of the CA superfamily, leading to the discovery of a series of CAIs active in the low μM range, but characterized by: (*i*) two unprecedented chemotypes as CAIs; (*ii*) an unprecedented selectivity profile for this class of molecules, with the ability to preferentially bind microbial CAs over the human ones; and (*iii*) good Ligand Efficiency and Binding Efficency Indexes (BEI)[9] with respect to that of marketed CAIs. Overall, the newly reported CAIs represent interesting hits, which can be further optimized as potential anti-microbial agents.

- **Results and Discussion**

Repurposing of the in-house 3-D chemical library. The knowledge of the main chemotypes/fragments present in a chemical library can direct the identification of new potential targets for compounds in that library. Therefore, we extrapolated and analysed the main fragments present in our in-house chemical library, composed of 12 main fragments/chemotypes, by applying the MCS approach.[7,8] The analysis reveals that, among others, the most abundant fragments are 2-aminothiazole (**1**), 2-phenylcyclopropane-1-amino (**2**), 2-hydroxypyridinium (**3**), pyridine-N-oxide (**4**), cyclopropan-carboxylic acid (**5**), and benzoic acid (**6**) (Chart 1). Taking into account that most of the compounds available in our lab were designed as anti-microbial agents[10–14] or inhibitor of metalloenzymes,[15] we decided to focus our attention on all those targets, which satisfied the following criteria: (*i*) are metalloenzymes ubiquitously expressed in most living organisms, and (*ii*) there are reported inhibitors characterized by fragments similar to those present in our library. An extensive knowledge-based literature search allowed us to identify the carbonic anhydrase superfamily as an eligible protein class.[16–20] Indeed, sulphonamides are the most common CAIs[16,18] (similar to fragment **12**, in Chart 1), but recently other CAIs chemotypes were disclosed such as 2-aminothiazole[16,18] (similar to **1**), phenols[21] (similar to **3**), hydroxypyridine(thi)ones[22,23] (similar to **4**), benzoic acid[2] (similar to **5**,**6** and **8**), coumarins[24,25](similar to **7**) and polyamines[26] (not present in our database).

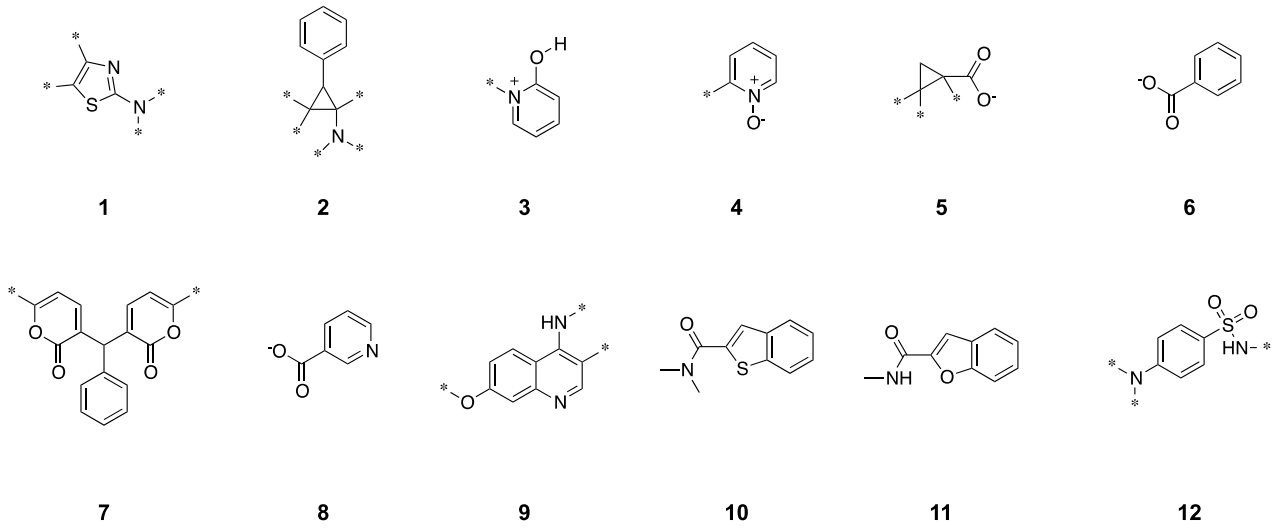


Chart 1. List of the main fragment identified in our compounds collection library, asterisks highlight the main point of substitutions for the reported scaffold.

Encouraged by these findings we set up a computational protocol aimed at the identification of potential CAIs among our compounds collection. To this aim, we collected all the CA X-ray crystal structures available in the PDB from different organisms, families, and isoforms (about 750 structures). A docking protocol was designed and optimized in a stepwise pipeline, aiming at: (*i*) identify the best docking parameters (by means of the re-docking procedure, see Supporting Information), (*ii*) identify the CA X-ray crystal structures more suitable for our docking purposes, and finally (*iii*) reliably screen the in-house collection library. Docking studies were performed by means of Glide and during the validation steps of the docking procedure we observed that several CA X-ray crystal structures have a glycerol molecule into the CA active site, establishing contacts with the co-crystallized ligand (Figure S1). As result, the stripping of the glycerol molecule (during the preparation phase of the docking protocol) led to a bad placement of the co-crystallised inhibitors during the re-docking phases (See Supplementary Information for details). Thus, all the CA X-ray crystal structures having co-crystallized molecules bound within the active site were discharged from further analysis. Once identified the best docking parameters and the proper X-rays dataset, we screened the compounds collection, using, when possible, more than one X-ray crystal structure per CA isoform, in order to include side chains and protein flexibilities in our calculations. In Figure 1 the heat maps scores for both human isoforms (Figure 1a) and microbial families (Figure 1b) are depicted. From the analysis of Figure 1a it can be noticed that: (*i*) several compounds have good Glidescores (> than 7.5 ± 0.1 Kcal/mol, dark blue) against all but *h*CA III isoforms (*h*CA III inset in Figure 1), apparently, *h*Ca III is not druggable by our chemotypes; (*ii*) some compounds display a preferential interaction with specific conformation of the same *h*CA isoform. For example, compound C_1 (Inset in Figure 1), shows good scores (> than 7.5 ± 0.1

Kcal/mol, dark blue) only for a reduced number of *h*CA II conformations, thus supporting the observation that small conformational changes into the CA active site can have important effects on the docking results.

When docked on the microbial CAs (Figure 1b), our compounds generally displayed low Glide scores. This can be explained by the fact that most of such CAs belong to the β-family, which is characterized by a narrower and more solvent-exposed active site (Figure S2). Exceptions are represented by those microbial CAs characterized by the α-family fold-type (i.e. the *Sulfurihydrogenibium* bacterial, highlighted by the black bar and arrow in Figure 1b), which show higher Glidescores with respect to the other ones.

