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Glycosidic carbonic anhydrase IX inhibitors: A sweet approach against cancer

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Dedicated to Professor Jean-Louis Montero on the occasion of his 70th birthday.

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ABSTRACT

Targeting tumour associated carbonic anhydrase (CA, EC 4.2.1.1) isoforms IX and XII is now considered as a pertinent approach for the development of new cancer therapeutics against hypoxic tumours. In the last period, with the help of X-ray crystallography, much progress has been achieved for the drug-design of selective CA IX inhibitors, by considering the three main structural elements that govern both potency and selectivity, that is, a zinc binding group (ZBG), an organic scaffold, and one or more side chains substituting the scaffold. The use of sugar moiety in the structure of sulfonamide-based CA inhibitors (CAIs), has allowed the discovery of very potent CA IX inhibitors able to impair the growth of both primary tumors and metastases. The search for specific CA IX inhibitors by using the sugar approach has become an important research field, leading to sulfonamides, sulfamates, sulfamides and coumarins with excellent in vitro activity and relevant potency in vivo, in animal models of cancer. This paper will review the latest development in this hot topic.

1. Introduction

1.1. CA IX as therapeutic target—relevance compared with other isoforms

Highly sophisticated molecular mechanisms are responsible for pH regulation in tumor cells, that is, maintaining a slightly alkaline intracellular (pHi) and and acidic extracellular (pHe) pH in tumor cells, which allow them to survive and proliferate in hypoxia, but also favoring metastasis.[1–6] They include both proteins which import weak bases (such as bicarbonate) within the cells, or others which extrude the weak acids generated during metabolism, such as CO_2/carbonic acid or lactic acid.[1] The main metabolic acids are carbonic acid, formed by the hydration of CO_2 which is the final product of all oxidative processes, and lactic acid which is formed through the glycolytic transformation of glucose in hypoxic conditions (glucose has an increased uptake in tumor cells, the so-called Warburg effect), not yet explained even 80 years after its discovery.[1,6] In addition, there are also molecular mechanisms by which the H^+ ions are directly extruded from the cells, either in exchange for other cations (such as Na^+) or through the energy furnished by the hydrolysis of ATP, by means of the vacuolar ATPase V-ATPase.[3–9] Among these proteins are also two carbonic anhydrases (CAs, EC 4.2.1.1), metalloenzymes catalyzing the hydration of CO_2 to bicarbonate and protons,[10] which are overexpressed in many tumors and present a limited expression in normal tissues.[11]

Indeed, α-CAs are widespread metalloenzymes in higher vertebrates, including humans.[10,12] Sixteen isozymes have been characterized to date in mammals, which differ in their subcellular localization, catalytic activity, and susceptibility to different classes of inhibitors. There are cytosolic isozymes (CA I, CA II, CA III, CA VII and CA XIII), membrane bound ones (CA IV, CA IX, CA XII, CA XIV and CA XV), mitochondrial (CA VA and CA VB) and secreted (CA VI) isoforms. Three acatalytic forms, called CA-related proteins (CARPs), CARP VIII, CARP X and CARP XI, are also known.[13,14] Most CAs are very efficient catalysts for the reversible hydration of carbon dioxide to bicarbonate and protons ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$), which is the only physiological reaction in which they are involved.[10,12,15] CA isoforms are involved in critical physiological processes such as respiration and acid–base regulation, electrolyte secretion, bone resorption, calcification and biosynthetic reactions which require bicarbonate as a substrate (lipogenesis, gluconeogenesis, and ureagenesis).[10,12] Two CA isozymes (CA IX and CA XII) are predominantly associated with and overexpressed in many tumors, being involved in critical processes connected with cancer progression and response to therapy.[10,12,16–18] CA IX is confined to few normal tissues (stomach and body cavity lining), but it is ectopically induced and highly overexpressed in many solid tumor types, through the strong transcriptional activation by hypoxia,

Abbreviations: hCA, human carbonic anhydrase; CARP, carbonic anhydrase related protein; HIF-1, hypoxia inducible factor 1.

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accomplished via the hypoxia inducible factor 1 (HIF-1) transcription factor.[2,9,12,16,18,19] Interestingly, CA IX is the most strongly overexpressed gene in response to hypoxia in human cancer cells[18] and it is also the most active CA isoform for the CO_2 hydration reaction.[19,20] Its X-ray crystal structure has recently been reported[21] evidencing a dimeric enzyme, unique among all CAs known so far. CA XII is also a transmembrane isoform with an extracellular active site, similar to CA IX[17] but its catalytic activity is lower compared to CA IX.[10,22] Similar to CA IX, CA XII is expressed in many tumors but it is also more diffuse in some normal tissues.[17] Since CO_2 is the main byproduct of all oxidative processes, being thus generated in large amounts in metabolically active tissues, and as its spontaneous hydration is a very slow process, the CAs play a fundamental role in acid–base equilibria in all systems, including tumors.[3,4,10,12] Considering the fact that these are relatively simple enzymes, rather well characterized biochemically, with a multitude of known inhibitors[10,12] it appeared of great interest to investigate whether their inhibition may lead to an antitumor effect. In fact, in the last years both CA IX and XII have been validated as antitumor targets.[1]

