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Diuretics with carbonic anhydrase inhibitory action: a patent and literature review (2005 – 2013)

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Introduction: The benzothiadiazines and high ceiling diuretics (hydrochlorothiazide, hydroflumethiazide, quinethazone, metolazone, chlorthalidone, indapamide, furosemide and bumetanide) contain primary sulfamoyl moieties acting as zinc-binding groups in the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). These drugs are widely used clinically and were recently shown to weakly inhibit isoforms CA I and II, but to possess stronger activity against isoforms involved in other important pathologies, for example, obesity, cancer, epilepsy and hypertension.

Areas covered: The class of clinically used diuretics, with CA inhibitory properties, is the main topic of the review. A patent literature review covering the period from 2005 to 2013 is presented.

Expert opinion: This section presents an overview of the patent literature in the sulfonamide diuretic field. Most of the patents deal with the combination of diuretic sulfonamide CA inhibitors with other agents useful in the management of cardiovascular diseases and obesity. Such combinations exert a better therapeutic activity compared to similar diuretics that do not inhibit CAs, raising the question of the polypharmacological and drug repositioning effects of these old drugs. These effects seem to be due to the potent inhibition of such drugs against CA isoforms present in kidneys and blood vessels, which explain both the blood pressure lowering effects as well as organ-protective activity of the drugs. An explanation of these data is provided by the fact that inhibition of the renal CAs leads to a large increase of the nitrite excretion in urine, suggesting that renal CAs are involved in nitrite reabsorption in humans. Important lessons for the drug design of sulfonamide CA inhibitors (CAIs) can be drawn from these data.

Keywords: carbonic anhydrase inhibitor, chlorthalidone, diuretic, furosemide and bumetanide, hydrochlorothiazide, hydroflumethiazide, indapamide, metolazone, quinethazone, sulfonamide

Expert Opin. Ther. Patents [Early Online]

1. Introduction

A number of 16 different α-carbonic anhydrase (CA, EC 4.2.1.1) isoforms have been described so far in mammals [1-5]. They catalyze the interconversion between CO_2, bicarbonate and protons, generating thus two of the ions (HCO_3^- and H^+) critical for pH regulation and many other physiological processes [6,7]:

$$CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$$

The human (h) CA isoforms possess a different catalytic activity, subcellular localization, tissue distribution and susceptibility to be inhibited by sulfonamides, the main class of CA inhibitors (CAIs) [1-5,7,8]. There are several cytosolic forms

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Article highlights.

- Many of the clinically used diuretics, such as hydrochlorothiazide, hydroflumethiazide, quinethazone, metholazone, chlorthalidone, indapamide, furosemide and bumetanide contain primary sulfamoyl moieties and inhibit CA isoforms from the kidneys and other organs.
- The CAIs clinically used, such as acetazolamide, methazolamide, ethoxzolamide and dichlorophenamide have different inhibition profiles compared to the thiazide- and high-ceiling diuretics incorporating SO_2NH_2 groups.
- A beneficial effect of indapamide/chlorthalidone/hydrochlorothiazide for the treatment of patients with hypertension and type 2 diabetes was observed, thought to be due to the potent inhibition of these drugs against CA isoforms present in kidneys and blood vessels, leading to blood pressure lowering effects as well as organ-protective activity of the drugs.
- Such polypharmacological/drug repositioning effects may be useful in therapy and in the drug design of sulfonamides possessing various biological activities correlated to the CA inhibition (antiobesity, antiepileptic and anticancer activity).
- There is a stringent need for new diuretic drugs to treat edema, hypertension, obesity and cardiovascular morbidities ensued by such conditions, since no new sulfonamides with such effects were launched clinically for a long period, although several recent patents report interesting furosemide derivatives.
- Most of the analyzed patents report the combination therapy of these drugs with other agents, mainly for the management of cardiovascular diseases.

This box summarizes key points contained in the article.