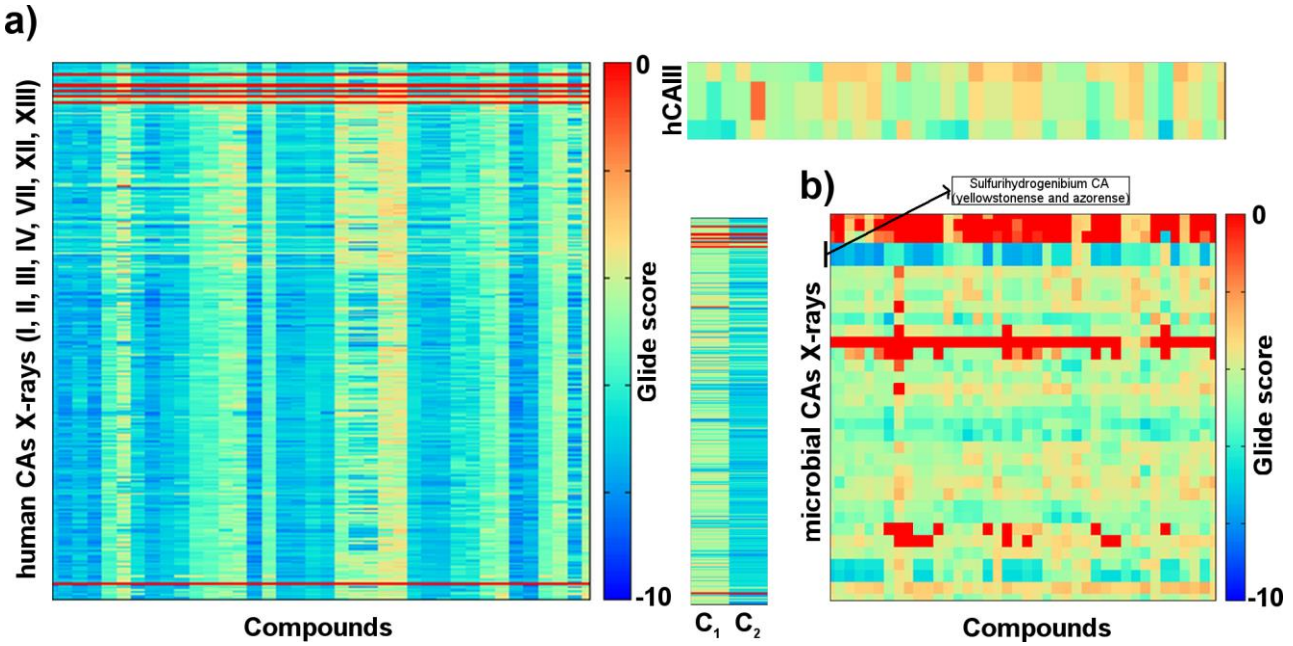


Figure 1. a) Heat map scores for the *h*CAs, the red lines indicate separation between the different *h*CAs in the following order: *h*CA I, II, III, IV, VII, XII and XIII. Highlighted in the insets the scores for the *h*CA III isoform and for two different compounds (C_1 and C_2). **b)** Heat map scores for microbial CAs. Scores are reported as Glide scores in Kcal/mol (colored bar beside the heat map).

In vitro assays against different CA isoforms and families. Accordingly to the preliminary computational observations, we selected a small set of ligands (Chart 2, see Material and Methods for compounds selection strategy) to be tested *in vitro* against three *h*CA isoforms (I, II, and III) and against *Ssp*CA (*Sulfurihydrogenibium yellowstonensis*). Table 1 lists the obtained K_i values for the selected compounds (Chart 2), along with the corresponding Glide scores produced by the docking calculations. The experimental results are in line with our computational prediction and compounds **13**, **15**, **17**, **18** are endowed with μM affinity against to *h*CA I and II. As predicted by our calculation none of the compounds was active against *h*CA III (> 100 μM). Again in line with the docking prediction, the compounds reported in Chart 2 showed K_i values in the low μM range for the bacterial α-family fold-type *Ssp*CA (Table 1).

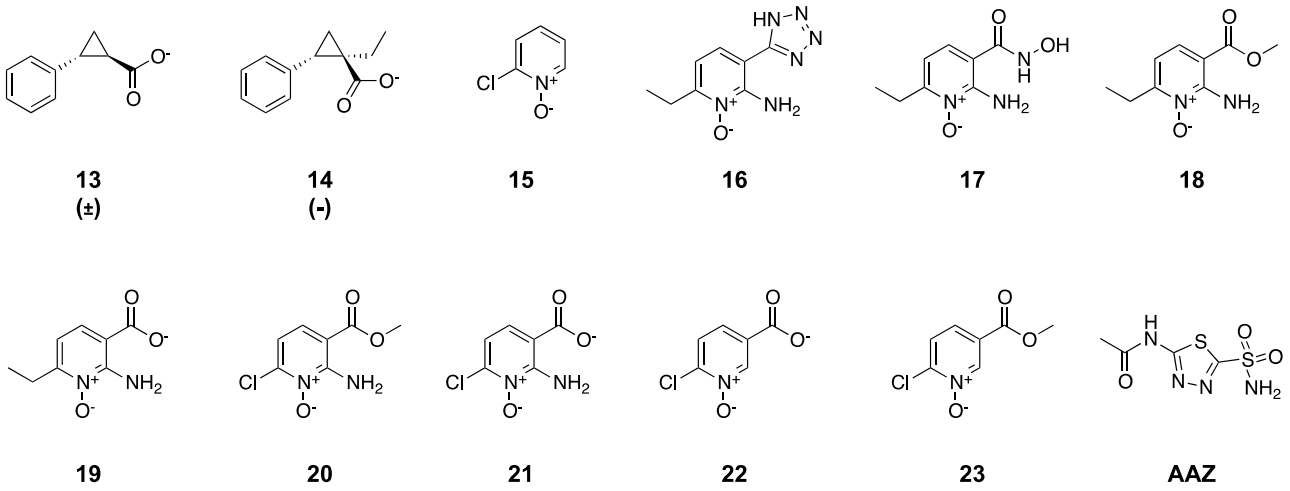


Chart 2. List of compounds selected to be tested *in vitro*.

Table 1. K_i (μM) values for *h*CAI, II and III and *Ssp*CA. Into the brackets the glide score (Kcal/mol) obtained for each ligand. For *h*CA I and II the average glide scores and its standard deviations (from three different assays), among different conformations, are reported, while for *Ssp*CA only one X-ray is available. In green, the glide scores greater than 7.5 ± 0.1 Kcal/mol.

	CA type *Ki* (μM)			
Compound	***h*CAI**	***h*CAII**	***h*CAIII**	***Ssp*CA**
13	70.6±6.3 (-7.72 ± 0.46)	70.3±5.9 (-7.37±0.42)	>100	2.80±0.21 (-9.01)
14	100±8.2 (-6.32 ± 0.37)	>100 (-6.41±0.45)	>100	1.71±0.11 (-7.73)
15	71±5.8 (-5.42±0.46)	>100 (-5.29±0.52)	>100	3.45±0.22 (-8.91)
16	100±6.3 (-4.84±0.52)	>100 (-4.55±0.38)	>100	36.30±3.5 (7.71)
17	90.3±8.0 (-8.02±0.43)	>100 (-7.35±0.42)	>100	5.96±0.42 (7.43)
18	91.7±8.3 (-7.55±0.65)	>100 (-4.81±0.54)	>100	25.70±2.4 (-8.94)
19	72±5.9 (-7.53±0.34)	>100 (-7.53±0.70)	>100	8.20±0.63 (-8.00)
20	92.7±7.5 (-7.25±0.24)	>100 (-7.12±0.59)	>100	1.89±0.13 (-9.60)
21	>100 (-7.28±0.30)	>100 (-7.58±0.67)	>100	3.47±0.20 (-7.82)
22	>100 (-7.49±0.37)	>100 (-7.18±0.45)	>100	2.59±0.16 (-7.21)
23	>100 (-5.57±0.34)	>100 (-5.62±0.42)	>100	35.20±1.8 (-8.62)
AAZ	0.25±0.008	0.012±0.001	0.17 ± 0.006	0.005±0.0001

Intrigued by the possibility to identify new CAI chemotypes with the ability to preferentially binding microbial CAs over the human ones we decided to challenge our compounds against another CA family, relevant for the treatment of human infections. Indeed, *Plasmodium falcimparum*, the causative agent of malaria, expresses a CA (*Pf*CA). The crystal structure of *Pf*CA

is not yet available, and for this reason this orthologous was not present in our initial structure dataset. Nevertheless, *Pf*CA represents an already identified target for treating malaria;[16,27,28] and it belongs to the η-family, which is the most similar to the α-family with respect to the other CA genetic families.[29,30] In Table 2 the K_i values for *Pf*CA are reported and, as expected, the compounds show inhibitory activities comparable to those observed for *Ssp*CA, and, even more interestingly, they all have a significant selectivity over the human CAs. The affinity of our compounds, either pyridine-N-oxides or phenylcyclopropane carboxylates, is much lower than that of reference compounds, such as acetazolamide (**AAZ**). Nevertheless, it must be stressed, however, that our compounds come from a recycling approach, and that they were optimized against very different targets.[10–15] We are confident that some of them can be still highly optimized against microbial CAs. For instances, two widely used metrics such as ligand efficiency (LE) and binding efficiency (BEI), reported in Table 2, indicate that most of our compounds have potential for large improvement in further hit-to-lead optimization cycles.

Table 2. K_i (μM) values for *Ssp*CA and *Pf*CA, with the corresponding selectivity folds and LE BEI indexes (from three different assays).