1.2. The sugar approach

One of the most successful approaches for designing CA inhibitors (CAIs) targeting all isoforms known to date, was denominated 'the tail approach'.[15] It has been initially developed for the synthesis of sulfonamide CAIs but later extended to many other classes of such compounds.[10,15] The tail approach originally consisted in attaching water-solubilizing tails to different scaffolds of well-known aromatic/heterocyclic sulfonamides possessing affinity for the CA active site, assuring in this way the possibility to modulate in greater details the physico-chemical properties of these pharmacological agents. Later, the nature of the 'tail' moieties was very much varied, incorporating all possible moieties (lipophilic, positively-charged, metal-coordinating groups, etc.) as it has been observed that the tails fragments bind towards the entrance of the active site of the CA isoforms,[23] a region which has the highest variability among the different isoforms, and this leads to compounds which are able to discriminate between the various isoforms and are thus isozyme-selective CAIs.[10,15] A very good example of such 'tails' is constituted by sugars, which represent a wide range of chemotypes, leading thus to a high number of new CAIs.[24] This has been denominated the 'sugar approach' by Winum et al. and led to highly successful drug design examples in the field of CAIs targeting many isoforms, but mostly the tumor-associated ones CA IX/XII. This topic will be discussed in the following part of this review.

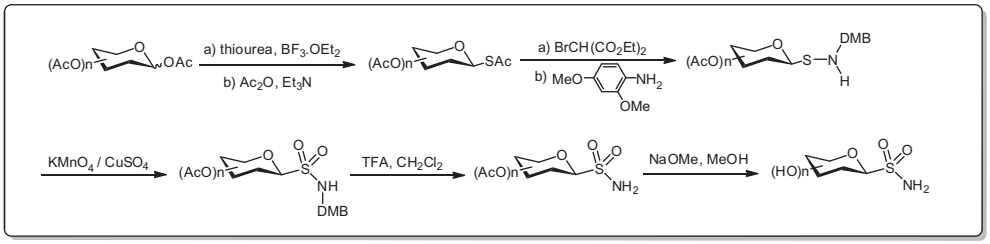
2. Inhibition of CAIX with glycosidic inhibitors

2.1. Anomeric sulfonamides

Anomeric sulfonamides are a class of glycosides which possess a sulfonamide moiety directly attached to the anomeric center of a carbohydrate. Only recently suitable synthetic strategies has been developed to prepare these chemical entities.[25] Poulsen's group has reported an efficient methodology for the synthesis of *S*-glycosyl sulfonamides through oxidation of 2,4-dimethoxybenzyl protected sulfenamides (Scheme 1).[26] These compounds have been prepared by reaction of glycosyl thioacetates with diethyl bromomalonate and 2,4-dimethoxybenzylamine. Subsequent oxidation with $KMnO_4/CuSO_4$ and removal of the protecting group under acidic conditions afforded the per-O-acetylated glycosyl sulfonamides (10–45% yields over three steps). The O-acetate groups of the carbohydrate moiety were removed using Zemplen's conditions to afford the fully deprotected *S*-glycosyl sulfonamides in good to high yields.

The anomeric sulfonamides were screened using the CO_2 hydration assay against the cytosolic hCA I and hCA II isozymes, as well as cancer-associated hCA IX and XII.[27] Strikingly the sulfonamides showed no isozyme selectivity with Kis in the micromolar range and neither the carbohydrate moiety nor the nature of the hydroxyl groups impacted to alter enzyme inhibition profile. Using protein X-ray crystallography, Poulsen and co-workers demonstrated that the shape of the glycosyl sulfonamides resulted in weak interaction of the inhibitor with the enzyme active site. The high resolution X-ray structures of hCA II in complex with galactose (**7**), lactose (**4**) and maltose (**10**) derivatives show that the binding mode of the sulfonamide moiety to the catalytic zinc ion is invariant (Fig. 1). Moreover the carbohydrate moieties are not causing a significant change in configuration of active site residues. Topiramate (**TPM**), an anti-epileptic fructopyranose sulfamate, has been shown to be a good inhibitor of CAs.[28] This compound presents a zinc binding function tethered to the anomeric pyranose center and comparison with its X-ray structure in complex with hCA II showed that anomeric sulfonamides were not able to form as many direct interactions as topiramate. The sugar moieties of sulfonamides do not provide sufficient transverse bulk to span the active site cleft as topiramate, which leads to a high degree flexibility of ligand conformations and to less fewer interactions of the inhibitor with the enzyme active site.