(CA I–III, CA VII and CA XIII [6-10] as well as the acatalytic CA VIII, X and XI [9]), five membrane-bound isozymes (CA IV, CA IX, CA XII, CA XIV and CA XV), two mitochondrial forms (CA VA and VB [11-15]), as well as one secreted CA isozyme, CA VI [2,3]. CO_2, bicarbonate and the protons generated in the CA-catalyzed reaction are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity and many other physiological/pathological processes [1,11-20].

2. Role of CAs in the kidney

Carbonic anhydrases (CAs) are highly abundant in the kidneys (a total concentration of about 8 – 10 μM has been estimated for this organ), and many isoforms have been shown to be present in various tissues of this organ [21-25]. In humans the CA isoforms present in kidneys are CA II, IV, VB, IX, XII and XIV and they play a crucial function in at least three physiological processes: i) the acid–base balance homeostasis (by secreting and excreting protons, due to the CO_2 hydration reaction catalysed by these enzymes); ii) the bicarbonate reabsorption processes and iii) the renal NH_4^+ output [25-29]. These important functions are well localized in the different segments of the nephron: bicarbonate reabsorption occurs in the proximal tubule, whereas urinary acidification and NH_4^+ output occur in the distal tubule and collecting duct [25-29].

Carbonic anhydrase localization in the kidneys has been extensively studied by histochemistry and immunohistochemistry [21,25,30-32]. Of the catalytically active CA isozymes, at least four (CA II, IV, XII, and XIV) were shown to be present in the epithelial cells of renal tubules [30-35]. In the intercalated cells of the late distal tubule, the collecting tubule and the collecting duct, high levels of CA II have been reported [25,30-35]. The CA II-positive segments along the mouse nephron and collecting ducts include the proximal convoluted (S1 segment) and straight tubules (S2 – S3 segments), the descending thin limb of Henle, the thick ascending limb of Henle, the distal convoluted tubule and the intercalated and principal cells of the collecting ducts [25,30-35]. On the basis of the distribution pattern described above, it is obvious that CA II is widely expressed in the kidney where it plays a key role in renal functions. Indeed, such a fundamental role of CA II in renal physiology was documented by Sly *et al.* [36] who reported renal tubular acidosis (as well as brain calcification and osteopetrosis) in patients with CA II-deficiency syndrome [37]. The membrane-associated isoforms CA IV, IX, XII and XIV are also present in various tissues of the kidneys: CA IV seems to be ubiquitous in this organ [37], whereas CA XII is expressed at the basolateral plasma membrane of the epithelial cells in the thick ascending limb of Henle and distal convoluted tubules, and in the principal cells of the collecting ducts [38]. CA XIV was found to be highly expressed in some segments of rodent nephron [24]. Strong signal was found in apical plasma membranes of the S1 and S2 segments of the proximal tubules, and weaker staining in the basolateral membranes as well as in the initial portion of the thin descending limb of Henle [24]. The positive staining in the proximal tubules suggested that CA XIV might also function in urinary acidification together with other isoforms present in this organ, that is, CA II, IV, XII and so on. Finally, CA IX is highly overexpressed in renal tumors [39-41]. A substantial amount of the renal physiology has been understood in detail by inhibiting CAs present in this organ by using sulfonamide CAIs [14,27,42,43].

3. Sulfonamide CAIs with diuretic action

Sulfonamides possessing the free SO_2NH_2 moiety act as potent inhibitors of the CAs and bind in the deprotonated form (as sulfonamidate anions) to the Zn(II) ion from the enzyme active site, thus impairing the catalytic cycle [1-4,44-46]. Many such compounds have been in clinical use for decades as antiglaucoma and diuretic agents [1-4,44-46], whereas a large number of recent patents deal with the development of