Compound	K_i (μM)		Selectivity Fold[a]		LE[b]		BEI[c]	
	SspCA	*PfCA*	*SspCA*	*PfCA*	*SspCA*	*PfCA*	*SspCA*	*PfCA*
13	2.80±0.21	5.82±0.23	25.11	12.08	0.648	0.611	0.048	0.045
14	1.71±0.11	6.73± 0.31	58.48	14.86	0.577	0.517	0.042	0.038
15	3.45±0.22	39.00±2.0	20.58	1.82	0.956	0.772	0.059	0.048
16	36.3±3.5	5.71±0.44	2.76	17.51	0.414	0.489	0.030	0.036
17	5.96±0.42	9.07±0.72	15.15	9.96	0.522	0.504	0.037	0.036
18	25.7±2.4	6.38±0.30	3.57	14.37	0.459	0.520	0.033	0.037
19	8.20± 0.63	6.09±0.51	8.78	11.82	0.548	0.562	0.039	0.040
20	1.89±0.13	37.60±1.84	49.04	2.46	0.616	0.477	0.040	0.031
21	3.47±0.20	39.80±2.16	28.82	2.51	0.637	0.513	0.041	0.033
22	2.59±0.16	41.30±4.01	38.61	2.42	0.711	0.558	0.045	0.035
23	35.0±1.8	8.07±0.62	2.84	12.39	0.520	0.594	0.033	0.038
AAZ	0.005±0.0001	0.17±0.003	2.40	0.07	0.894	0.729	0.052	0.043

[a]Calculated as *h*CA-K_i (with the lowest K_i determined)/(*Ssp* or *Pf)*CA-K_i
[b]Ligand Efficiency = RTpK_i/N, where N= number of non-hydrogen atoms
[c]Binding Efficiency Index = RTpK_i/MW, where MW = Molecular weight (kDa)

Among the compounds reported in Chart 2 compound **13** was available in our lab as racemic mixture, while compound **14** as a single enantiomer (*1R,2S*). Therefore, we proceeded to the resolution of the racemic mixture of **13 [24**(*1S,1S*) and **25**(*1R,1R*)**]**, and to the synthesis of the enantiomer *1S,2R* of **14** (**26**). Moreover, to further expand the exploration around the pyridine-N-oxide scaffold, 2-amino-pyridine-N-oxide was also synthetized (**27**). The compounds were,

therefore, tested against all the CA families and isoforms previously described, and the data are reported in Table 3. Interestingly, the analysis of the data indicate that *h*CA isoforms show an appreciable stereospecificity, since compound **24** is active against the *h*CA II, while compound **25** against the *h*CA I, thus highlighting the possibility to obtain isoform selectivity by modulating the stereochemistry of the phenylcyclopropane carboxylate scaffold. The introduction of the benzyl moiety, on the aforementioned scaffold, has detrimental effects on the activity against the human isoforms, since both compounds **14** and **26** are completely inactive against the human ones. On the other hand such a modification of the phenylcyclopropane carboxylate scaffold lead to a slight improvement of the activity for *Ssp*CA, since **14** is more active than the **24**, **25**, and **26** (**14**>**26** >**25** >**24** see Table 2 and 3).

On the contrary, *Pf*CA seems to be not affected by the stereochemistry and the modification of the main core (phenylcyclopropane carboxylate), since compound **13**, **14,** **24**, **25**, and **26** show comparable activity against *Pf*CA (Table 2 and Table 3).

Finally, compound **27** shows comparable activity against all the CAs considered in this study when compared to **15**. This observation suggested that the chlorine atom or the amino group in position 2 of the pyridine-N-oxide ring are well tolerated, while substitutions of the other positions of the main core play a crucial role in terms of selectivity and activity.

Table 3. K_i (μM) values for *h*CAI, II and III and *Ssp*CA (from three different assays).

	CA type *Ki* (μM)				
Compound	***h*CAI**	***h*CAII**	***h*CAIII**	***Ssp*CA**	***Pf*CA**
24	> 100	41.2±2.0	> 100	8.44±0.42	6.87±0.37
25	66.3±4.8	> 100	> 100	6.19±0.25	7.48±0.39
26	> 100	> 100	> 100	3.66±0.12	8.24±0.50
27	83.5±7.1	> 100	> 100	6.36±0.33	49.8±3.9

Binding mode and selectivity against the different CA isoforms and families. Among the tested compounds, **18** is quite a promising one, since it shows the best combination of selectivity over *h*CAs, LE and BEI parameters, and, interestingly, was devoid of any activity towards the target for which was originally designed,[15] thus highly reducing the risk of cross-interaction with other receptors/enzymes.

In Figure 2, the proposed binding mode of **18** into the *h*CA I-III and *Ssp*CA structures are depicted. Compound **18** nicely accommodates into the *Ssp*CA active site, with the N-oxide moiety chelating the Zn^{2+} ion. The 2-amino and 3-methyl-ester groups accommodate into the hydrophilic half of the active site, defined by the Asn62, Thr65, Gln67 residues, while the 5-ethyl moiety protrudes into

the hydrophobic half, which is defined by Val110, Val120, Val173, Val182. Finally, the pyridine ring engages a π-h-bond interaction with Thr175 (Figure 2a). Comparing the binding mode of the same compound into the *h*CA I (Figure 2b), and *h*CAIII (Figure 2c) it is clear that aminoacidic differences at both hydrophilic and hydrophobic halves (Figure 2d) of the active site account for:(*i*) the reduced activity of **18** for *h*CA I, and (*ii*) the lack of activity toward *h*CA III. Indeed, **18** in *h*CA I is not anymore able to establish the π-h-bond interaction observed in *Ssp*CA. Moreover Asn62 and Val120 are substituted with Val62 and Ala121 in the human isoform, leading to less favourable interaction of **18** with the *h*CA I active site. On the other hand, **18** cannot adopt a similar binding mode in *h*CA III due to two important amino acid differences: (*i*) His64 (in *Ssp*CA) is substituted with Lys64 (in *h*CA III), which clashes with the 3-methyl-ester group, and (*ii*) the *h*CA III active site is further narrowed by the presence of Phe198, which hamper the proper accommodation of the 6-ethyl moiety as observed in *Ssp*CA or *h*CA I.

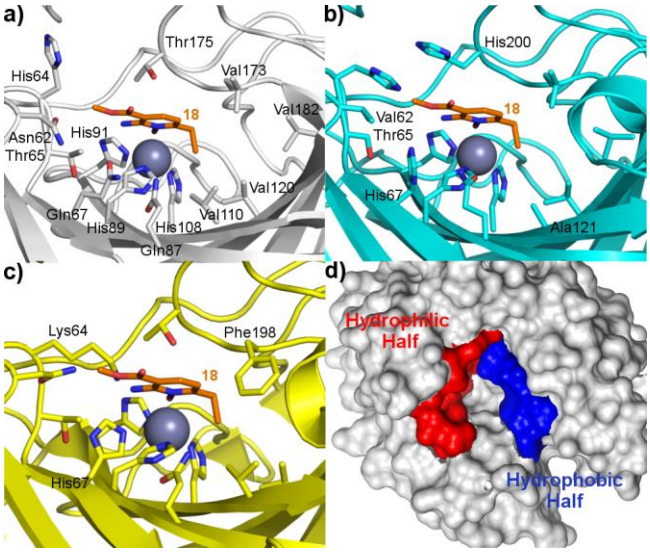


Figure 2. a) Binding mode of compound **18** into the *Ssp*CA active site (white sticks and cartoon), into the *h*CA I (**b**, cyan sticks and cartoon) and into the *h*CA III (**c**, yellow sticks and cartoon); **d)** hydrophilic (red surface) and hydrophobic (blue surface) halves of *h*CA I (grey surface).

