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Published

2014

Book Title

Carbonic anhydrase: Mechanism, Regulation, Links to Disease, and Industrial Applications

DOI

10.1007/978-94-007-7359-2_16

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Natural products that inhibit carbonic anhydrase

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Keywords: carbonic anhydrase, natural products, coumarin, phenol, polyamine, sulfonamide, sulfamate

Abstract

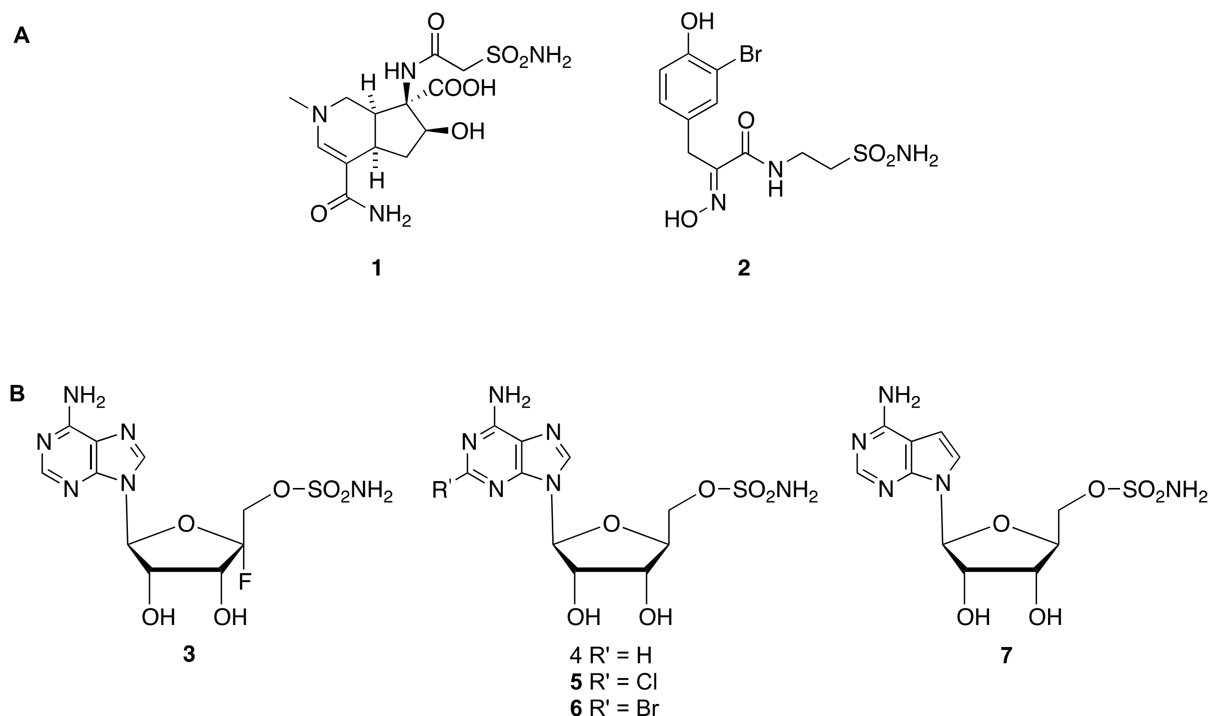
The chemical diversity, binding specificity and propensity to interact with biological targets has inspired many researchers to utilize natural products as molecular probes. Almost all reported carbonic anhydrase inhibitors comprise a zinc binding group in their structure of which the primary sulfonamide moiety (-SO_2NH_2) is the foremost example and to a lesser extent the primary sulfamate (-O-SO_2NH_2) and sulfamide (-NH-SO_2NH_2) groups. Natural products that comprise these zinc binding groups in their structure are however rare and relatively few natural products have been explored as a source for novel carbonic anhydrase inhibitors. This chapter will highlight the recent and growing interest in carbonic anhydrase inhibitors sourced from nature, demonstrating that natural product chemical space presents a rich source of potential alternate chemotypes for the discovery of novel drug-like carbonic anhydrase inhibitors.

Introduction

Carbonic anhydrases (CAs) are zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and a proton (1). The active site zinc cation is the implied target for small molecule inhibitors to block the endogenous CA catalyzed reaction. Almost all reported CA inhibitors comprise a zinc binding group (ZBG) of which the primary sulfonamide moiety (-SO_2NH_2) is the foremost example, and to a lesser extent the primary sulfamate (-O-SO_2NH_2) and primary sulfamide (-NH-SO_2NH_2) groups. Compounds sourced from nature that comprise either a primary sulfonamide, sulfamate or sulfamide moiety in their structure are exceedingly rare. A literature search of the Dictionary of Natural Products (DNP) database (2) (a comprehensive and fully-edited database on natural products (NPs)) revealed just two NP primary sulfonamide compounds, (-)-altemicidin **1** and psammaplin C **2**, and five NP primary sulfamate compounds, nucleocidin **3**,(3) 5'-*O*-sulfamoyl adenosine **4**,(4) 5'-*O*-sulfamoyl 2-chloroadenosine **5**,(5) 5'-*O*-sulfamoyl 2-bromoadenosine **6** (5, 6) and 5'-*O*-sulfamoyl tubercidin **7** (Figure 1) (7, 8). (-)-Altemicidin **1** is a marine alkaloid isolated from the actinomycete strain *Streptomyces sioyaensis* (9). This compound exhibited potent acaricidal activity as well as strong inhibition of tumor cell growth (10). A total synthesis of **1** as well as the isolation of two secondary sulfonamide analogues of **1** have subsequently been reported (9). Psammaplin C **2** is a bromotyrosine amino acid derivative isolated from the marine sponge *Pseudoceratina purpurea*;(11, 12) no bioactivity for this alkaloid has been reported to date. The sulfamate nucleosides **3**-**7** were isolated from actinomycete species belonging to the genus *Streptomyces.* These structurally related sulfamates are reported to have a range of biological effects including cytotoxicity,(4) herbicidal activity,(6-8, 13) inhibition of blood platelet aggregation,(5) antibacterial

activity (5, 14) and antitrypanosomal activity (14). Nucleocidin **3**, isolated from the fermentation broth of *Streptomyces calcus,* is of particular note since it was the first NP to contain either a fluorinated carbohydrate or a primary sulfamate group. This molecule became an attractive synthetic target owing to its novel structural features, with the first total synthesis reported in 1976 (15). Whilst NP **3** has been shown to exhibit broad spectrum antibacterial effects as well as potent antitrypanosomal activity, its potential use in the clinic has been limited due to toxicity (16). The NPs **1-7** have not been investigated for CA inhibition properties, however it is likely that these compounds would inhibit CA activity owing to the presence of an unhindered primary sulfonamide or primary sulfamate moiety within their structure. Finally, our search of the DNP failed to identify any NP primary sulfamides.

