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Design and Development of Sulfonylurea Derivatives as Zinc Metalloenzyme Modulators

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Sulfonamide derivatives are an important class of zinc metalloenzyme inhibitors. While the sulfonylurea group is a bioisostere of sulfonamide, their effect on the modulation of metalloenzymes has never been studied. In the present work we synthesize and screen new sulfonylurea derivatives towards the zinc metalloenzymes, human carbonic anhydrase II (hCA II) and histone deacetylase 1 (HDAC 1). Surprisingly sulfonylurea derivatives exhibit activation of HDAC 1 rather than inhibition. The activation of HDAC 1 is not uniform across the derivatives tested suggesting a specific mode that depends on structural features of the compounds. Specific sulfonylurea derivatives are found to inhibit hCA II with IC_{50} in the nano molar to micro molar range. Docking studies indicate the binding of the inhibitors to the mouth of the active site cavity thereby blocking access to the enzyme.

Zinc containing metalloenzymes are implicated in a wide array of functions such as regulation of blood pH, digestion and transcription regulation.[1] Defects in zinc metalloenzymes or shortcomings in the modulation of their activity have been associated with clinical conditions such as cancer, glaucoma, heart diseases and HIV.[2-4] Zinc metalloenzymes like carbonic anhydrase (CA), angiotensin converting enzyme (ACE), and histone deacetylase (HDAC) are prominent pharmaceutical targets for the treatment of specific disease conditions.[5] Inhibitors and activators that target zinc metalloenzymes associated with the above disorders could be used for the treatment of the corresponding conditions. Notable zinc metalloenzyme inhibitors that have been developed for specific targets include Acetazolamide as carbonic anhydrase inhibitor (CAI), suberoylanilide hydroxamic acid (SAHA or Vorinostat) as histone deacetylase inhibitor (HDACI) and Captopril as Angiotensin Converting Enzyme Inhibitor (ACEI) (see Fig. 1).

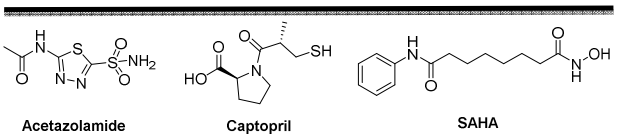

Fig. 1 Zinc Metalloenzyme Inhibitors

These inhibitors generally comprise of two main parts, a zinc binding group (ZBG) and a backbone. Hydroxamic acid is the most common ZBG followed by carboxylic acids, thiols, phosphonates and sulfonamides.[5] Sulfonamide derivatives are an important class of zinc metalloenzyme inhibitor especially for HDAC[6] and carbonic anhydrase.[7] While the sulfonylurea group is a bioisostere of sulfonamide, their effect on the modulation of metalloenzymes has never been studied to the best of our knowledge. In this work, we investigate the ability of sulfonylurea derivatives to modulate the activity of zinc metalloenzymes histone deacetylase and carbonic anhydrase.

Histone deacetylase (HDAC) is a prominent example of zinc-containing enzymes that have emerged as possessing significant clinical relevance. By catalyzing the reversible acetylation of histones, these enzymes affect chromatin structure and ultimately play a crucial role in gene regulation.[8] The covalent modification effected by HDACs is not limited to histones alone and non-histone proteins such as transcription factors can also undergo modification thereby permitting further regulation of gene expression.[9] Inhibitors of HDACs (also called histone deacetylase Inhibitors or HDACIs) have been the subject of intense research as they have been found to cause growth arrest, apoptosis or differentiation of tumor cells.[10] The beneficial role of an HDAC activator in the treatment of chronic obstructive pulmonary disease (COPD) has also been explored.[11] The rational design of HDAC modulators continues to hold promise not only in the context of anti-tumor agents but also for enhancing the understanding of HDAC-mediated gene regulation and its implication in various disease conditions.