Compound **18** can be considered a prototype structure for a new class of zinc chelating agents as CAIs. Most of the CAIs so far reported have a sulphonamide group playing the principal role in the binding to the CA active sites. Due to the prominent role played by the sulphonamide in the interaction with CA active sites, modifications at the core structure have only reduced effects in terms of affinity/selectivity. We can hypothesize than being the N-oxide a weaker Zn-Chelating Group (ZCG)), compounds bearing this functionality will be more amenable of modification of the organic core with a much higher potential for better control of the affinity and selectivity profile, with respect to the sulphonamide structures. As a matter of fact, the elucidation of the binding mode of **18** shed light on the structural basis underlying its selectivity profile toward the different CAs

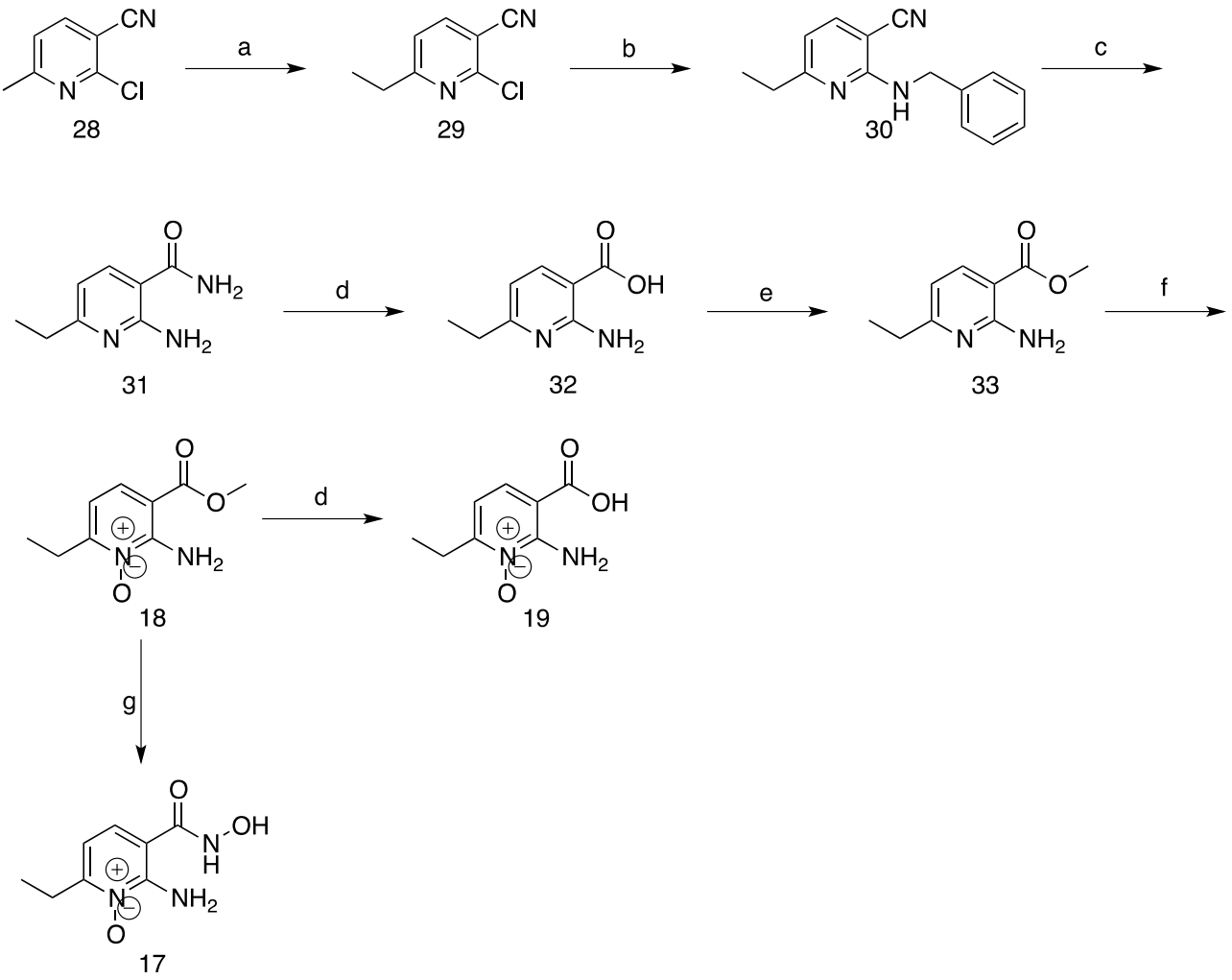
considered (Figure 2). The engagement of specific interaction with strategic residues inside the CA active site can lead to the design of selective CAIs, which preferentially bind microbial CAs over the human ones.

Chemistry. Compounds **17**, **18** and **19** were obtained starting from the commercially available 2-chloro-6-methylnicotinonitrile which reacted with methyl iodide in presence of NaH, as base, obtaining compound **29**.[15] The chlorine in position 2 was substituted by an amino group through the reaction of compound **29** with benzylamine, conducted in a microwave oven, leading to compound **30**. This compound was debenzylated with H_2SO_4 98%. Subsequently a basic hydrolysis in presence of KOH 10% at reflux temperature,[15] gave the acid **32,** which was methylated by esterification reaction in the presence of TMS-diazomethane.[15] The desired compound **18** was obtained by the oxidation of pyridine nitrogen with MTO in catalytic amount;[15] subsequently a basic hydrolysis of the ester gave the desired compound **19**. Finally, starting from compound **18**, a reaction with hydroxylamine and sodium methoxide as base,[31] gave compound **17** with high yield (**Scheme 1**).

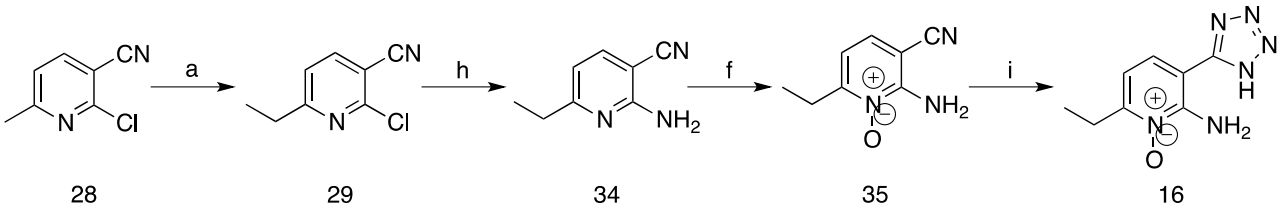
For the synthesis of compound **16** a protocol already reported was followed,[15] where the last step was conducted in a sealed tube in the presence of sodium azide (**Scheme 2**). Compounds **20, 21, 22** and **23** were obtained through a synthesis already reported (**Scheme 3**).[15]

The oxidation of compounds **40** and **41**, commercially available, was performed with *m*-perbenzoic acid in dichloromethane and gave the desired compounds **15** and **27** (**Scheme 4**).[22]

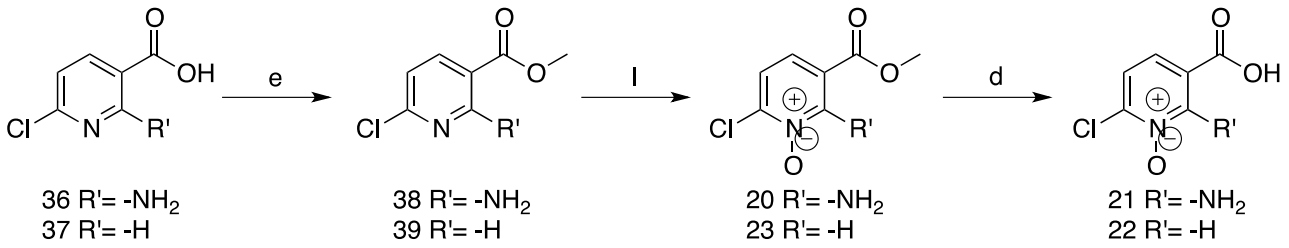
Compounds **14** and **26** were prepared through a straightforward protocol already reported (**Scheme 5**).[14] Compound **13** was commercially available. The enantiopure compounds **24** and **25** were obtained starting from compound **13** as racemic mixture that reacted with R)-(−)-2-phenylglycinol[32] to obtain the two diastereoisomers **(+)46** and **(-)46**. **(+)46** and **(-)46** were separated through flash chromatography and hydrolysed in acidic conditions[32] leading to compounds **24** and **25** as enantiopure compounds (**Scheme 6**).