Figure 1. Natural products (NPs) that comprise a primary sulfonamide or sulfamate moiety in their structure. A.) NP primary sulfonamides: (-) altemicidin **1** and psammaplin C **2**. B.) NP primary sulfamates: nucleocidin **3**, 5'-*O*-sulfamoyl adenosine **4**, 5'-*O*-sulfamoyl 2-chloroadenosine **5**, 5'-*O*-sulfamoyl 2-bromoadenosine **6** and 5'-*O*-sulfamoyl tubercidin **7**.



At the time of writing the Protein Data Bank (PDB) contained X-ray structures of ~160 sulfonamide ligands ($R\text{-}SO_2NH_2$) in complex with hCA II (h = human). The binding mode of the

sulfonamide anion ($R-SO_2NH^-$) to the Zn^{2+} cation is invariant in these structures, with the sulfonamide anion coordinated to the active site Zn^{2+}. Primary sulfamates and sulfamides, ZBG isosteres of primary sulfonamides, contribute an additional ~35 X-ray structures of ligands in complex with hCA II in the PDB. Of the remaining PDB protein-ligand structures most comprise very simple ligands such as anions or small organic molecules, there are however several structures comprising more complex alternate CA ligands, some of which are NPs.

NPs comprise a vast collection of diverse chemical structures and have proven to be an invaluable source of new chemotherapies (17-22). Plant NPs have been the basis of traditional medicine for thousands of years and continue to actively contribute to contemporary drug discovery (22). In more recent times, marine macro- and micro-organisms along with terrestrial microbes have been the source of numerous lead molecules or drugs (23). The significance of NPs in drug discovery is most evident in the anticancer and anti-infective therapeutic areas (24-27). For example, between 1940 and 2011 48.6% of all new anticancer small molecule therapies approved by the FDA were either NPs or NP derivatives (27). Furthermore between 1981 and 2011 75% of all antibacterial new chemical entities were either NPs or their derivatives (27). The success of NPs and their semi-synthetic derivatives as therapeutic agents is intrinsically linked to the fact that NPs have been biologically pre-validated and selected during evolution to bind to biosynthetic enzymes (28-33). It has been hypothesized that this inherent capacity to bind in biological space allows NPs to also recognize human therapeutic targets (28, 30, 33). Furthermore, computational studies have shown that NPs occupy complementary areas of chemical space compared with synthetic compounds, and thus should be implemented to increase the chemical complexity and drug-likeness of screening libraries (29-32). The chemical diversity, binding specificity and efficiency, and propensity to interact with biological targets have inspired many researchers to utilize NPs as molecular probes. These studies go beyond the identification of potential new lead or drug molecules, and have increased our understanding of biological pathways and systems (34, 35). While the primary sulfonamide and sulfamate moieties are poorly represented in NP chemical space, this space does provide a rich source of alternate chemotypes for the discovery of CA inhibitors with a different enzyme binding mode to the typical ZBGs. The remainder of this chapter will highlight the recent and growing interest in novel CA inhibitors sourced from nature.

Natural products that inhibit carbonic anhydrase

Several classes of novel CA inhibitors have been identified from screening collections of NPs,

most notable are coumarin and phenol containing NPs (36). NPs comprising these fragments display diverse profiles of CA inhibition when compared to classical ZBGs and these will be discussed further in the following sections. In addition a selection of NP-derived CA inhibitors have been synthesized where the NP scaffold has been synthetically modified to incorporate the classical ZBG of CA inhibitors i.e. sulfonamide, sulfamate or sulfamide. A summary of these hybrid molecules will also be presented.

1. Coumarins

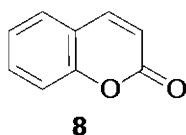
Coumarin compounds are abundant secondary metabolites in plants and are found to a lesser extent in microorganisms and animal sources. Plant coumarins are phytoalexins, defense compounds produced when the plant is under threat from other organisms, and have attracted interest owing to a range of biological activities including antimicrobial, molluscicidal, acaricidal, antiviral, anticancer, antioxidant and anti-inflammatory properties (37). The coumarin structure comprises a benzopyrone core, with NP coumarins categorized as a) simple coumarins, b) furanocoumarins, c) pyranocoumarins, d) bis- and triscoumarins; or e) coumarinolignans (37). Simple coumarins, including coumarin **8** (Figure 2) are highly abundant in several plant species belonging to the taxonomic families Umbelliferae, Rutaceae and Compositae (37). A recent review of coumarin-based drugs highlights the growing interest in the coumarin compound class to deliver new therapeutics,(38) with potential therapeutic applications driving efforts towards the isolation and the structural characterization of further novel bioactive coumarin derivatives. In recent years it was discovered that NP coumarins inhibit CA enzymes via an alternate and unprecedented mechanism to classical sulfonamides,(39) these findings are described next.

Nature Bank is a unique drug discovery resource that encompasses a diverse collection of >50,000 biota samples of plants, fungi and marine invertebrates collected from Australia, China and Papua New Guinea along with biota extracts, semi-purified fractions and pure compounds (40). A selection of *Leionema ellipticum* (family Rutaceae) extracts was sourced from Nature Bank and screened using Fourier transform ion cyclotron resonance electrospray ionization mass spectrometry (FTICR ESI MS) for binding to bovine CA II (bCA II) (39). From this study the NP coumarin, 6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one **9** was identified as a ligand for bCA II as it formed a noncovalent complex that could be detected by ESI MS. In follow on studies it was demonstrated that coumarin **9** inhibits a spectrum of human CAs in an unprecedented time dependent manner.(41) The usual enzyme assay conditions to investigate small molecule inhibition of CA activity is to incubate the test compound with the CA protein of

interest for 15 minutes prior to monitoring the effect on CA-mediated CO_2 hydration. Under these conditions this coumarin had only weak CA inhibition prompting us to extend the pre-incubation time. Following 6 hours of pre-incubation with hCA II the K_i of coumarin **9** dropped to 60 nM (with a similar reduction in K_i at other CA isozymes also observed). As the coumarin chemotype lacked a classic ZBG typical of known small molecule CA inhibitors and displayed unusual time-dependent inhibition it was important to understand how this chemotype binds to and inhibits CAs. Using protein X-ray crystallography the crystal structure of hCA II with this NP was obtained at a resolution of 2.0 Å (Figure 3). The coumarin **9** was not observed, instead the hydrolysis product of **9**, the cinnamic acid derivative **10** was identified (41). Esterase activity is known for CAs (42-44) and the observation of cinnamic acid **10** rather than NP coumarin **9**, although unexpected, could be rationalized as a consequence of hCA II esterase activity leading to hydrolysis of the lactone of **9**. The bulky hydrolysis product **10** then plugged the CA active site cavity entrance, exhibiting no interactions with the catalytic zinc ion. This unusual inhibition mode is previously unobserved for CAs and together with the coumarin pedigree in medicinal chemistry is suggestive of a potential new avenue for drug development compared to the ZBGs of classical CA inhibitors. Reactive Michael acceptors are a general structural alert in drug discovery, however it has been demonstrated that simple coumarins exhibit poor protein binding characteristics compared to other carbonyl containing Michael acceptors (45, 46). The lower reactivity of the coumarin double bond compared with other Michael acceptors, has been attributed to it being part of a pseudoaromatic system (47).