The structure activity relationship (SAR) study of HDACIs has suggested a modular architecture comprising a ZBG to chelate zinc ion, a cap to sit at the mouth of a tunnel shaped cavity and a linker to connect a ZBG and cap (see Fig. 2). Further, the shape of the active site places an emphasis on the length of the linker. A shorter chain keeps the ZBG group hanging above the zinc ion in the active site while a longer chain restricts the cap to sit on the mouth of the tunnel shaped cavity.[12]

We have designed a series of novel molecules with sulfonylurea positioned as a ZBG, along with an alkyl linker and phenyl cap (see Fig. 2). Notably, the ZBGs that have been explored previously have resided at a terminal section of the molecule. Since the sulfonylureas used in present work are substituted at both ends, the size of the groups presented in the terminal part of the molecule is expected to play an important role. Analyses of crystal structure of HDAC (mainly class I HDACs) suggests the constrained nature of space near the zinc ion in the active site.[13, 14] Thus,

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presence of a bulky group adjacent to the ZBG may lead to steric problems for the prospective enzyme modulators. The linker length was designed to possess four carbons so as to mimic the natural substrate of HDAC namely *N*-acetylated lysine in which a chain of four carbons occupies the tunnel.

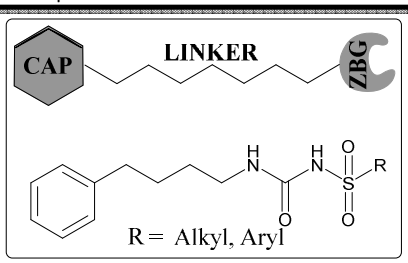
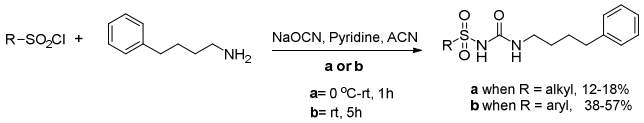


Fig. 2 Comparison of designed molecules with general pharmacophore for HDACIs

Synthesis of sulfonylureas with aliphatic sidechains is challenging owing to the limited literature on the subject.[15, 16] Our attempts to use common methods for the synthesis of sulfonylureas, namely treatment of sulfonamide with isocyanate in presence of base or conversion of sulfonamide into their carbamate derivatives followed by treatment with amines, did not yield desired aliphatic sulfonylureas. The synthesis of sulfonylurea by treatment of sulfonyl chloride with amine in presence of cyanate has been reported previously.[17, 18] We achieved synthesis of designed aliphatic sulfonylureas by treatment of the corresponding sulfonyl chloride with sodium cyanate in presence of base followed by addition of amine to yield the desired compounds (Scheme 1). The same principle has been suitably modified to generate a series of novel aromatic sulfonylurea (Scheme 1). All the synthesized compounds reported in Table 1 (except Compound **6**) are novel.



Scheme 1 Synthesis of sulfonylurea derivatives.