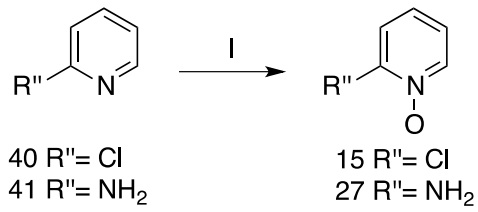
Scheme 1. *Reagents and conditions.* ***a)*** NaH, CH_3I, DMF, 15' 0°C, 4h r.t., 68% yield; ***b)*** benzylamine, µw 150W, 200°C, 15', 86% yield; ***c)*** H_2SO_4 98%, r.t., 18h, 98% yield; ***d)*** KOH 10%, 100°C, 3h, 98% yield; ***e)*** TMS-diazomethane, Toluene/methanol, 0°C, 30', 95% yield; ***f)*** MTO, 35% aqueous H_2O_2, EtOH, rt, 3 h, 78% yield; ***d)*** KOH 10%, 100°C, 3h, 98% yield; ***g)*** NH_2OH HCl, MeONa, MeOH, r.t., 18 h; 76% yield.



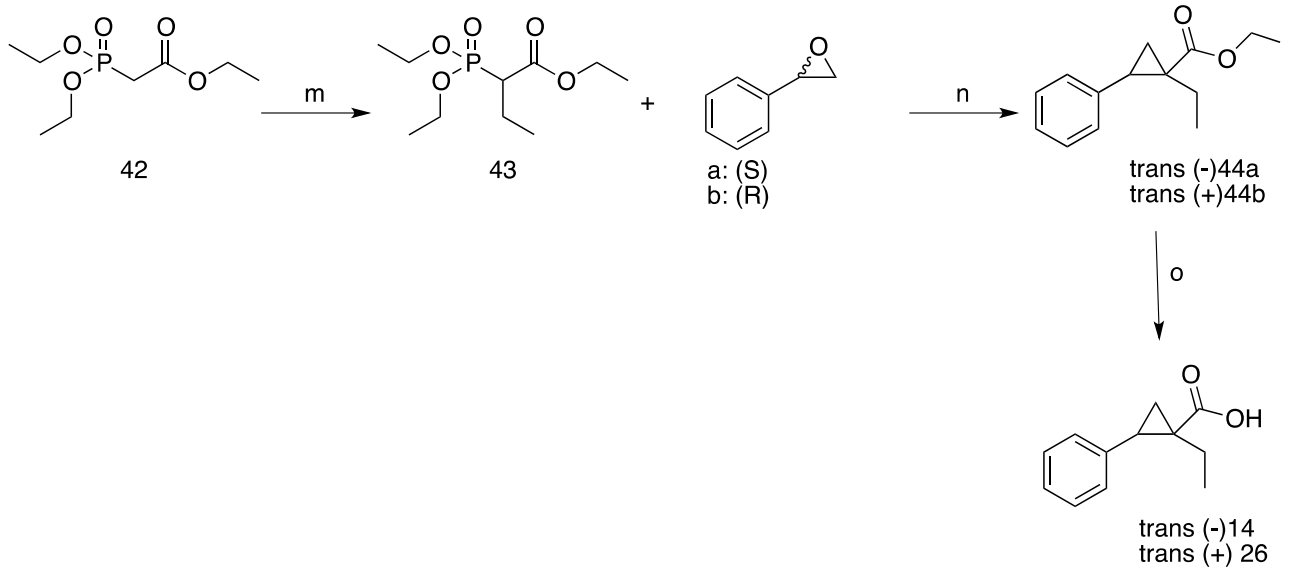
Scheme 2. *Reagents and conditions.* ***a)*** NaH, CH_3I, DMF, 15' 0°C, 4h r.t., 68% yield; ***h)*** EtOH/NH_3, sealed tube, 24h, 200°C, 45% yield; ***f)*** MTO, 35% aqueous H_2O_2, EtOH, rt, 3h, 78% yield; ***i)*** NaN_3, NH_4Cl, DMF, 130°C, 3h, 68% yield.



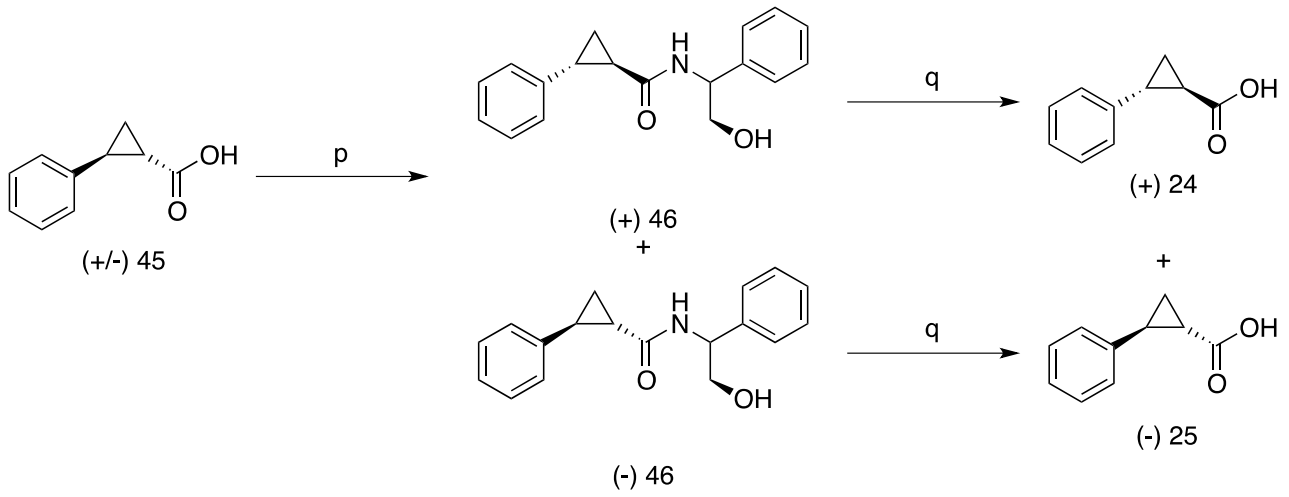
Scheme 3. *Reagents and conditions.* ***e)*** TMS-diazomethane, Toluene/methanol, 0°C, 30', 95% yield; ***l)*** *m*-CPBA, DCM, 4h, rt, 67% yield; ***d)*** KOH 10%, 100°C, 3h, 98% yield.



Scheme 4. *Reagents and conditions.* ***l)*** *m*-CPBA, DCM, 4h, rt, 67% yield.



Scheme 5. *Reagents and conditions.* ***m)*** NaH, CH_3CH_2I, DME, 2h rt, 2h 60°C, 65% yield; ***n)*** *n*-BuLi, DME, 30', rt, 18h, 90°C, 73% yield; ***o)*** LiOH, THF, MeOH, H_2O, μw, 10', 100°C, 85% yield.



Scheme 6. *Reagents and conditions.* ***p)*** (R)-(−)-2-phenylglycinol, TBTU, EDC HCl, TEA, DCM, 1h 0°C, 4h r.t., 75% yield; H_2SO_4 3N, dioxane, 18h, 100°C, 98% yield.

- **Conclusion**

In the context where the economic downturn and the reduced successful rate in drug development program are hampering classical drug discovery activities medicinal chemists have to identify alternative strategies to overcome productivity problems.[33,34] In academia, one possibility can be represented by the ability to disclose new-purposes for compound libraries already available in-house. Applying a rational-target-focus repurposing approach we were able to identify a new purpose for some compounds of our whole collection. First of all, we proceeded to the analysis of the fragments and chemotypes present in our libraries by applying the MCS approach.[7,8] The analysis of the data available in literature, for similar class of chemical structures, allowed us to identify the Carbonic Anhydrase (CA, EC 4.2.1.1) metalloenzyme family as potential target of our compound libraries. Modelling studies together with *in vitro* assays allowed us to identify new CAI chemotypes, which are characterized by a low μM affinity for microbial CA. The obtained modest K_i values can be reasonable since the molecules used where not designed to primarily targeting CAs. Even if, the activity profile of the compounds needs to be improved, the identified molecules can represent excellent hits to be further optimized in hits-to-lead campaigns. Indeed, compound **18** seems to be the most promising one and the analysis of its binding mode in the different CAs considered allowed us to pave the way for the design of potential selective CAIs, which preferentially inhibit microbial CAs. Indeed, the combination of the herein identified ZCG, with organic cores properly decorated can further increase the activity and selectivity against CAs of human pathogens characterized by the α- and η-families fold type.

- **Experimental Section**

Chemistry.