In the development of anti-cancer compounds that target selectively the membrane bound isoform CA IX versus the ubiquitous isoform CA II, the design of membrane non-permeant inhibitors is crucial. Poulsen's group had calculated the lipophilicity and



1 R=Ac
6 R=H

2 R=Ac
7 R=H

3 R=Ac, R^1=CH_3
8 R=R^1=H

4 R=Ac
9 R=H

5 R=Ac
10 R=H

Scheme 1. Synthesis of anomeric sulfonamides.

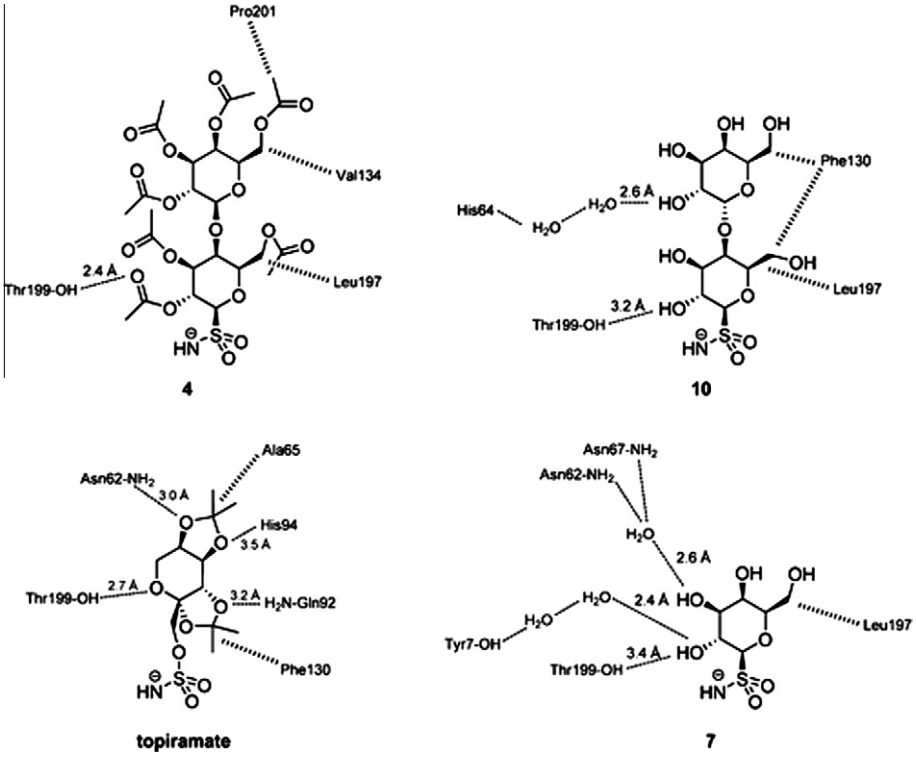


Figure 1. Binding of anomeric sulfonamides **4**, **7**, **10** and topiramate to hCA II active site. The figures represent distances in Å.

topological polar surface area for the S-glycosyl sulfonamides showing that all compounds fall within the range indicative of molecules with poor membrane permeability. The authors also measured apparent in vitro effective permeability (Pe) using a parallel artificial membrane artificial assay (PAMPA).[29] Although it was not possible to measure Pe of anomeric sulfonamides due to the analytical limits of the method, the results suggested that the compounds have poor passive membrane permeability. Though anomeric sulfonamides showed no selectivity for the cancer associated CAs, their physicochemical properties would lead to preferential inhibition of the transmembrane CA IX over cytosolic CA II.