Figure 2. NP coumarin CA inhibitors. A.) Coumarin **8**. B.) CA hydrolysis of coumarin **9** to form the cinnamic acid compound **10**.

A



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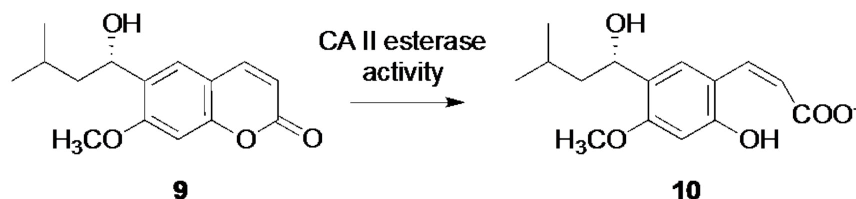
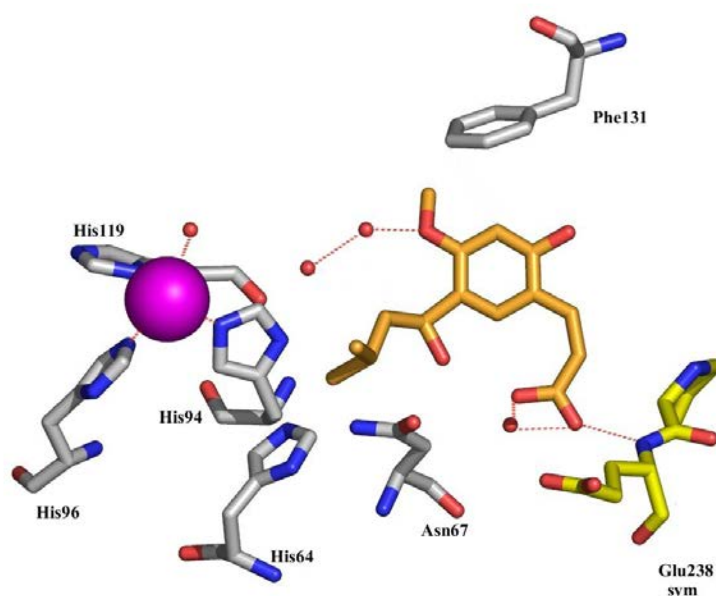


Figure 3. Detailed interactions between hCA II and NP coumarin **9** hydrolysis product **10** from a protein X-ray crystal structure. The catalytic site showing the tetrahedral Zn^{2+} cation (violet sphere) with the three coordinated His ligands (His94, His96, and His119) and a water molecule (red sphere). The cinnamic acid **10** (gold) interacts with three active site ordered water molecules (red spheres), with Phe131 and Asn67 (CPK colors) from the active site as well as with Glu238sym (yellow) from a symmetry related enzyme molecule. The proton shuttle residue His64 is shown (CPK colors). Reprinted with permission from Maresca, A.; Temperini, C.; Vu, H.; Pham, N. B.; Poulsen, S.-A.; Scozzafava, A.; Quinn, R. J.; Supuran, C. T. *J. Am. Chem. Soc.* 2009, *131*, 3057. Copyright 2009 American Chemical Society.



Following the findings outlined above we performed a substructure search of the Nature Bank (40) pure compound repository against the bare coumarin scaffold **8**. A set of 81 coumarins were identified and from this a subset of 27 coumarins were sourced in sufficient quantity and purity for follow up evaluation as CA inhibitors (48). These NP coumarins, compounds **11–37** (Figure 4) comprise 24 plant coumarins (compounds **11**-**34**) and three marine coumarins (compounds **35**-**37**). Specifically, the plant NPs comprise avicennin **11**,(49, 50) trans-avicennol **12**,(51, 52) calanolide B **13**,(40, 53) dihydrogeiparvarin **14**,(54) geiparvarin **15**,(54, 55) dehydromarmin **16**,(54) xanthyletin **17**,(56) xanthoxyletin **18**,(51, 56) ceylantin **19**,(57) alloxanthoxyletin **20**,(56) fraxidin **21**,(58) fraxin **22**,(59) scopoletin **23**,(60) 6,7,8-trimethoxycoumarin **24**,(61) 5,7,8-trimethoxycoumarin **25**,(61) 7-hydroxy-8-methoxycoumarin **26**,(60) isoscopoletin **27**,(62)

fraxoside **28**,(63) scopolin **29**,(64) murralongin **30**,(65) (+)-isomurralonginol nicotinate **31**,(66) isophellodenol C **32**,(67) ellagic acid **33** (68) and nasutin B **34** (69). The ascidian NP coumarins include lamellarins E **35**,(70) B **36**,(71) and G 8-sulfate **37** (72). A variety of bioactivities have been reported for these coumarins, for example calanolide B **13** isolated from the tropical rainforest tree *Calophyllum lanigerum*, displayed protection against HIV-1 replication and cytopathicity (EC_{50} =0.4 μM).(53) Dihydrogeiparvarin **14** and geiparvarin **15**, both isolated from *Geijera parviflora*,(54, 55) possessed significant *in vitro* activity against human carcinoma of the nasopharynx.(73, 74) Xanthoxyletin **18**,(51, 56) purified from a variety of *Citrus* species, acts as a DNA-damaging agent,(75) while several synthetic derivatives have been shown to exhibit toxicity towards L-1210 leukemia cells with IC_{50} values ranging from 0.9 to 60.3 μM (75). The inhibition activity data for the NP coumarins **9** and **11-37** against hCA I and II (off-target isozymes), as well hCA IX and XII (isozymes of interest in cancer drug development) is presented in Table 1. Data for the simplest coumarin **8** and standard CA inhibitors (Figure 5) is included for development of structure-activity relationships (SAR).

Figure 4. NP coumarin library (**11-37**) sourced from Nature Bank (40, 48).