Chemicals, Materials and methods: all the reagents were purchased from SigmaAldrich, Alfa-Aesar and Enamine at reagent purity and, unless otherwise noted, were used without any further purification. Dry solvents used in the reaction were obtained by distillation of technical grade materials over appropriate dehydrating agents. MCRs were performed using CEM Microwave Synthesizer-Discover model. Reactions were monitored by thin layer chromatography on silica gel-coated aluminium foils (silica gel on Al foils, SUPELCO Analytical, SigmaAldrich) at both 254 and 365 nm wavelengths. Where indicated, intermediates and final products were purified through Merck silica gel 60 flash chromatography (silica gel, 0.040e0.063 mm), using appropriate solvent mixtures.

^{1}H NMR and ^{13}C NMR spectra were recorded on a BRUKER AVANCE spectrometer at 300 and 75.5 MHz respectively. ^{1}H NMR spectra are reported in this order: multiplicity and number of protons. Standard abbreviation indicating the multiplicity was used as follows: s . singlet, d . doublet, dd . doublet of doublets, t . triplet, q . quadruplet, m . multiplet and br . broad signal.

HPLC/MS experiments were performed with HPLC: Agilent 1100 series, equipped with a Waters Symmetry C18, 3.5 mm, 4.6 mm _ 75 mm column and MS: Applied Biosystem/MDS SCIEX, with API 150EX ion source, or, in alternative, HPLCeMS experiments were performed on an Acquity UPLC apparatus, equipped with a diode array and a Micromass SQD single quadruple (Waters).

HRMS experiments were performed with LTQ ORBITRAP XL THERMO.

All compounds were tested as 95% purity samples or higher (by HPLC/MS).

General procedure for the esterification of Nicotinic Acids. (Trimethylsilyl)diazomethane, 2.0 M in diethyl ether (2.00 equiv.) was added dropwise, over a period of 15 min, to a stirred, cooled (0°C) suspension of substrate (1.00 equiv.) in dry toluene/methanol, 3/2 (v/v, 10 mL/mmol), under a nitrogen atmosphere. The reaction mixture was kept under stirring at this temperature for 30 min. At the end of this time, the reaction mixture became a clear, yellow solution. TLC, eluting which chloroform/methanol, 9/1, showed complete consumption of the starting material. The reaction was stopped.

Solvents were evaporated under reduced pressure. The crude, solved in chloroform, was washed with saturated aqueous $NaHCO_3$ and brine. The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The desired product was purified by silica flash chromatography, eluting with chloroform, then chloroform/methanol, 95/5 (yield 75-95%).

Compounds **33**, **38** and **39** were obtained by this method. Analytical data for this compounds matched the data already published.[15]

General procedure for the Oxidation of the Pyridine Nitrogen. Method A. A 1/2 (v/v) solution of 35% (w/w) aqueous H_2O_2 in absolute ethanol was stirred over anhydrous Na_2SO_4 (5g/30ml) at room temperature for 3h. After filtration, methyltrioxorhenium (VII) (MTO, 0.10 equiv.) was added to this oxidant solution (4mL/mmol), followed by substrate (1.00 equiv.). The reaction mixture was stirred at room temperature for 4h, after which TLC, eluting with chloroform/methanol, 9/1, showed complete consumption of starting material. The reaction was stopped.

Brine was added to the reaction mixture, and it was extracted with chloroform. The organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The desired compounds were purified by silica gel flash chromatography, eluting with chloroform, then chloroform/methanol, from 99/1 to 95/5 (yield: 68-88%). Compounds **18** and **35** were obtained by this method. Analytical data for this compounds matched the data already published.[15]

General procedure for the Oxidation of the Pyridine Nitrogen. Method B.

The appropriate substrate (1 eq) was combined with m-chloroperoxybenzoic acid (m-CPBA, 2 eq) in dichloromethane (4ml/mmol) and allowed to stir at room temperature for 4h. After this time, TLC eluting with dichloromethane/methanol, 95/5, showed complete consumption of starting material.

The solution was washed with saturated aqueous NaHCO3, and the solvent evaporated under reduced pressure. The product was purified by silica gel flash chromatography eluting with 95:5 dichloromethane/methanol (yield: 56-67%). Compounds **15**, **20**, **23** and **27** were obtained by this method. Analytical data for this compounds matched the data already published.[15]

General procedure for the Hydrolysis of Methyl Esters and 2-amino-6-ethylnicotinamide (33) to the corresponding Carboxylic Acids. A fine suspension of the substrate in 10% (w/v) aqueous KOH (1 mL/mmol) was heated to reflux and stirred at this temperature for 3h. TLC after this time, showed complete consumption of the starting material. The reaction was stopped.

After cooling at room temperature, the clear, colourless solution so obtained was cooled to 0°C and carefully acidified to pH 4-5, with 3 N aqueous HCl. A white precipitate immediately formed. The desired product was collected by filtration in vacuo in quantitative yield. Compounds **32**, **19**, **21** and **22** were obtained by this method. Analytical data for this compounds matched the data already published.[15]

Synthesis of 2-chloro-6-ethylnicotinonitrile (29). Sodium hydride, 60% dispersion in mineral oil (1.50 equiv.), was carefully added under a nitrogen atmosphere, to a stirred, cooled (0°C) solution of 2-chloro-6-methylnicotinonitrile (**28** 1.00 equiv) in dry *N,N'*-dimethylformamide (2.5 mL/mmol). After stirring at this temperature at this temperature for 15 minutes, iodomethane (4.00 equiv) was added. The reaction mixture was allowed to warm to room temperature and stirred for 4 h under nitrogen. TLC after this time, eluting with petroleum ether/ethyl acetate, 7/3, showed almost complete consumption of the starting material. The reaction was stopped.

The reaction mixture was cooled to 0°C, then water was carefully added. The mixture was extracted with diethyl ether. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude was purified by silica gel flash chromatography, eluting with petroleum ether/ethyl acetate, from 9/1 to 8/2, affording the target product in 68% yield as a colourless oil. Analytical data for this compounds matched the data already published.[15]

Synthesis of 2-(benzylamino)-6-ethylnicotinonitrile (30). A 10 mL microwave tube was charged with 2-chloro-6-ethylnicotinonitrile (**29**, 1.00 equiv) and benzylamine (2.00 equiv). The tube was placed in a microwave and irradiated at 200°C for 15 min (maximum power input 150 W; maximum pressure, 160 PSI; power max, ON; stirring, ON). TLC after this time, eluting with petroleum ether/ethyl acetate, 9/1, showed almost complete consumption of the starting material. The reaction was stopped.

The reaction mixture was cooled to 0°C, then HCl 1N aqueous solution was carefully added. The mixture was extracted with chloroform. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude was purified by silica gel flash chromatography, eluting with petroleum ether/ethyl acetate, from 98/2 to 9/1, affording the target product in 86% yield as colourless oil.

Synthesis of 2-amino-6-ethylnicotinamide (31). H_2SO_4 95% was carefully added to compound **30** and the dark mixture was stirred at room temperature for 18 h. TLC after this time, eluting with petroleum ether/ethyl acetate, 9/1, showed almost complete consumption of the starting material. The reaction was stopped.

The reaction mixture was cooled at 0°C, then NaOH 1M aqueous solution was carefully added. The mixture was extracted with chloroform. The organic phase was washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude was used for the next step

without further purification.

Synthesis of 2-amino-6-ethyl-3-(hydroxycarbamoyl)pyridine 1-oxide (17). Compound **18** (1.00 eq) was added carefully, dropwise through dropping funnel, at 0°C, to a suspension of NH_2OH HCl (1.5 eq) and MeONa (1.5 eq) in MeOH. The reaction was stirred at room temperature for 18 hours. TLC after this time, eluiting with DCM/MeOH 85/15, showed complete consumption of the starting material. The reaction was stopped. The reaction mixture was cooled to 0°C and HCl 1N aqueous solution was carefully added. The mixture was extracted with chloroform. The organic phase was washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude was purified by silica gel flash chromatography, eluting with chloroform/methanol/formic acid (8.7/1/0.3), affording the target product in 76% yield as white solid.