2.2. Anomeric sulfamides

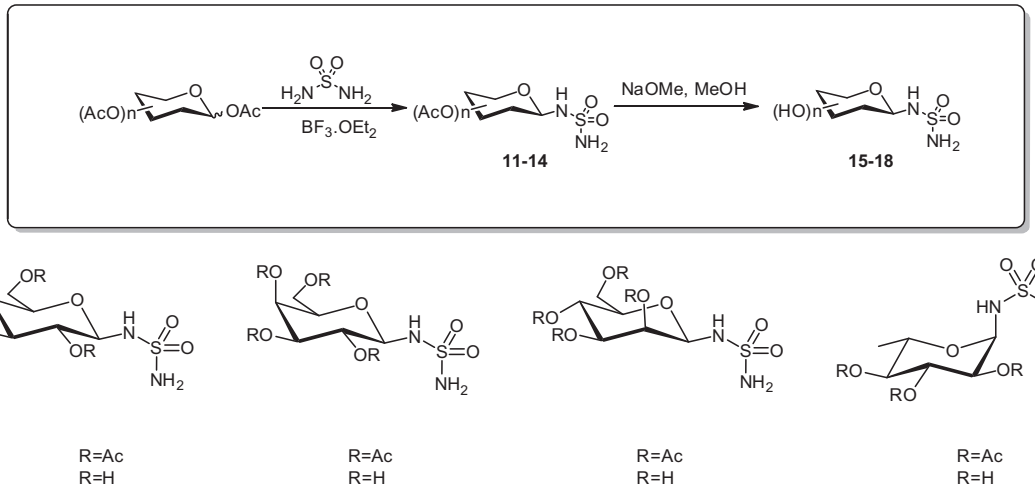
An unusual glycosidase resistant replacement for the glycosidic linkage is the sulfonamide group corresponding to the union of a glycosylamine carrying a sulfonyl group at nitrogen. However, glycosylamines are prone to hydrolysis and anomerization. To overcome this problem one of our groups has developed several sulfonamidoglycosylations of sugar derivatives.[30–39] Previous studies demonstrated that the sulfamide group is a suitable zinc binding function (ZBF) for CA inhibitors. Very recently we have reported the synthesis of *N*-glycosyl sulfamides, a new class of compound wherein a sulfamide moiety is directly attached to the anomeric center of a monosaccharide.[34]

Per-O-acetylated pyranose derived from the monosaccharides D-glucose, D-galactose, D-mannose and D-rhamnose were reacted with boron trifluoride diethyl etherate and sulfamide to provide the β-sulfamideglycosides in very good yields (Scheme 2). Next Zempleńs conditions were applied to afford the fully deprotected *N*-glycosyl sulfamides in nearly quantitative yields. Enzyme inhibition data was determined for physiologically dominant hCA I and II and cancer associated hCA IX and XII.[35] Per-O-acetylated compounds were micromolar inhibitors of hCA I, while deprotected carbohydrate derivatives showed a diminished affinity. A similar pattern was found for the dominant isoform hCA II although acetylated glycosyl sulfamides were quite effective inhibitors in the micromolar range. These compounds (**11**–**14**) also were shown to be very good hCA IX inhibitors with inhibition constants clustered below 8 nM (5.0–7.7 nM) with very good selectivity over CA I (480- to 1800-fold) and up to 17-fold selectivity against CA II. On the other hand the deacetylated sulfamideglycosides were weaker inhibitors of CA IX (0.9–6.5 μM). Deprotected sulfamides inhibited selectively this isoform over CA II (7- to 52-fold) with the exception of glucosyl sulfamide **15**. Calculated physicochemical properties of sulfamide glycosides (topological polar surface area and lipophilicity) showed that all compounds fall within the range indicative of molecules with poor membrane permeability and thus would lead to preferential inhibition of CA IX over the ubiquitous cytosolic hCA II in vivo.

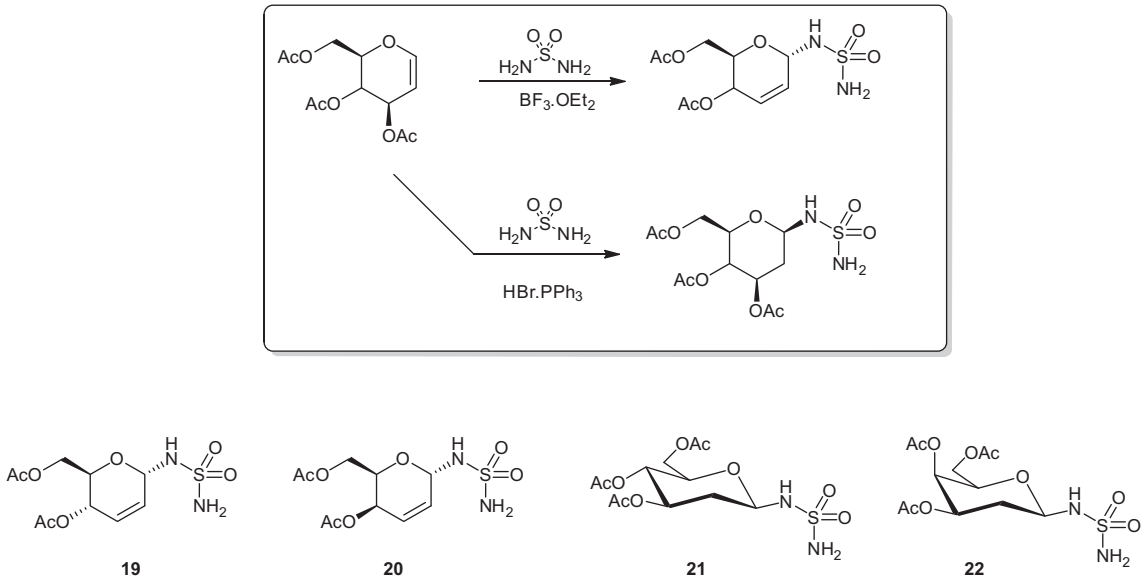
A small series of *N*-glycosyl sulfamides have been prepared by reaction of per-O-acetylated glycals with sulfamide in the presence of two different catalyst. Ferrier sulfonamidoglycosylation catalyzed by boron trifluoride diethyl etherate afforded the 2,3-unsaturated glycosyl sulfamides **19** and **20** in good yields with excellent α-stereoselectivity (Scheme 3).[33] Alternatively reaction in the presence of triphenylphosphine hydrobromide provided the β-*N*-2-deoxy-glycosyl sulfamides **21** and **22**.[30] The sulfamide glycosides were potent inhibitors of hCA II, IX and XII in the nanmolar range but weaker inhibitors of hCA I (0.42–0.83 μM).[36] Erythro derivative **19** was a very effective hCA IX inhibitor (9 nM) and showed sixfold selectivity against hCA II. Probably the 4-acetyl moiety participates in a clash with an amino-acid residue within the hCA II active site, which leads to this decreased affinity. On the other hand, in its threo epimer **20**, the negative interaction is not present, and the inhibitor shows no selectivity.