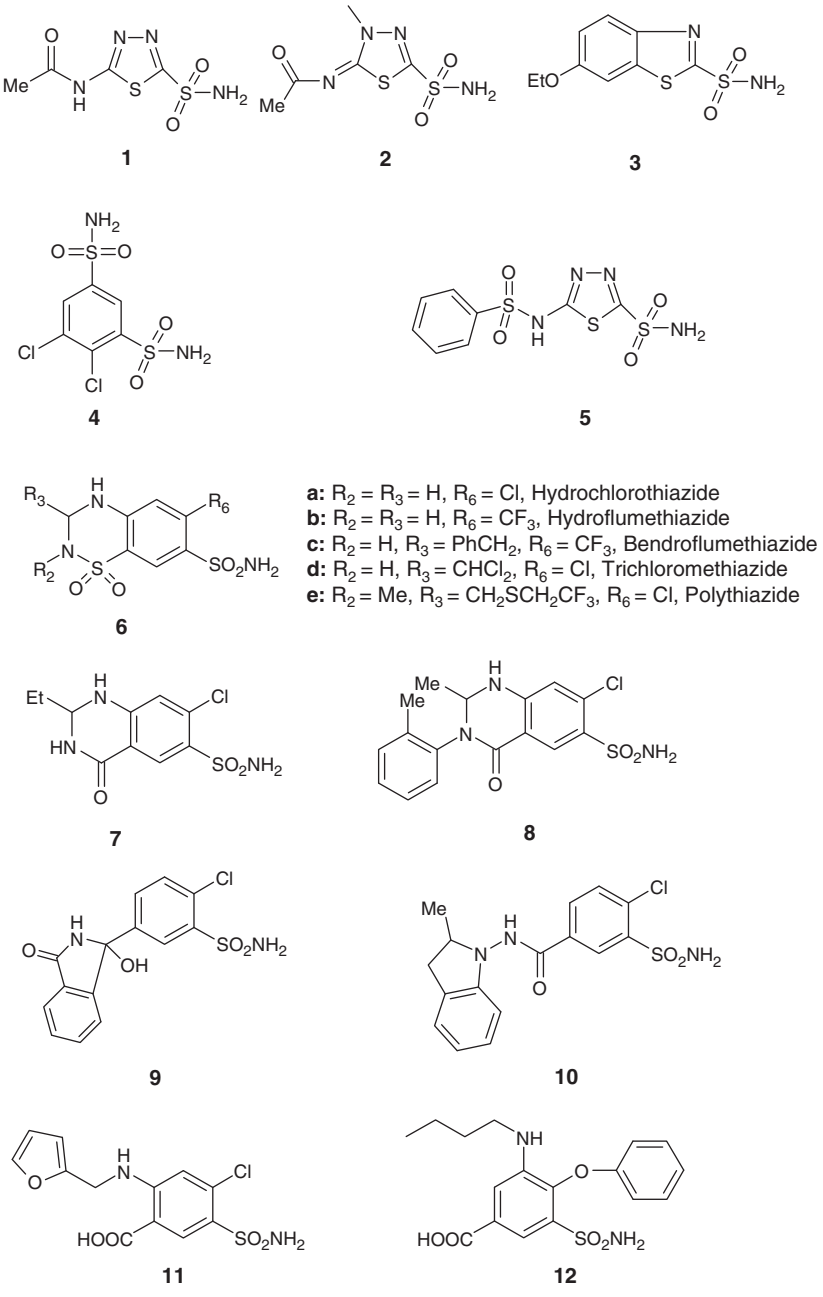

Figure 1. The classical carbonic anhydrase inhibitors (CAIs) 1 – 4, the orphan drug benzolamide 5 and the diuretics incorporating sulfonamide groups and possessing CA inhibitory properties 6 – 12.

sulfonamide CAIs for a range of applications, mainly as antitumor agents or diagnostic tools [16-20,47-55].