The compounds listed in Table 1 were screened for their inhibitory activity for histone deacetylase 1 (HDAC1) (using HDAC1 inhibitor assay kit from Cayman Chemicals). Test compounds were first incubated with enzyme and substrate followed by the addition of developer. Fluorescence was recorded using excitation wavelength of 350 nm and emission wavelength at 450 nm according to manufacturer instructions. Surprisingly all the compounds exhibit higher fluorescence compared to a negative control (with buffer) while a positive control (with SAHA) shows nearly complete inhibition of enzyme. The percentage increase in HDAC activity for the synthesized compounds at 10 uM and 100 uM concentrations are listed in Table 1. In order to verify that the increase in fluorescence was not due to non-specific interference with the screening assay, we tested our compounds against the developer, substrate and buffer. The results obtained from these tests were in the same range as that of other negative controls (wells with fluorescent developer, substrate and buffer) nullifying the chance of increase in fluorescence due to either the compound itself or interaction of compound with fluorescent substrate. Further, the observed variation in percentage activation for each compound points towards he specific interaction of the compounds with enzyme. We have located only one previous report on HDAC activators and the compounds described therein have a similar type of scaffold to sulfonylurea.[11] While all the compounds display significant percentage of activation, compound **6** appears to be an exception. Compound **1** with the smallest R group in the form of a methyl shows greatest activation. Increase in length of R group as present in compounds **2**, **3** and **4** results in decrease in activation of HDAC. Presence of aromatic R group in compounds **6**, **7**, **8**, **9** and **10** results in a lower percentage activation of HDAC. Docking studies were performed to obtain an insight into the binding mode of sulfonylurea derivatives with HDAC. Lack of crystal structures of HDAC1 led us to use HDAC2 for the docking studies with nearly 80% similarity in the structure and activity of the two isoforms.[19, 20] Docking of **1** clearly indicates interaction of the sulfonylurea group with the active site zinc ion of HDAC2. In addition to interactions of **1** with Gly 154 and Tyr 308, the terminal phenyl ring acts as a cap and sits at the entrance of the cavity. These features appear to facilitate occupation of the enzyme cavity by **1** (see Fig. 3). The variation in activation by the sulfonylureas is interesting and is currently being investigated in our lab along with a mechanistic study of activation performed by compound **1.** Since our designed molecules contained a four-carbon linker by way of mimicking a lysine substrate, we performed docking studies and calculated MMGBSA based binding energy to evaluate the effect of linker length on interaction with HDAC. Compounds designed with 1-3 carbon linker lengths displayed significantly lower docking scores and poor binding energy compared to compound **1**. While the binding pose of these shorter-linker compounds suggest proximity and interaction of the sulfonylurea group with active site zinc ion, the poor docking score is attributed to their ineffective capping ability owing to the shorter linker lengths (see Supporting Information, Table S1 and Fig S1).

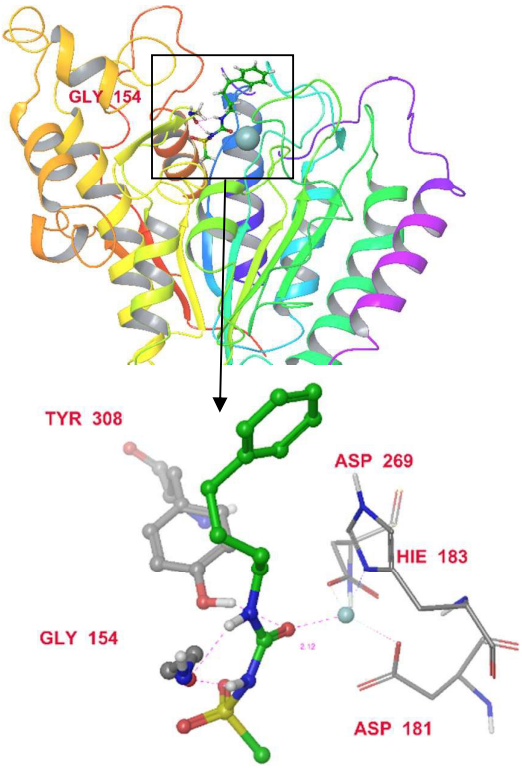


Fig. 3 Binding mode of compound **1** in active site of HDAC2

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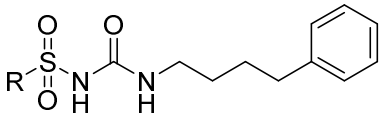
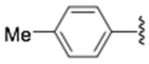
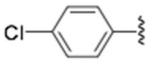
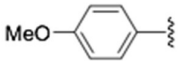
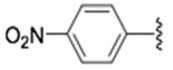
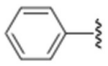


Fig. 4 General structure of compounds **1**-**10**

Table 1 List of compounds synthesized and their percentage activation of HDAC-1 at 10 uM & 100 uM concentration.