Compound	R_1	R_2
17	H	H
18	OCH_3	H
19	OCH_3	OCH_3

Compound	R_1	R_2	R_3	R_4
21	OH	OCH_3	OCH_3	H
22	OH	OH	OCH_3	H
23	H	OH	OCH_3	H
24	OCH_3	OCH_3	OCH_3	H
25	OCH_3	OCH_3	H	OCH_3
26	OCH_3	OH	H	H
27	H	OCH_3	OH	H
28	O″-D-Glc	OH	OCH_3	H
29	H	O″-D-Glc	OCH_3	H

Compound	R_1	R_2	R_3
33	H	H	H
34	CH_3	CH_3	CH_3

Compound	R_1	R_2	R_3	R_4	R_5	R_6
35	H	CH_3	CH_3	H	CH_3	OH
36	H	CH_3	H	CH_3	CH_3	OCH_3
37	CH_3	H	CH_3	H	SO_3Na	H

Figure 5. Standard CA inhibitors: acetazolamide **AZA**, zonisamide **ZNS** and topiramate **TPM**.

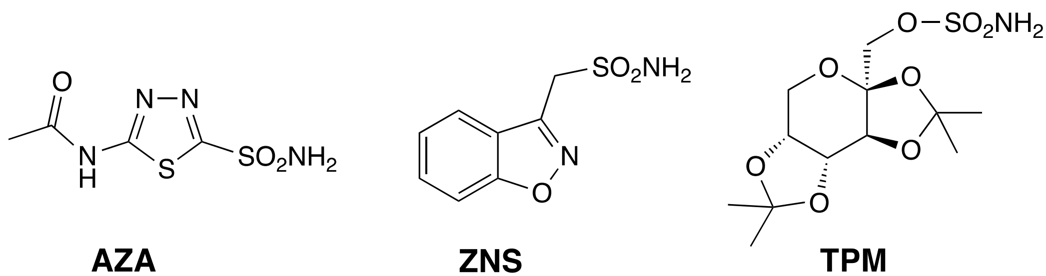


Table 1. Inhibition data for coumarins **8, 9**, **11-37** against hCA isozymes I, II, IX and XII.(48) Standard inhibitors (**AZA**, **ZNS** and **TPM**) are included for comparison (76-78).

Compd	K_i (μM)[a-c]			
	CA I	**CA II**	**CA IX**	**CA XII**
8	3.10	9.20	>1000	>1000
9	0.08	0.06	54.5	48.6
11	7.66	>100	0.62	0.79
12	8.46	>100	0.78	0.77
13	9.31	50.7	0.83	0.81
14	59.2	63.4	0.89	0.60
15	9.75	>100	0.60	0.83
16	7.81	>100	4.03	0.70
17	21.5	>100	7.51	25.7
18	7.71	>100	0.74	0.96
19	9.21	49.3	0.86	8.35
20	5.60	>100	3.50	9.10
21	9.89	>100	0.85	7.84
22	4.86	94.3	0.61	7.70
23	10.56	>100	0.96	4.05
24	0.0097	>100	6.58	18.2
25	4.31	9.65	0.76	0.83
26	36.4	>100	0.85	9.12
27	14.0	>100	7.37	4.14
28	5.04	>100	0.37	7.45
29	5.93	>100	8.72	0.78
30	9.11	>100	8.12	7.44

31	5.84	>100	0.67	7.39
32	7.52	78.9	9.75	0.77
33	68.2	>100	79.8	8.15
34	44.1	>100	17.4	7.42
35	6.45	>100	3.22	9.07
36	40.1	>100	6.33	8.51
37	6.55	>100	3.27	1.79
AZA	0.25	0.012	0.025	0.0057
ZNS	0.056	0.035	0.005	11
TPM	0.25	0.005	0.058	0.0038

[a]This inhibition data was acquired following a 6 h incubation time with enzyme using a stopped flow assay that monitors the CA catalyzed hydration of CO_2 (79). [b]Errors in the range of □ 5% of the reported value, from three determinations. [c]All proteins were recombinant.

Since the discovery of the NP coumarin **9** synthetic libraries of coumarins and thiocoumarins have been prepared and evaluated as CA inhibitors (80-82). The complexity and diversity of NP coumarin structures far exceeds that described for synthetic coumarin CA inhibitors. Coumarin **8**, the simplest coumarin, is not an appreciable inhibitor of CA IX or XII however it is a weak inhibitor of off-target CA I and CA II, with K_is of 3.1 and 9.2 μM, respectively. The NP coumarins are substituted at any of six available sites, with many fused to form tricyclic, tetracyclic or larger ring systems. This diversity does not readily allow simple SAR to be defined, however several trends surrounding CA inhibition are evident. Most obvious is that the NP coumarin library members are very weak CA II inhibitors, most have K_is > 100 μM, the only exception being the trimethoxycoumarin **25** (K_i = 9.65 μM). When compared to the structurally related methoxy/hydroxy coumarins **21-24**, **26** and **27**, compound **25** differs only in the pattern of substituents, this SAR indicates that it may be a combination of interacting substituents that directs the CA inhibition profile at CA II. At CA I, IX and XII many of the NP coumarins have K_is in the range of 1 – 10 μM, this tight grouping of K_is reflects minimal isozyme selectivity with these coumarins, however there are a few outliers to this general trend and these compounds represent interesting structures owing to their CA isozyme selectivity characteristics. At CA I there was one stand out compound being compound **24**, a nanomolar CA I inhibitor. This

trimethoxy coumarin is the most potent of any of the NP coumarins at CA I and is a structural isomer of **25**, the only potent CA II coumarin of the study. Around half of the NP coumarins have submicromolar inhibition of the isozymes CA IX and XII, some of these coumarins (**11**, **12**, **13**, **14**, **15**, **18** and **25**) are submicromolar at both CA IX and XII, while the remainder are submicromolar at either CA IX (**19**, **21-23**, **26**, **28** and **31**) or CA XII (**16**, **29** and **32**). This subset of NP coumarins has viable selectivity characteristics that warrant further studies in cell-based models of CA in cancer.

2. Phenols

The first single crystal X-ray structure of phenol **38** and a CA protein (hCA II) was reported in 1994 and identified that **38** binds in an unprecedented way within the enzyme active site.(83) It was shown that the phenolic OH interacts with the zinc-bound water molecule/hydroxide ion through a hydrogen bond while a second hydrogen bond formed between the phenolic OH and the NH amide of Thr199, an amino acid critical for the catalysis and inhibition of various CAs. NPs containing the phenol fragment **38** are highly abundant in nature. A substructure search of the DNP (2) against the phenol fragment identified >50,000 NPs from the 246,994 database entries that contain this fragment (~20% of all entries). Early CA inhibitory studies focused on simple, commercially available mono-, di- or tri-substituted phenols that are also found in nature (2) such as pyrocatechol **39**, resorcinol **40**, hydroquinol **41**, salicylic acid **42**, *p*-hydroxybenzoic acid **43**, *p*-coumaric acid **44**, caffeic acid **45**, ferulic acid **46**, gallic acid **47** and syringic acid **48** (Figure 6) (84, 85). A number of phenolic-NPs containing more complex scaffolds **49-57** have since been sourced from the Davis open-access compound repository housed at the Queensland Compound Library (QCL),(86) and screened against selected CAs (Figure 6) (87, 88). These phenolic-derivatives include the endophytic fungal metabolites, (-)-xylariamide A **49**,(89) and its synthetic enantiomer (+)-xylariamide A **50**,(89) xanthones **51** and **52**,(90) the marine ascidian-derived alkaloids, polyandrocarpamines A **53** and B **54** (91, 92) and the plant secondary metabolites, endiandrins A **55** (93) and B **56**,(94) and (-)-dihydroguaiaretic acid **57** (93, 94).