2.3. 6-Sulfamoyl carbohydrates

Recently Poulsen's group has developed a synthesis of carbohydrate sulfamates from mono- and disaccharides through selective



Scheme 2. Synthesis of *N*-β-glycosyl sulfamides.

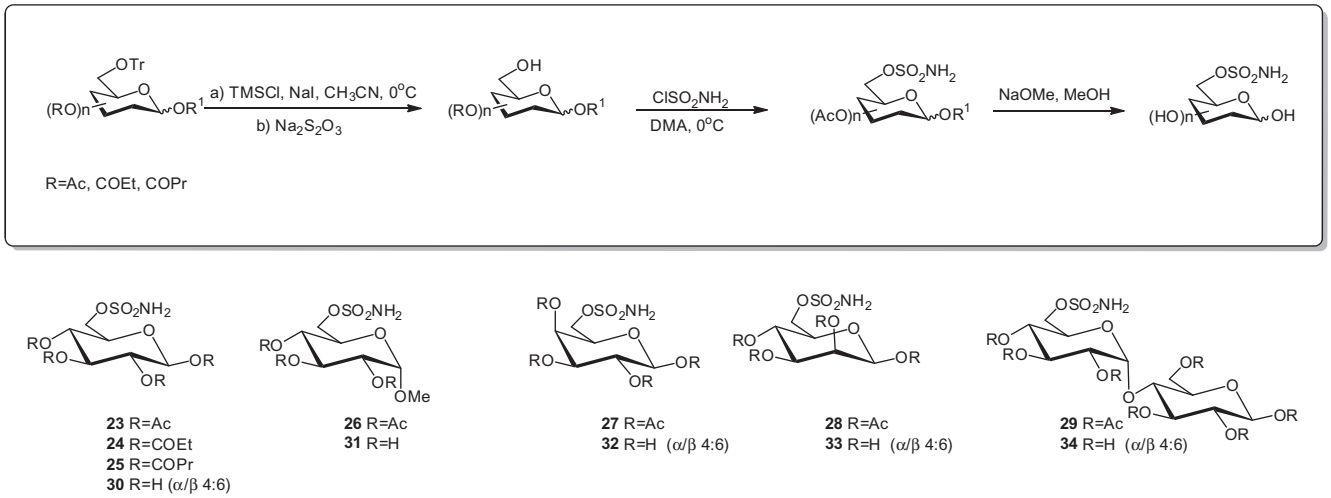


Scheme 3. Synthesis of glycosyl sulfamides.