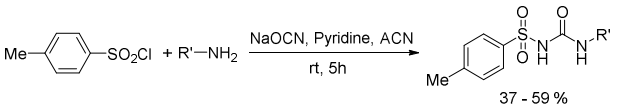
Compound	R	% Yield	% Activation of HDAC 1 (10 uM)[a]	% Activation of HDAC 1 (100 uM)[a]
1		18	57	83
2		14	22	31
3		16	23	33
4		15	20	30
5*		12	-	-
6		54	06	05
7		57	25	43
8		55	11	20
9		38	28	48
10		52	18	23

* Compound not involved in Biological assay. [a] Average of triplicate

The surprising activation of HDAC by sulfonylurea derivatives prompted us to investigate the effect of these compounds on carbonic anhydrase (CA). Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc metalloenzymes which catalyse the interconversion of carbon dioxide and bicarbonate ion. This interconversion plays a critical role in a variety of physiological processes such as CO_2/bicarbonate transport, pH maintenance, CO_2 homeostasis, electrolyte secretion in various tissues, biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification and tumorigenicity.[4] In mammals, 16 different isozymes of CAs have been reported that show wide variation in their tissue distribution and subcellular localization.[21] Modulation in activity of individual or multiple isoforms is well established in several disease conditions such as oedema,[22] glaucoma,[23] obesity,[24] cancer,[25] epilepsy and osteoporosis.[26] carbonic anhydrase inhibitors (CAIs) have been used clinically for the treatment of glaucoma and as a diuretics drug.[27] Association of membrane bound CA isoforms IX and XII to tumours has been well established and selective inhibitors of these CA isozymes could be potential clinical candidates for the treatment of cancer.[4] CAIs developed so far mainly comprise of sulphonamide or its bioisostere such as sulfamates and sulfamides. These bind in their deprotonated state to zinc metal ion present inside the active site of CA. Recently a new class of coumarin based natural products were reported as CA Inhibitors.[28] These compounds show a completely new mode of inhibition by binding to residues at the entrance of active site thereby resulting in its blockage.[29]

A few reports on modulation of CA activity discuss the coupling of sulfonylurea mainly as a backbone with either sulphonamide or coumarin (as ZBG) group. The inhibitory activity of such compounds has been primarily attributed to binding of the sulphonamide group with active site zinc ion.[30] To the best of our knowledge there is no report describing modulation of CA activity by sulfonylurea derivatives alone.

CA is known to possess a 15 Å deep cavity with the zinc ion seated at the bottom.[7] The tosylurido group has been suggested as conferring isoform specificity and greater affinity.[30] We thus incorporate the tosyl group at one end of the designed sulfonylurea derivatives. Synthesis of all designed compounds was achieved by using Scheme 2 with p-toulenesulfonyl chloride as one of the starting materials and by using various amines. Among the amines tested, the heterocyclic amines like 2-amino benzothiazole and 2-amino pyrazine were transformed into compounds **11** and **12**. Further, reaction with an aromatic amine such as aniline gives compound **13**, while aliphatic amines such as propyl amine, isobutyl amine and cyclohexyl amine give compounds **14**, **15** and **16**, respectively. Phenylethyl amine is used to prepare compound **17**. The secondary amine piperidine produces the corresponding sulfonylurea **18**. The compounds synthesized along with their percentage yield are listed in Table 2.



Scheme 2 Synthesis of sulfonylurea derivatives for CA

Compounds **11 – 18** as well as **1** - **10** were screened for their CA modulation by monitoring esterase activity using *p*-nitrophenyl acetate as a substrate.[5] hCA II was selected for studies since it is the prime target for anti-glaucoma drugs and also a physiologically abundant isoform.[21] All compounds were incubated with human carbonic anhydrase II (hCA II) at 37 ^{o}C for about 10 min followed by addition of substrate to start the reaction. Absorption was recorded at 405 nm and percentage inhibition of CA activity by all test compounds was calculated and listed in Table 2.