Synthesis of 2-amino-6-ethylnicotinonitrile (34). A solution of the substrate in ammonia saturated ethanol (1ml/mmol) was placed in a sealed tube, heated to 200°C, and maintained at this temperature for 24h. After cooling to room temperature, solvent was removed by evaporation in vacuo and the desired product was purified by silica gel flash chromatography, eluting with petroleum ether/ethyl acetate, from 9/1 to 6/4, affording the target product in 45% yield. Analytical data for this compound matched the data already published.[15]

Synthesis of 6-ethyl-3-(1H-tetrazol-5-yl)pyridin-2-amine (16). A sealed tube was charged with 2-amino-3-cyano-6-ethyl pyridine 1-oxide (**35**) (1.00 eq), NaN_3 (3.00 eq), NH_4Cl (3.00 eq) and DMF dry (1ml/mmol). The reaction was stirred at 130°C for 3 hours. TLC after this time, eluting with DCM/MeOH 85/15, showed complete consumption of the starting material. The reaction was stopped. The reaction mixture was cooled at 0°C and water was added. The mixture was extracted with chloroform and the organic phase was washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude was purified by silica gel flash chromatography, eluting with chloroform/methanol/formic acid (8.7/1/0.3), affording the target product in 68% yield as white solid.

Synthesis of ethyl 2-(diethoxyphosphoryl)butanoate (43). Ethyl 2-(diethoxyphosphoryl)acetate (**42**) (1.00 eq) was added dropwise to a cooled suspension of NaH (1.1 eq) in dry DME (2 mL/mmol). After stirring at 25 °C for 2 h, ethyl iodide (1.1 eq) was added, and the mixture was heated at 60 °C for 2 h. The reaction mixture was poured into ice water and extracted with ethyl acetate and the combined organic layers were washed with H2O and brine, dried over Na_2SO_4, and

evaporated under reduced pressure. The crude material was purified through flash chromatography eluting with dichloromethane/diethyl ether 95:5 to give the desired compounds as colourless oil in 65% yield. Analytical data for this compounds matched the data already published.[14]

Synthesis of (1R,2S)-ethyl 1-ethyl-2-phenylcyclopropanecarboxylate (44a). To a solution of compound **38** (2 eq) in dry DME (5 mL/mmol) at 25 °C, n-buthyllithium (2.5 M in hexane, 2 equiv) was added dropwise over 5 min. After stirring at the same temperature for 30 min, (S)-(−)-Styrene oxide (1 eq) was added portionwise and the mixture heated at 90 °C for 18 h. After cooling, saturated aqueous solution of NH4Cl was added and the organic layers were extracted with ethyl acetate, washed with brine, dried over anhydrous Na2SO4, and evaporated under reduced pressure. The oil obtained was purified through flash column chromatography eluting with petroleum ether/ethyl acetate (95:5), to give the desired product as yellowish oil in 85% yield. Analytical data for this compounds matched the data already published.[14]

Synthesis of (1S,2R)-ethyl 1-ethyl-2-phenylcyclopropanecarboxylate (44b). To a solution of compound **38** (2 eq) in dry DME (5 mL/mmol) at 25 °C, n-buthyllithium (2.5 M in hexane, 2 equiv) was added dropwise over 5 min. After stirring at the same temperature for 30 min, (R)-(+)-Styrene oxide (1 eq) was added portionwise and the mixture heated at 90 °C for 18 h. After cooling, saturated aqueous solution of NH4Cl was added and the organic layers were extracted with ethyl acetate, washed with brine, dried over anhydrous Na2SO4, and evaporated under reduced pressure. The oil obtained was purified through flash column chromatography eluting with petroleum ether/ethyl acetate (95:5), to give the desired product as yellowish oil in 85% yield. Analytical data for this compounds matched the data already published.[14]

Synthesis of (1R,2S)-1-ethyl-2-phenylcyclopropanecarboxylic acid (14). Compound **44** (1 eq) and LiOH·H2O (4 eq) were dissolved in a solution of THF/MeOH/H2O (3/1/1, 1 mL/mmol) and heated under stirring in a microwave oven at 100 °C for 7 min. The reaction mixture is then evaporated in vacuo, and the crude is taken up with H_2O, acidified with HCl 1 N, and extracted with ethyl acetate, that is in turn washed with brine and dried over anhydrous Na2SO4. After the evaporation of the solvent the crude material was purified through flash column chromatography eluting dichloromethane/methanol (95:5), to give the desired product as a white solid in yield 85%. Analytical data for this compounds matched the data already published.[14]

Synthesis of (1S,2R)-1-ethyl-2-phenylcyclopropanecarboxylic acid (26). Compound **44b** (1 eq)

and LiOH·H2O (4 eq) were dissolved in a solution of THF/MeOH/H2O (3/1/1, 1 mL/mmol) and heated under stirring in a microwave oven at 100 °C for 7 min. The reaction mixture is then evaporated in vacuo, and the crude is taken up with H_2O, acidified with HCl 1 N, and extracted with ethyl acetate, that is in turn washed with brine and dried over anhydrous Na2SO4. After the evaporation of the solvent the crude material was purified through flash column chromatography eluting dichloromethane/methanol (95:5), to give the desired product as a white solid in yield 85%. Analytical data for this compounds matched the data already published.[14]

Synthesis of N-(2-hydroxy-1-phenylethyl)-2-phenylcyclopropanecarboxamide (+,− 46). To a solution of compound **40** (1 eq) in dichloromethane (1ml/mmol) were added (R)-(−)-2-phenylglycinol (1.5 eq), TBTU (1 eq), EDC HCl (1.5 eq) and TEA (1 eq). The reaction mixture was stirred 1h at 0°C and then 4h at room temperature. TLC after this time, eluting with dichloromethane/methanol, 9/1, showed almost complete consumption of the starting material. The reaction was stopped.

A NH_4Cl aqueous solution was carefully added to the reaction mixture and then was extracted with chloroform. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude was purified by silica gel flash chromatography, eluting with dichloromethane/methanol, from 99/1 to 95/5, affording the two diastereoisomers (+)**46** and (−)**46** as white solid in 43% and 31% yields rispectively.

General procedure for the Hydrolysis of N-(2-hydroxy-1-phenylethyl)-2 phenylcyclopropanecarboxamide. A solution of substrate in H_2SO_4 3N and dioxane (1:1, 1ml/mmol) was stirred at 100°C for 18h. TLC after this time, eluting with dichloromethane/methanol, 9/1, showed almost complete consumption of the starting material. The reaction was stopped.

Water was carefully added to the reaction mixture and then was extracted with chloroform. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude was purified by silica gel flash chromatography, eluting with dichloromethane/methanol, from 99/1 to 95/5, affording the target compound as white solid in 98% yield. Compounds **24** and **25** were obtained by this method.

2-(benzylamino)-6-ethylnicotinonitrile. (30) ^{1}H NMR (300 MHz, $CDCl_3$): 7.58 (d, J=7.8, 1H); 7.41-7.30 (m, 5H); 6.51 (d, J=7.8, 1H); 5.45 (br, 1H); 4.75 (d, J=5.6, 2H); 2.76-2,68 (q, 2H); 1.30-

1.25 (t, 3H). HPLC/MS: found *m/z* $[M + H]^+$ 238.30.

2-amino-6-ethylnicotinamide. (31) ^{1}H NMR (300 MHz, DMSO): 7.86 (d, J=7.7, 1H) 7.80 (br, 2H); 7.16 (br, 2H); 6.43 (d, J=7.7); 2.55-2.50 (q, 2H); 1.19-1.14 (t, 3H). HPLC/MS: found *m/z* $[M + H]^+$ 166.19.

2-amino-6-ethylnicotinic acid. (32) ^{1}H NMR (300 MHz, DMSO): 13.48 (br, 1H); 7.94 (d, J=7.7, 1H); 7.18 (br, 2H); 6.46 (d, J=7.7, 1H); 2.57-2.50 (q, 2H); 1.19-1.14 (t, 3H). HPLC/MS: found *m/z* $[M + H]^+$ 167.18.

methyl 2-amino-6-ethylnicotinate. (33) ^{1}H NMR (300 MHz, DMSO): 7.96 (d, J=8.0, 1H); 7.11 (br, 2H); 6.51 (d, J=8.0, 2H); 3. 79 (s, 3H); 2.61-2.54 (q, 2H); 1.20-1.15 (t, 3H). HPLC/MS: found *m/z* $[M + H]^+$ 181.20.

methyl 2-amino-6-ethylnicotinate 1-oxide. (18) ^{1}H NMR (300 MHz, DMSO): 7.73 (br, 2H); 7.68 (d, J=8.4, 1H); 6.71 (d, J=8.4, 1H); 3.86 (s, 3H); 2.88-2.80 (q, 2H); 1.24-1.89 (t, 3H). ^{13}C NMR (75.5 MHz, DMSO): 168.27; 154.71; 152.15; 128.22; 109.61; 106.73; 52.76; 24.65; 10.84. HPLC/MS: found *m/z* $[M + H]^+$ 197.20.