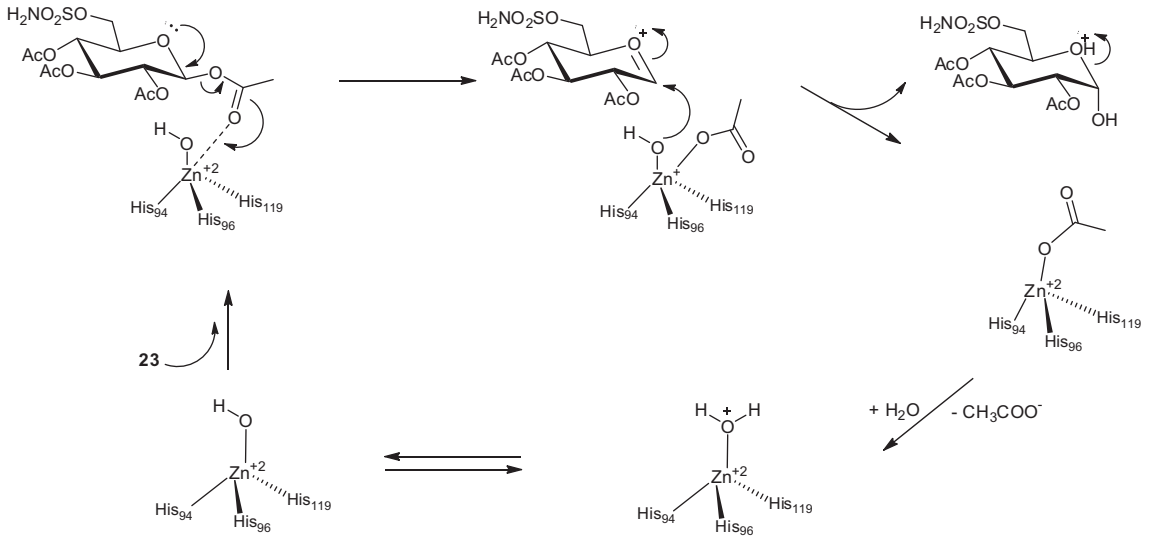
sulfamoylation of the C-6 hydroxyl group (Scheme 4).[37] The first step involved selective protection of 6-position of the monosaccharides (or 6′ for disaccharides). The remaining hydroxyl groups were acylated with different carboxylic acid anhydrides (acetic, propionic or butyric). Deprotection of C-6 (or C-6′) primary hydroxyl groups followed by selective reaction with sulfamoyl chloride generated in situ, afforded the per-O-acylated carbohydrate sulfamates. Zemplen's conditions were employed to remove the ester protecting groups to prepare the fully deprotected compounds.

The new compounds were weak inhibitors of CA I in the micromolar range. The carbohydrate sulfamates behaved as quite effective inhibitors of the dominant isoform CA II in the low nanomolar range (11.3–307 nM), especially the acetylated mannose derivative **28** (K_i = 11.3 nM). Several sulfamate compounds were very good hCA IX inhibitors and exhibited a wide range of Kis from 7.8 to 86 nM. The glucose derivatives **26**, **30** and **31** were also selective inhibitors of CA IX in the range of 8- to 14-fold compared to CA II. Poulsen et al. have also calculated several physicochemical properties of the new sulfamates. Acyl protected **23**–**29** compounds were predicted to be orally available and may be used as ester prodrugs. Once in the body, the esters groups could be readily hydrolyzed by ubiquitous esterases to give deprotected parent sulfamates that would selectively target the extracelullar CA IX due to their poor membrane permeability.

Very recently the same group had demonstrated that sulfamate carbohydrates are not only inhibitor but also substrate for hCA II.[38] Protein X-ray crystallography was used to study the interaction of per-O-acetylated sulfamate carbohydrates with active site residues of hCA II. Surprisingly hydrolysis of the hydroxyl protecting groups was observed. In all cases hydrolysis of C-1 and C-2 acetyl group was observed, while hydrolysis of C-3 acetate was observed in D-galactose derivative. Using bioaffinity mass spectrometry methodology (BAMS) the authors proposed a mechanism for the hydrolysis of the sugar acyl groups where the first step involves cleavage of the most labile anomeric acetate with formation of the oxocarbenium cation (Scheme 5). The zinc bound hydroxyl then reacts to afford the axial anomer. The zinc bound hydroxyl is regenerated after acetate displacement by water and the hydrolysis may continue with the next nearest carbonyl group. The results suggested that initially, the substrate binding mode dominates, but



Scheme 4. Synthesis of carbohydrate sulfamates.



Scheme 5. Proposed mechanism for the sugar acyl group hydrolysis.

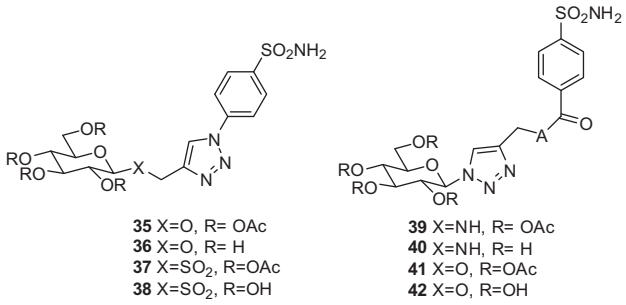
following hydrolysis, the sulfamate carbohydrate can also bind as a pure inhibitor.

3. Inhibition of CA ix with conjugated sugar-based inhibitors

Targeting of trans-membrane CAs over cytosolic one by incorporation a carbohydrate tail group into the inhibitor structure has been developed by several research groups and much progress has been made these last 5 years through modifications of the inhibitors scaffold structure.