The phenols **38-57** have been evaluated for their inhibition of human cytosolic isoforms CA I and II (off-target) and mitochondrial isozymes CA VA and CA VB, Table 2. The latter have been recognized as potential targets for designing anti-obesity agents that act with a novel mechanism of action (95, 96). The simple phenolic secondary metabolites **38-48** have also been tested against hCA III, IV, VI, VII, IX, XII, XIII and XIV (84, 85). These data (not shown) indicate

that the phenol class of NP CA inhibitor exhibits complex SAR, with small chemical changes leading to large effects on CA enzyme inhibition. The chemical diversity of phenolic NPs is vast; so far investigation of this chemotype for its interaction with CAs is in its infancy.

Figure 6. NP phenols **38-57** tested as CA inhibitors (84, 85, 87, 88).

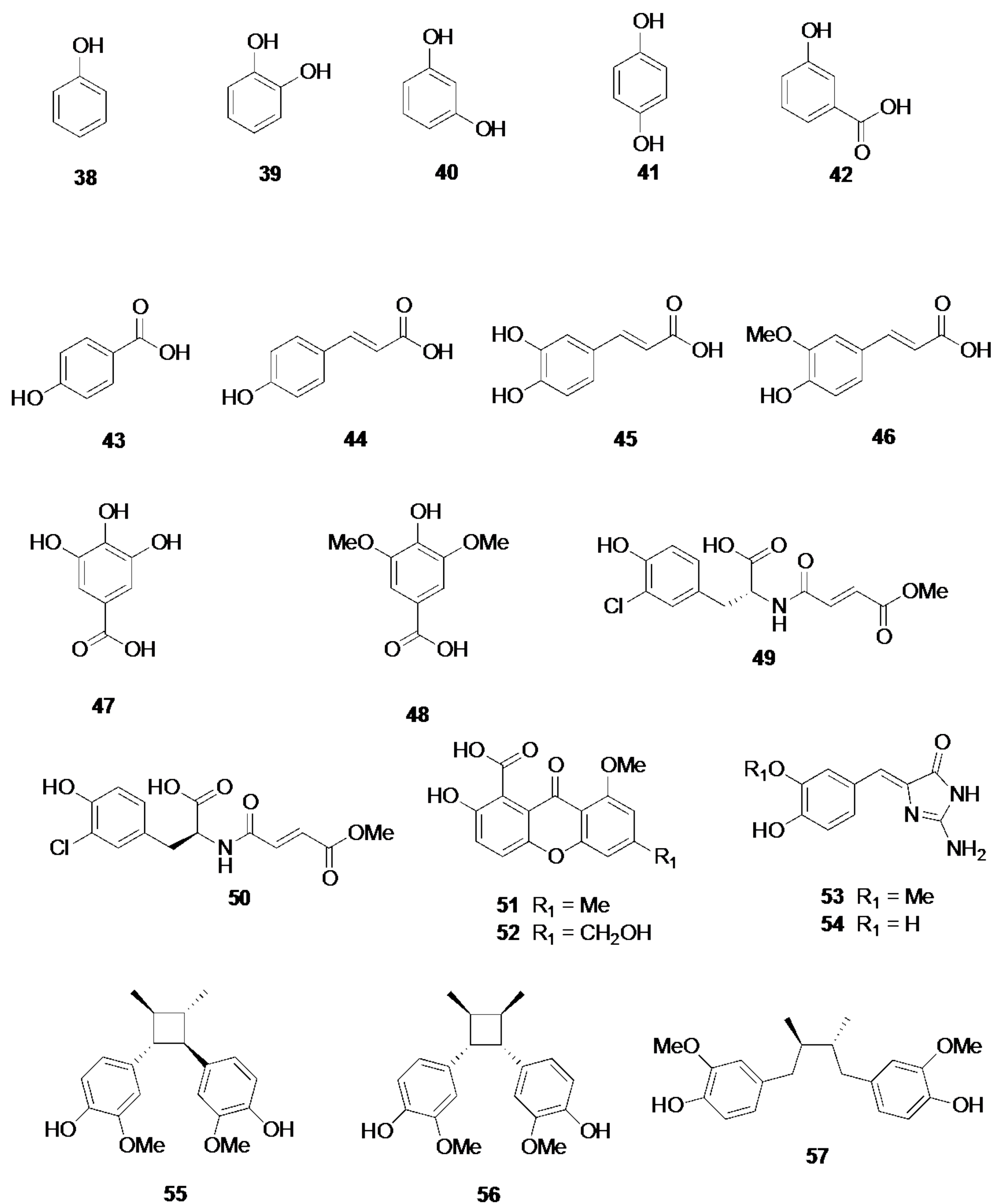


Table 2. Inhibition data for phenolic NPs **38-57** against hCA isozymes I, II, VA and VB (84, 85, 87, 88). Standard inhibitors **AZA**, **ZNS** and **TPM** are included for comparison (76-78).

Compd	K_i (μM)[a,b]			
	CA I	**CA II**	**CA VA**	**CA VB**
38	10.2	5.5	218	543
39	4003	9.9	55.1	4.2
40	795	7.7	8.7	7.1
41	10.7	0.090	14.1	12.5
42	9.9	7.1	678	355
43	9.8	10.6	9.2	10.5
44	1.07	0.98	5.9	7.7
45	2.38	1.61	6.5	9.1
46	2.89	2.40	7.0	10.5
47	3.20	2.25	4.1	9.9
48	4.15	3.19	6.3	35.4
49	239	8.3	0.095	0.114
50	231	8.0	0.108	0.102
51	201	8.4	0.093	0.103
52	374	9.2	0.094	0.102
53	10.5	9.6	0.099	0.070
54	355	13.1	0.101	0.076
55	368	11.7	0.093	0.069
56	354	12.1	0.098	0.079
57	307	230	0.085	0.071
AAZ	0.25	0.012	0.063	0.054
TPM	0.25	0.010	0.063	0.030
ZNS	0.056	0.035	0.020	6.3

[a]Errors in the range of □5% of the reported value, from three determinations. [b]All proteins were recombinant.