2-amino-6-ethyl-N-hydroxynicotinamide 1-oxide. (17) ^{1}H NMR (300 MHz, DMSO): 11.20 (br, 1H); 9.34 (br, 1H); 7.73 (br, 2H); 7.62 (d, J=8.4, 1H); 6.47 (d, J=8.4, 1H); 2.79-2.73 (q, 2H); 1.21-1.17 (t, 3H). ^{13}C NMR (75.5 MHz, DMSO): 161.22; 155.68; 151.98; 127.74; 110.03; 106.74; 24.65; 10.84. HPLC/MS: found *m/z* $[M + H]^+$ 198.19.

6-ethyl-3-(1H-tetrazol-5-yl)pyridin-2-amine 1-oxide. (16) ^{1}H NMR (300 MHz, DMSO): 11.72 (br, 1H); 7.88 (br, 2H); 7.81 (d, J=8.2, 1H); 6.87 (d, J=8.2, 1H); 2.91-2.85 (q, 2H); 1.27-1.23 (t, 3H). ^{13}C NMR (75.5 MHz, DMSO): 160.20; 157.33; 151.71; 126.98; 110.54; 106.74; 24.65; 10.84. HPLC/MS: found *m/z* $[M + H]^+$ 206.4

2-chloropyridine 1-oxide. (15) ^{1}H NMR (300 MHz, DMSO): 8.44 (d, 1H); 7.81-7.72 (t, 1H); 7.57-7.53 (t,1H); 7.40 (d, 1H). ^{13}C NMR (75.5 MHz, DMSO): 125.34; 111.25; 105.76. HPLC/MS: found *m/z* $[M + H]^+$ 114.15.

pyridin-2-amine 1-oxide. **(27)** ^{1}H NMR (300 MHz, DMSO): 8.38 (d, 1H); 7.97 (br,1H); 7.67-7.62

(t, 1H); 7.51-7.48 (t,1H); 7.35 (d, 1H). ^{13}C NMR (75.5 MHz, DMSO): 124.32; 110.34; 104.23. HPLC/MS: found *m/z* $[M + H]^+$ 95.11.

(1R,2R)-N-(2-hydroxy-1-phenylethyl)-2-phenylcyclopropanecarboxamide. (+)46 ^{1}H NMR (300 MHz, DMSO): 8.57 (d, 1H); 7.33-7.12 (m, 10H); 4.91-4.87 (m, 1H); 3.56-3.52 (t, 2H); 2.26-22.21 (m, 1H); 2.06-2.02 (m, 1H); 1.32-1.28 (m, 1H); 1.20-1.18 (m- 1H). HPLC/MS: found *m/z* $[M + H]^+$ 282.35.

(1S,2S)-N-(2-hydroxy-1-phenylethyl)-2-phenylcyclopropanecarboxamide. (-)46 ^{1}H NMR (300 MHz, DMSO): 8.57 (d, 1H); 7.33-7.12 (m, 10H); 4.91-4.87 (m, 1H); 3.56-3.52 (t, 2H); 2.26-22.21 (m, 1H); 2.06-2.02 (m, 1H); 1.32-1.28 (m, 1H); 1.20-1.18 (m- 1H). HPLC/MS: found *m/z* $[M + H]^+$ 282.74.

(1R,2R)-2-phenylcyclopropanecarboxylic acid. (+)24 ^{1}H NMR (300 MHz, DMSO): 11.06 (br, 1H); 7.35-7.22 (m, 5H); 2.67-2.60 (m, 1H); 1.96-1.91 (m, 1H); 1.72-1.68 (m, 1H); 1.47-1.40 (m, 1H). ^{13}C NMR (100.6 MHz, $CDCl_3$): 129.29; 128.21; 126.87; 33.32; 31.17; 21.48. HPLC/MS: found *m/z* $[M - H]^-$ 161.19. α = +47.62.

***In Vitro* assays.**

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic/inhibition of various CA isozymes as reported by Khalifah.[35] The CA-catalysed CO_2 hydration reaction was followed for a period of 5-10 s. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.4) as buffer and 0.1 M Na_2SO_4 or $NaClO_4$ for maintaining constant the ionic strength (these anions are not inhibitory in the used concentration). Saturated CO_2 solutions in water at 25 °C were used as substrates. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in the assay buffer) and dilutions up to 0.1 nM done with the same assay buffer. 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 the Cheng-Prusoff equation, as reported earlier,[36,37] and represent the mean from at least three different determinations. CA II was purchased by Sigma-Aldrich and used without further purification, whereas all the other CA isoforms were recombinant ones obtained in-house as reported earlier.[30,37,38] The concentration of the enzyme in the assay

system were: 9.2 nM for hCA I; 7.1 nM for hCA II; 13.6 nM for hCA III; 8.4 nM for SspCA and 12.0 nM for pfCA, respectively.

Computational Methods.

Maximum Common Scaffold decomposition. The Maximum Common Scaffold decomposition was performed by using the Library MCS tool of ChemAxon[39] and the default parameters.

Identification and validation of the docking protocol. The best docking parameters and the X-ray structures to be used in the study were identified applying a two steps pipeline protocol. In the first step a re-docking procedure was performed on a small set CA of X-ray crystal structures (10 CA X-ray crystal structures), by using Glide.[40] The default docking parameters were modified until the identification of the combination, which turned out the best performance in terms of ligand re-docking r.m.s.d. In this case the 10 CA X-ray crystal structures were chose so as to account for the different nature of the CAIs so far crystallized. The final docking procedure is defined by the following steps: (*i*) protein preparation using the metal-binding site function and removal of co-crystallization factors (i.e. Glycerol); (*ii*) the grid was cantered on the catalytic Zn^{2+} metal atom, with a box dimension of 15x15x15 Å; (*iii*)ligand preparation using *LigPrep*[40] in combination with the;[40] (*iv*) each docking run was carried out with the standard precision (SP) method, and the van der Waals scaling factor of nonpolar atoms was set to 0.8; (*v*) final refinement of the obtained docking pose applying the extra-precision (XP) method; (*vi*) computation of the re-docked ligand r.m.s.d with respect to the crystal structureIn the second step, the docking protocol identified at the first step was challenged against a greater number of CA X-ray structures (258 *h*CA X-ray Crystal Structures). This final step allowed as to robustly assess the performance of the selected protocol and to identify issues related to the X-ray crystal structures (i.e. the presence of co-crystallization factor into the CA active site). Detail about the docking procedure, the performance and the 10 CA X-ray crystal structures used are discussed in Supplementary Information.

Docking. Once concluded the validation step of the docking protocol we apply the identified method to screen our in house library against the CA X-ray crystal structure data set generated. In this case the pdb data set was defined by: (*i*) X-ray crystal structure of the CA α-family [for different organisms, such as human (I, II, III, IV, VII, XII and XIII isoforms), bacterial, protozoa, etc….], excluding those X-ray structures with co-crystallization factor inside the CA active site; and (*ii*) X-ray crystal structure of the CA β-family (for different organisms).For a total of 530 out of 753

CA X-ray crystal structures. One pose per ligand was retained and the glide score was evaluated by using the heat map representation.

Compounds selection for in vitro assays. Among the different compounds screened during the docking studies only those: (*i*) showing a glide score greater than 7.5 Kcal/mol (when more of one X-ray crystal structures was available for the same enzyme the average and its standard deviation were considered) and (*ii*) physically available in the lab, were selected to be tested *in vitro*.

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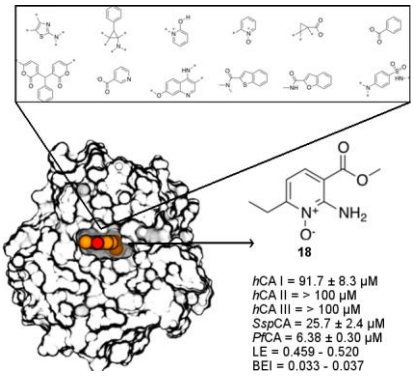

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