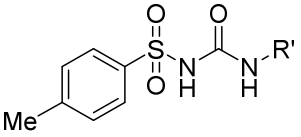


Fig. 5 General structure of compounds 11-18

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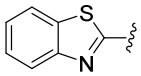
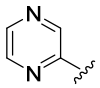
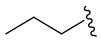
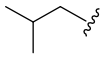
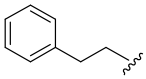
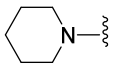
Table 2 List of compounds synthesized and their percentage Inhibition of hCA II at 10 uM concentration and their IC_{50}

Compound	R'	Yield	% Inhibition of hCA II (10 uM)[b]	IC_{50} (uM)[c]
11		41	10	NA
12		45	95	0.59 ± 0.02
13		59	86	0.58 ± 0.02
14		53	48	≥50
15		52	78	3.03 ± 0.4
16		37	21	NA
17		59	37	NA
18		55	79	2.07 ± 0.35

NA= Not Active. [b] Average of triplicate. [c] Values were the average of three different experiments run in triplicate ± SD.

SAR studies suggest that compounds with a four carbon linker between the amine *N* and phenyl ring (Compounds **1**-**10**) do not show any significant modulation of hCA II activity (see Supporting Information, Table S2). Variation in linker length between amine *N* and phenyl ring shows that compounds with linker length 4 and 2 do not show any significant inhibitory property, while the compound with no linker group namely **13** exhibits nearly complete inhibition of hCA II. Steric effects are clearly at play in the inhibition as evident from the behaviour of compounds **14** and **15**. While **14** and **15** have minor differences in structure they exhibit dramatically different inhibitory activities. Comparison of the activities of compounds **13**, **16** and **18** indicate planarity of the ring to be an important factor. Loss of partial planarity in compound **18** compared to compound **13** results in a drop in inhibitory activity which diminishes significantly in case of compound **16**.

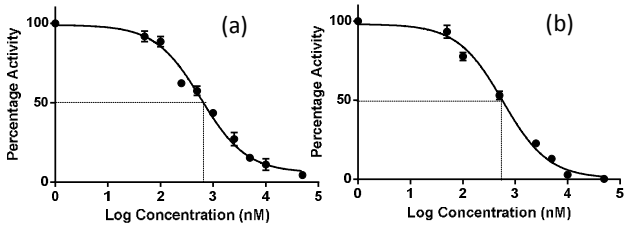


Fig. 6 Screening of compound **12** (a) and compound **13** (b) against hCA II using its esterase activity at various concentrations.

IC_{50} value of compounds showing at least 50% inhibition of hCA II activity at 10 uM concentration were calculated and reported in Table 2. These compounds display a strong dose dependent response (see Fig. 6).

Determination of IC_{50} values of other compounds was attempted but was unsuccessful due to their poor inhibitory property. It may be noted that compounds **11** – **18** displayed insignificant activation of HDAC1 (see Supporting Information, Table S3).The biological assays highlight sulfonylurea derivatives with specific substituents as exercising inhibitory action. To get insight into the mode of binding and inhibition of our sulfonylurea derivatives for hCA II, we performed a FRET-based assay using dansylsulfonamide. The assay is based on the formation of a FRET pair between an active site tryptophan and dansylsulfonamide that binds in the active site.[31, 32] First dansylsulfonamide (25 uM) was incubated with hCA II and FRET was observed by exciting the tryptophan at 280 nm and recording dansylsulfonamide emission at 470 nm. Addition of acetazolamide (competitive inhibitor with better potency) is expected to completely replace dansylsulfonamide from the active site. The significantly lower FRET upon addition of acetazolamide agrees with the expected behaviour. Next, the same scheme was used for testing compounds **12** and **13**. Based on the changes in FRET, compound **13** is able to displace dansylsulfonamide in a manner comparable to that of acetazolamide. In contrast compound **12** only shows partial displacement of dansylsulfonamide over a period of time (see Table 3). These results reflect the differences in ability of the compounds **12** and **13** to bind the enzyme active site. Notably, compounds **12** and **13** display similar magnitude of inhibition. Further, while the sulfonylurea derivatives are likely binding in the active site, their mechanism of inhibition could be by direct chelation of zinc akin to sulphonamides or by blocking access to the active site such as that performed by coumarins.