The β-CAs from *Helicobacter pylori*, *Candida albicans, Candida glabrata*, *Cryptococcus neoformans* and *Brucella suis* are essential for growth and have proven susceptible to inhibition with several compound classes including sulfonamides, carboxylates and boronic acids (97-104). A positive correlation from enzyme assays to a cell-based anti-infective phenotype assay demonstrates that the β-CAs from these pathogens are potential druggable targets for anti-infective therapies. Mammals possess only α-CAs, whilst many pathogenic organisms, such as

bacteria and fungi encode β-CAs. Similarly to α-CAs, a zinc cation defines the location of the active site of the β-CA enzymes. Phenols **49-57** (93, 94) along with the fungal NP phenols **58-62** (105-107) (Figure 7) have been screened for enzyme inhibition against selected pathogen β-family CAs, Table 3. CAs from *Mycobacterium tuberculosis*, *Candida albicans* and *Cryptococcus neoformans* were studied and selectivity towards the pathogen isozymes over human CAs was assessed.

Figure 7. Phenolic NPs **58-62** with activity against mycobacterial and fungal CAs (87).

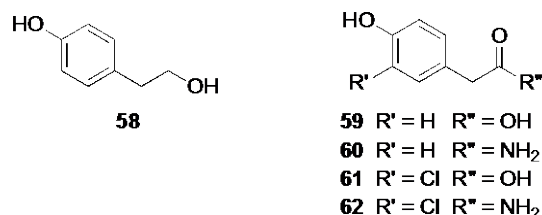


Table 3. Enzyme inhibition of pathogenic *M. tuberculosis* β-CA isozymes Rv3273 and Rv1284, *C. albicans* isozyme Nce103, *C. neoformans* isozyme Can2 and human α-CA isozymes I and II with the NP phenols **49-62** (87) and standard CA Inhibitors **AZA**, **ZNS**, and **TPM**.

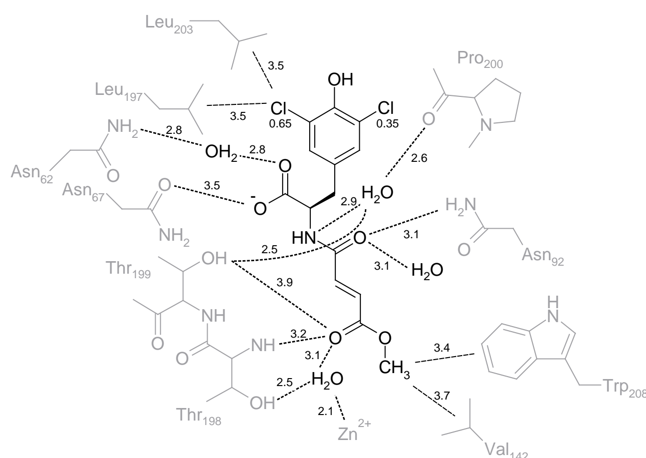
Compd	K_i (μM)[a,b]					
	CA I	**CA II**	**Rv3273**	**Rv1284**	**Nce103**	**Can2**
38	10.1	5.5	79.0	64.0	17.3	25.9
49	239	8.3	11.3	0.84	1.03	1.15
50	231	8.0	10.9	0.71	1.06	1.11
51	201	8.4	11.4	10.5	1.06	1.12
52	374	9.2	10.9	0.99	1.01	1.08
53	10.5	9.6	0.91	11.8	0.92	0.89
54	355	13.1	0.92	0.91	0.90	0.95
55	368	11.7	8.92	0.82	0.73	0.77
56	354	12.1	0.89	0.80	0.70	0.95
57	307	230	9.10	0.85	0.62	0.81
58	430	8.7	12.1	0.85	1.10	1.08
59	309	10.3	11.4	10.8	1.02	0.90
60	309	11.2	9.12	0.85	0.91	0.84

61	265	8.6	10.8	10.3	1.08	1.12
62	237	131	11.2	10.5	1.00	0.85
AAZ	0.25	0.012	0.10	0.48	0.13	0.01
TPM	0.25	0.010	3.02	0.61	1.11	0.37
ZNS	0.056	0.035	0.21	286.8	0.94	0.97

[a]Errors in the range of 5 % of the reported value, from [b]All proteins were recombinant.

These studies showed that several phenolic NPs were selective inhibitors of mycobacterial and fungal β-CAs, with the two best performing NPs identified as (-)-dihydroguaiaretic acid **57** and 3-chloro-4-hydroxyphenylacetamide **62**. Specifically, **57** was a sub-micromolar β-CA inhibitor with up to 495-fold selectivity over hCA I and 371-fold selectivity over hCA II. Compound **62** was also a low micromolar inhibitor of the fungal CAs and displayed 130- to 280-fold selectivity over the two human CAs. These compounds were the first non-sulfonamide inhibitors that display β over α-CA enzyme selectivity. In order to determine how the phenolic-based NPs **38-62** interacted with CAs, soaking and co-crystallization studies were undertaken with the readily available protein hCA II. While the most selective NPs **57** and **62** did not yield co-crystals with CAs suitable for X-ray diffraction studies, compound **50** [(+)-xylariamide A] did at a resolution of 2 Å. While it was predicted that the phenolic moiety present in **50** would play a major role in the hCA II binding it was discovered that instead the ester carbonyl of **50** interacts with a zinc-bound water molecule and is further engaged in a hydrogen bond donated by the backbone amide group of Thr198 (Figure 8). In this crystal structure the electron density of the inhibitor is well defined, allowing unambiguous placement of the ligand. This was a totally new binding mode to CAs.

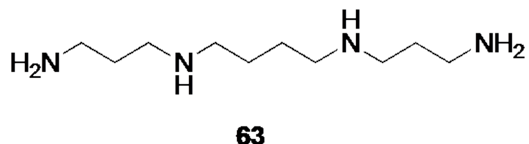
Figure 8. The ligand-protein interactions are illustrated schematically for hCA II in complex with compound **50** [(+)-xylariamide A]**.** Protein residues are shown in grey; hydrogen bond interactions are shown as dotted lines, van der Waals interactions as dashed lines. Distances are given in Å. Two conformations of the phenol moiety were observed, giving rise to the appearance of the chloro substituent on both sides of the phenolic hydroxyl group; the refined occupancies for both positions are noted at each position. Reprinted with permission from Davis, R. A.; Hofmann, A.; Osman, A.; Hall, R. A.; Mühlschlegel, F. A.; Vullo, D.; Innocenti, A.; Supuran, C. T.; Poulsen, S. A. *J. Med. Chem.* 2011, *54*, 1682. Copyright 2011 American Chemical Society.