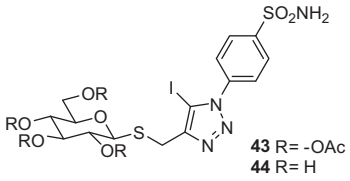
3.1. Sulfonamide series

Previous work has allowed to Poulsen and Supuran research groups to observe that the CA active sites are tolerant to diverse structural characteristics within the tail moiety of inhibitors glucoconjugate CA inhibitors wherein the glucosyl moiety is either a free sugar or a peracetylated sugar and spacer linkage between sugar moiety and phenylsulfonamide pharmacophore which can be either 1,2,3-triazoles with O-glucoside **35**, **36** or sulfonyl glucoside **37**, **38**, or with amide **39**, **40** or ester **41**, **42** covalent linkages (Scheme 6).[24]

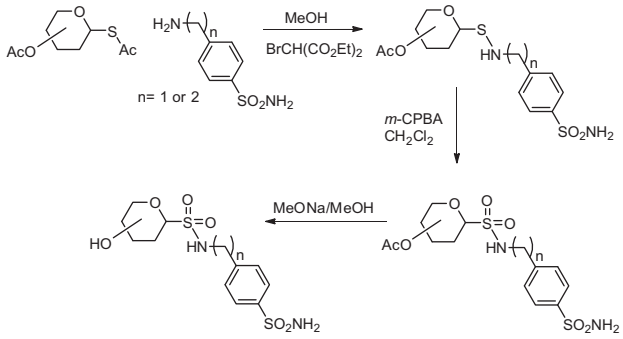


Scheme 6. Previously reported glucoconjugate CA inhibitors.

Based on the same scaffold, Poulsen and co-workers have reported very recently new series of inhibitors where the H atom at the 5-position of the triazole moiety is substituted by an iodine atom.[39] Among these compounds, two very effective CA IX and CA XII inhibitors **43** and **44** were identified (Scheme 7). These inhibitors were studied in two fibroblast cell lines—one lacking endogenous expression of CA IX and one overexpressing CA IX. It was



Scheme 7. Glucoconjugate in *S*-glucosyl series with iodine atom at the 5 position of the triazole.



Scheme 8. *S*-Glycosyl sulfonamide and *S*-glycosylsulfenamide synthesis.

reported that these two new inhibitors can selectively blocked CA IX-induced survival and extended studies with the per-acetylated analogue **43** showed that it reduced membrane-associated CA activity to a similar level as that obtained in cells lacking CA IX.

Lopez et al. described recently the synthesis of sulfonamide-linked neoglycoconjugates developing two new series of inhibitors that comprise both *S*-glycosyl sulfenamides and *S*-glycosyl sulfonamides derived from monosaccharide glucose, galactose, glucuronic acid and from disaccharide lactose and maltose.[40] A general model of these compounds is depicted in Scheme 8. Synthesis of this family of inhibitor is described starting from the corresponding peracetylated glycosyl thioacetate and reaction with aminobenzyl or aminophenethyl sulfonamides to give the per -O-acetylated sulfonamide glycoconjugates. These later were then oxidized to lead to per-O-acetylated glycosylsulfonamidyl benzenesulfonamides. Fully deprotected sugar analogues can be then obtained under Zemplen's conditions (Scheme 8).

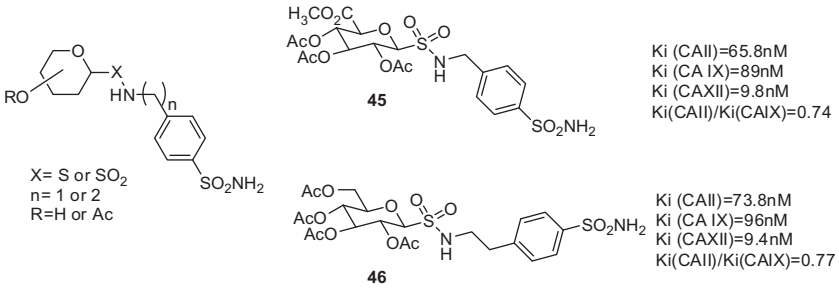
The CA inhibition profile of the library of 30 neoglycoconjugates, demonstrated inhibitory activity in the range of 80–120 nM against CA IX and below 20 nM against CA XII with in both case selectivity ratio below 1 against the physiologically abundant CA II. Two examples of inhibitors **45** and **46** are shown in the Scheme 9.

This class of compound exhibited poor selectivity against CAIX versus CAII on purified protein. No in vivo data are currently available in literature for these compounds. Nevertheless, considering the physicochemical properties of these compounds, especially the poor lipophilicity of free sugar moiety, the fully deprotected neoglycoconjugates would be expected to have poor passive membrane permeability and thus may preferentially inhibit the transmembrane CAs IX and XII over cytosolic CAs in the in vivo environment.