Table 3 Percentage FRET observed for dansylsulfonamide in presence of Acetazolamide, Compound **12** and **13**

Sl No	Inhibitor (10 uM)	% FRET (0 min)[d]	% FRET (10 min)[d]	% FRET (20 min)[d]
1	-	100	100	100
2	Acetazolamide	5	5	5
3	Compound **12**	86	63	57
4	Compound **13**	8	12	14

[d] Errors are in range of 5-10% from three separate experiments

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Molecular docking studies were performed to understand the mode of binding of sulfonylurea derivatives in the active site of hCA. Docking studies were performed with compounds **12**, **13**, **15** and **18** using GLIDE (Schrodinger, LLC, NY, USA). The results show that all four compounds bind to residues at the entrance of the cavity. This mode of binding is similar to that of recently reported coumarin derivatives, albeit the residues involved in the interactions are different.[29] Binding pose analyses reveals that these compounds form a network of H-bonds with water present inside the cavity owing to their multiple hydrophilic residues. They also form H-bonds with residues Gln 92 and Asn 67 (see Fig. 7 and Fig S2-S4). Docking results shed light on the inhibitory action of specific sulfonylurea derivatives. In particular our results show that sulfonylurea derivatives bind to residues present at the entrance of the active site cavity thereby preventing access of substrate to the active site. The compounds that fit precisely on the mouth of cavity exhibit the best inhibition. The sulfonylurea derivatives with bulkier or smaller substituents do not fit into the mouth of the cavity and thus lack the inhibitory property. This mechanism explains the differences in inhibitory activity of various sulfonylurea derivatives and especially the difference in activity of compounds **14** and **15**.

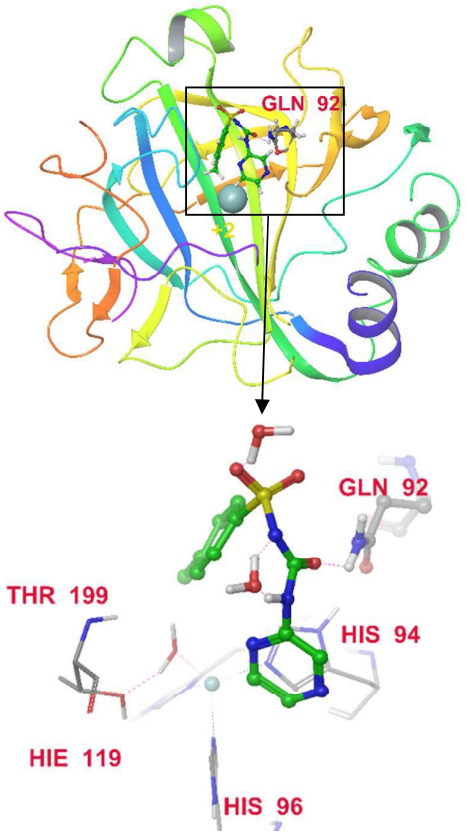

Fig. 7 Binding mode of compound **12** in active site of hCA II

Conclusions

We have explored the modulation of zinc-metalloenzymes HDAC-1 and hCA II in the present study. A series of novel sulfonylurea derivatives (compounds **1**-**10,** except compound **6**) were synthesized. The designed molecules were screened for their HDAC inhibition but surprisingly display activation of HDAC. A new set of sulfonylurea derivatives were synthesized with tosylurido group as common scaffold. Screening of sulfonylurea derivatives against CA reveals that compounds **12**, **13**, **15** and **18** exhibit nearly complete inhibition of CA II activity at 10 uM concentration and compound **12** and **13** shows IC_{50} of approximately 600 nM. FRET based assay using dansylsulfonamide indicates that the sulfonylurea derivatives bind in the active site region. Molecular docking studies revealed that all active sulfonylurea derivatives bind to residues at the entrance of the active site thereby blocking access and resulting in enzyme inhibition. The results presented in this work are promising due to the modular character of the compounds being prepared as well as indications of their desirable steric and electronic properties. Further exploration of the current strategy will enable stronger modulators of the metalloenzymes to be developed.

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