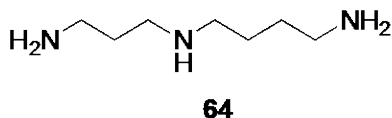


3. Polyamines

Polyamines belong to an alkaloid structure class and have been reported from various natural sources including terrestrial and marine animals, plants, fungi and bacteria (2). Two of the simplest polyamines isolated to date include spermine **63** and spermidine **64** (Figure 9). A substructure search of the DNP against **63** and **64** identified >400 NPs from the 246,994 database entries that comprise these alkaloid fragments (2). The polyamine chemotype has been shown to modulate multiple biological processes including gene expression, cell proliferation, translation, cell signaling, membrane stabilization and ion channel inhibition as well as antibacterial activity (108-114). Despite the myriad bioactivities reported for polyamine NPs until recently no CA inhibition had been reported. Carta *et al.* showed that **63**, **64** and several semi-synthetic polyamine analogues inhibited hCA I-XIV with K_i values ranging from low nanomolar to millimolar (115). A single crystal X-ray structure of spermine **63** with hCA II (at a resolution of 2.0 Å) was also reported (115) showing compound **63** anchored to the zinc bound water ligand (as for phenol **38**) through a network of hydrogen bonds. The terminal amine moiety of **63** is hydrogen bonded with residues Thr200 and Pro201. Notably **63** binds differently to hCA II when compared to either sulfonamides, phenols or coumarins and thus polyamines have the potential for the identification and development of additional CA inhibitors with a unique mechanism of binding and CA selectivity profile. This alkaloid structure class warrants further investigation and we expect that NPs will provide future opportunities to study additional polyamine alkaloids for CA inhibition.

Figure 9. NP polyamine CA inhibitors spermine **63** and spermidine **64** (115).





4. Semi-synthetic NPs modified to incorporate a ZBG and inhibit CA

4.1 Carbohydrate-ZBG hybrid molecules

Carbohydrates represent an abundant group of NPs and a selection of naturally occurring mono- and disaccharides have been modified to incorporate CA recognizing ZBG's to give glycosyl primary sulfonamides (sugar-SO_2NH_2),(116) glycosyl primary sulfamides (sugar-NH-SO_2NH_2) (117) and glycoconjugate sulfamates (sugar-O-SO_2NH_2) (118). Compounds **65-74** derived from the monosaccharides D-glucose, D-galactose, D-mannose and the disaccharide maltose are shown (Figure 10). An aromatic group, which is typical for classical CA inhibitors, is absent from these compounds and instead they comprise the hydrophilic mono- or disaccharide fragment directly attached to the ZBG. These NP-ZBG hybrid molecules have been evaluated as CA inhibitors, Table 4. All carbohydrate-ZBG hybrid compounds behaved as weak inhibitors of hCA I, with the anomeric sulfonamides **65-67** and sulfamides **68-70** also weak micromolar inhibitors of hCA II, IX and XII. In contrast the C-6 sulfamates **71-74** delivered good activity, particularly monosacchrides **71-73**, which showed K_i <10 nM against hCA XII. The glucose sulfamate **71** also has a K_i <10 nM at hCA IX and displayed selectivity for inhibiting the tumor-associated isoforms CA IX and XII over cytosolic CA I and II. The interested reader is directed to crystal structures for anomeric sulfonamides and glycoconjugate sulfamates in complex with hCA II in the PDB (accession codes: 3HKN, 3HKQ, 3HKT, 3HKU, 3T82, 3T83, 3T84, and 3T85).

The membrane permeability properties were measured for selected carbohydrate-ZBG hybrid CA inhibitors, the results confirm that the compounds are expected to have poor passive membrane permeability. cLog P is an indicator of passive diffusion through cell membranes and values <0 are indicative of molecules with poor membrane permeability. The cLog P values of the hybrid molecules **65-74** range from -2.7 for monosaccharides to -5.5 for dissacharides, Table 4. The compound design, employing a deliberate approach towards CA IX/XII isozyme selectivity by changing the physicochemical properties to impart poor membrane permeability, is consistent with these cLog P values. Membrane permeable ester 'prodrugs' of the carbohydrate-ZBG hybrids were also synthesized, this allows for potential oral administration, with the polar

carbohydrate-ZBG hybrid molecules ‘unmasked’ *in vivo* enabling targeting of extracellular CA IX and XII.

Figure 10. Carbohydrate-ZBG hybrid molecules **65-74**: glycosyl primary sulfonamides (sugar-SO_2NH_2), glycosyl primary sulfamides (sugar-NH-SO_2NH_2) and glycoconjugate sulfamates (sugar-O-SO_2NH_2) (116-118).