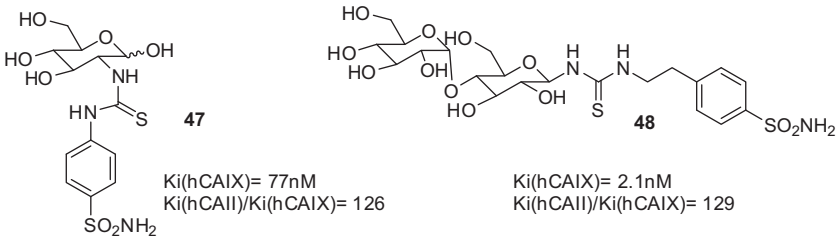
Very recently, the same group reported a series of glycosyl-thioureido sulfonamides where the thioureido function is connected on the anomeric position of the sugar moiety.[41] This work based on previously reported structure by our group[42] where the thioureido function is linked on the position 2 of the sugar, gave new compounds with low nanomolar binding to CAs IX and XII (such as **47**) but with the best selectivity ratios of 129-fold detected for the maltosyl derivative **48** (Scheme 10).

3.2. Coumarin series

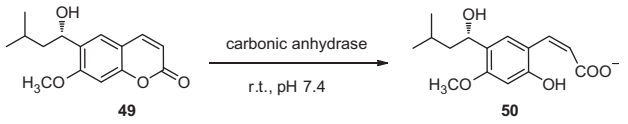
Coumarins are a class of widely spread natural compounds which was reported to possess carbonic anhydrase inhibitory properties. They exhibited a completely unprecedent inhibition mechanism, which was only recently deciphered. A natural product coumarin **49**, was shown to be carbonic anhydrase inhibitor (Scheme 11).[43] By means of X-ray of its adduct with hCA II, it was evidenced the presence of a 2-hydroxy-cinnamic acid derivative **50** in the enzyme active site, which is the hydrolysis product of the original coumarin.[44] The 2-hydroxy-cinnamic acid formed



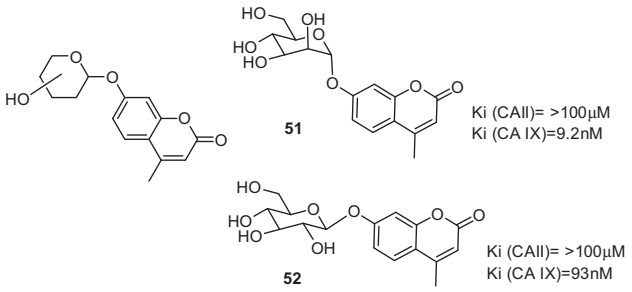
Scheme 9. *S*-Glycosyl sulfonamide and *S*-glycosylsulfenamide model.



Scheme 10. Glycosyl-thioureido sulfonamides.



Scheme 11. Formation of 2-hydroxy-cinnamic acid by CA mediated hydrolysis of coumarin.



Scheme 12. Glycosylcoumarins models.

from the original coumarin was found to occlude the entrance to the enzyme active site, a mechanism never evidenced before for CA inhibition.

Recently our group described the synthesis of 7-substituted coumarins incorporating various glycosyl moieties (Scheme 12). Investigation of their inhibition profile against physiologically relevant CA isoforms revealed that these compound are weak inhibitor of the housekeeping, offtarget isoform CA I and CA II but very effective against CAIX and CAXII with activity in the nanomolar range.[45] A very interesting feature of some of these new derivatives was their potent inhibition of growth of primary tumours and metastasis in a human and mouse model of orthotopic, CA IX-positive breast cancer. Such inhibitors as **51** and **52** thus constitute interesting candidates for the development of conceptually novel anticancer drugs based on a novel mechanism of action that takes advantage of the overexpression of CA IX and CA XII in hypoxic tumours.[46]

4. Conclusion—future prospect

The regulation of pH in tumors involves the interplay of many proteins, among which two carbonic anhydrases (CA IX and XII), which are overexpressed in tumors via the HIF-1 pathway, being also bad prognostic factors for most of these tumors. The concerted action of these proteins assures a slightly alkaline internal pH (pHi) and an acidic external pH (pHe) within the tumors, which favors proliferation of the primary tumor and formation of metastases. Inhibition studies of one or more of them with specific inhibitors of the sulfonamide, sulfamate, sulfamide and coumarin types was shown to lead to the return of both pHi and pHe towards normal values with the consequent impairment of the tumor growth. This constitutes an antitumor mechanism not exploited by the classical anticancer drugs. Thus, both CA IX and CA XII are validated antitumor targets, and their inhibition with antibodies, sulfonamide and coumarin inhibitors was undoubtedly proven to reverse the effect of tumor acidification, leading to the inhibition of the cancer cells growth. This approach may be useful for both imaging and treatment purposes of tumors overexpressing these two enzymes, and some sugar-containing inhibitors seem to be the most promising such tools at this moment. The paper reviewed the field of the drug design of sulfonamide/sulfamate/sulfamides as well as coumarin CA IX/XII inhibitors and their application for developing novel antitumor therapies.

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