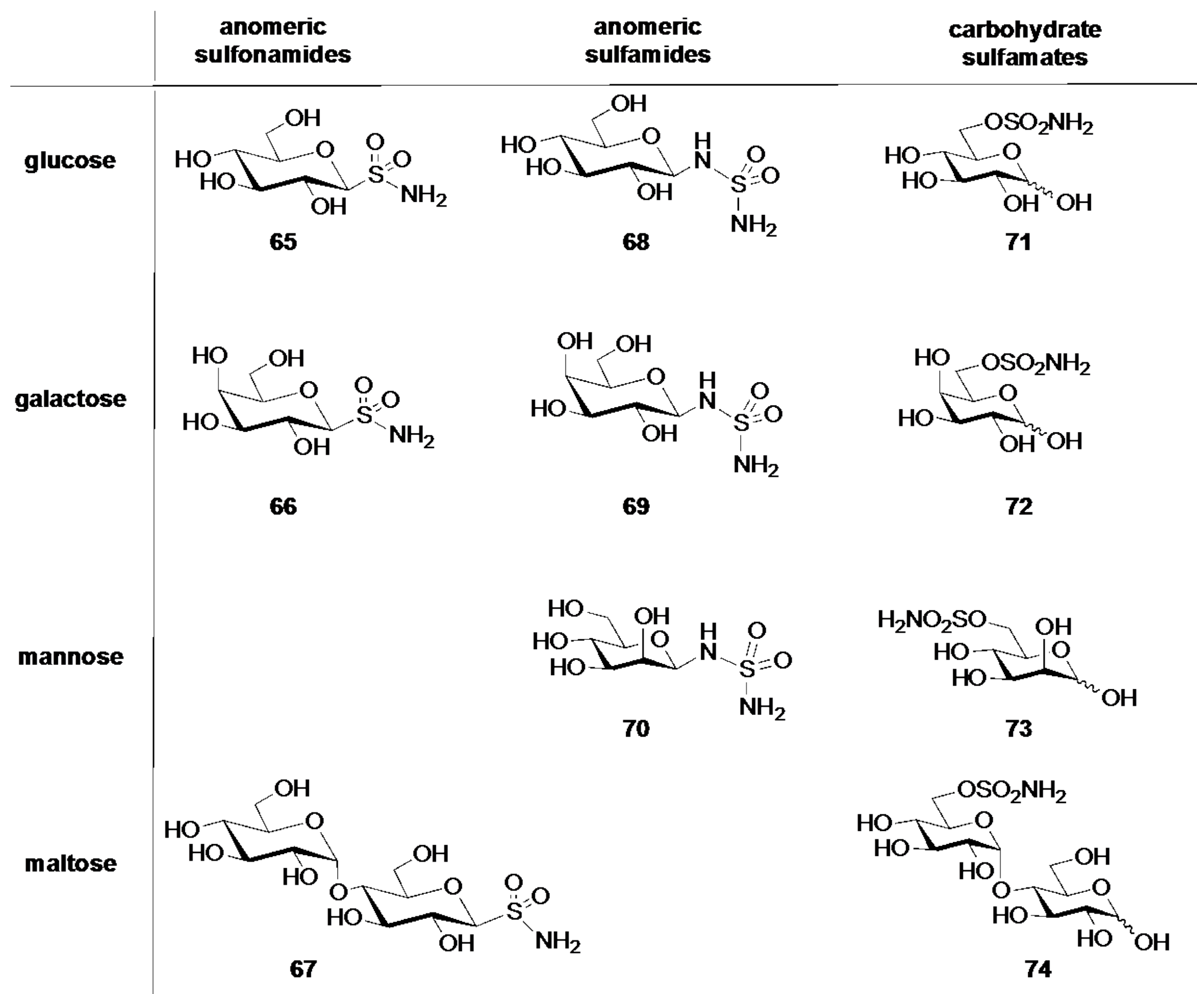


Table 4. Inhibition data of hCA Isozymes I, II, IX and XII and cLog P values for carbohydrate-ZBG hybrid molecules **65-74** (116-118).

Compd	K_i (nM)[a]				cLog P[d]
	CA I[b]	CA II[b]	CA IX[c]	CA XII[c]	

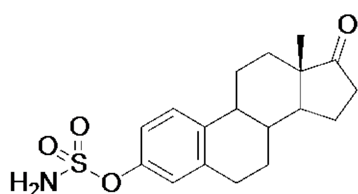
65	3,900	4,910	4,050	4,690	-2.8
66	3,930	4,550	4,190	4,800	-2.8
67	4,150	4,100	4,220	4,840	-5.0
68	75,400	4,680	6,470	1,970	-2.7
69	65,800	48,500	940	8,230	-2.7
70	91,900	21,200	1,790	5,440	-2.7
71	1180	82	8.6	7.3	-3.3
72	4500	93	62	7.6	-3.3
73	5960	104	53	9.5	-3.3
74	8750	513	497	138	-5.5

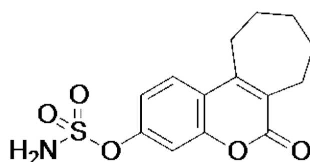
[a]Errors in the range of [illegible]05% of the reported value, from three determinations. [b]Human (cloned) isozymes. [c]Catalytic domain of human (cloned) isozymes. [d]cLog P data calculated using ChemBioDraw Ultra 11.0.

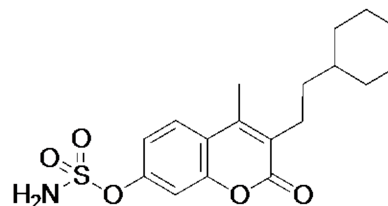
4.2 Coumarin-ZBG and steroid-ZBG hybrid molecules

A selection of NP-derived sulfamates are potent inhibitors of the cancer drug target steroid sulfatase (STS) and are being developed as a therapy for hormone-dependent breast cancer (119). This includes the steroidal sulfamate oestrone-3-*O*-sulphamate (EMATE) **75** and two coumarin based sulfamates, COUMATE-667 **76** and STX-118 **77** (Figure 11), which at a simpler structural level may also be considered phenolic sulfamates. These NP hybrids, modified with the sulfamate ZBG, are also potent CA inhibitors, Table 5 (120-122). It is hypothesized that dual steroid sulfatase/CA inhibitors may represent a novel method for treating hormone dependent breast cancer tumors, with the reversible binding of the sulfamates to erythrocyte CA II increasing the metabolic stability of the compounds by protecting the sulfamate moiety from rapid degradation (123). This indirect improvement of biopharmaceutical properties may persist alongside the direct effect of modulating the activity of cancer-associated CA IX and XII. The X-ray crystal structure of hCA II with both **75** (124) and **76** (123) are reported. These structures conform to the classical ZBG interactions with the sulfamate moiety binding to the active site Zn^{2+} cation. The steroid fragment of **75** and the coumarin fragment of **76** interact with the residues in the hydrophobic half of the CA II active site.

Figure 11. Steroidal sulfamate oestrone-3-*O*-sulphamate (EMATE) **75** and two coumarin based sulfamates, COUMATE-667 **76** and STX-118 **77**.







75 **76** **77**

Table 5. Inhibition data of hCA Isozymes I, II, IX and XII and cLog P values for steroid- and coumarin-ZBG hybrid molecules **75-77.** (nd = not determined)

Compd	***K*i (nM)**			
	CA I	**CA II**	**CA IX**	**CA XII**
75(120)	37	10	30	nd
76(121)	3450	21	34	12
77(122)	nd	IC_{50} = 59	nd	nd

Conclusion

Contemporary drug discovery is under increased pressure to identify more suitable small molecules as chemical starting points for drug development and finding novel compounds as starting points for optimisation is one of the major challenges in drug discovery research. NPs already provide a significant portion of FDA approved drugs and have emerged as an effective way to sample chemical diversity. The chemical diversity within NPs is vast and while the investigation of NP chemotypes for interaction with CAs is in its infancy, an encouraging start has been made. The NP compounds presented here (phenols, coumarins and polyamines) are suggestive of a tremendous opportunity that NPs provide for the discovery of novel chemotypes for selectively targeting either human or pathogen CAs. It will be imperative for future efforts to further evaluate the NP or NP-hybrid compounds in cell-based models of CA associated disease alongside classical control compounds for validation. The identification of unique CA binding for any NPs might offer possibilities for future rational drug discovery design and development. Thus the use of NPs in the search for new CA inhibitors has a strategic advantage since nature's unique chemical diversity has only been superficially explored in this particular field of research. We predict additional NP structures classes will be identified as binding to and perturbing CA function as further research is undertaken.

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