5-Acetamido-1,3,4-thiadiazole-2-sulfonamide, or acetazolamide **1**, was the first CAI to be used clinically (**Figure 1**) [14]. Acetazolamide is a potent but promiscuous inhibitor against most CA isoforms from mammals, but was the first non-mercurial diuretic to be used clinically, starting with 1956 [14]. Acetazolamide represents the prototype of a class of pharmacological agents with apparently limited therapeutic usefulness nowadays, but played a major role in the development of fundamental renal physiology and pharmacology, as well as in the design of many of the presently widely used diuretic agents, such as thiazide and high ceiling diuretics, among others [1-4,56-60]. Following the administration of a CAI, such as acetazolamide **1**, the urine volume promptly increases, and its normally acidic pH (of 6) becomes alkaline (around 8.2) [14]. An increased amount of bicarbonate is thus eliminated into the urine (120 times higher than the amount eliminated normally), together with Na^+ and K^+ as accompanying cations, whereas the amount of chloride excreted is diminished [14]. The increased alkalinity of

Table 1. Inhibition data with some of the clinically used sulfonamides 1 – 12 against isozymes I – XV (the isoforms CA VIII, X and XI are devoid of catalytic activity and probably do not bind sulfonamides as they do not contain Zn(II) ions) [56-60].

Isozyme	K_I* (nM)*												
	1	2	3	4	5	6a	6b	7	8	9	10	11	12
hCA I‡	250	50	25	1200	15	328	2840	35000	54000	348	51900	62	4930
hCA II‡	12	14	8	38	9	290	435	1260	2000	138	2520	65	6980
hCA III‡	2.10^5	7.10^5	$1\ 10^6$	$6.8.10^5$	$1.4.10^5$	$7.9.10^5$	$8.7.10^5$	nt	$6.1.\ 10^5$	$1.1.\ 10^4$	$2.3.10^5$	$3.2\ 10^6$	$3.4\ 10^6$
hCA IV‡	74	6200	93	15000	nt	427	4780	nt	216	196	213	564	303
hCA VA‡	63	65	25	630	37	4225	10200	nt	750	917	890	499	700
hCA VB‡	54	62	19	21	34	603	429	nt	312	9	274	322	nt
hCA VI‡	11	10	43	79	93	3655	8250	nt	1714	1347	1606	245	nt
hCA VII‡	2.5	2.1	0.8	26	0.45	5010	433	nt	2.1	2.8	0.23	513	nt
hCA IX‡	25	27	34	50	49	367	412	nt	320	23	36	420	25.8
hCA XII‡	5.7	3.4	22	50	3.5	355	305	nt	5.4	4.5	10	261	21.1
mCA XIII‡	17	19	50	23	nt	3885	15400	nt	15	15	13	550	2570
hCA XIV‡	41	43	25	345	33	4105	360	nt	5432	4130	4950	52	250
mCA XV‡	72	65	58	95	70	135§	141§	nt	79§	143§	234§	176§	431§

*h: Human; m: Murine isozyme; nt: Not tested, data not available.

‡From [56-60].

§Data reported here for the first time (unpublished data from our laboratory).

the urine is accompanied by a decrease in the excretion of titratable acid and ammonia, and in consequence by metabolic acidosis [14,56]. This sequence of events is due to the inhibition of the various CA isozymes in the proximal tubule (see the preceding section), which leads to inhibition of H^+ secretion by this segment of the nephron [14]. Inhibition of both cytosolic (CA II) as well as membrane-bound (CA IV, XII and CA XIV) enzymes seems to be involved in the diuretic effects of the sulfonamides [1-4,14,56-60]. Inhibition of such CAs decreases the availability of protons for the $Na^+ - H^+$ antiporter, which maintains a low proton concentration in the cell. The net effect of these processes is the transport of sodium bicarbonate from the tubular lumen to the interstitial space, followed by movement of the isotonically obligated water and whence augmented diuresis [4,14,56-60]. Carbonic anhydrase inhibitors also increase phosphate excretion (by an unknown mechanism) but have little or no effect on the excretion of calcium or magnesium ions [4,14,56-60].

Acetazolamide **1** and structurally related sulfonamides (**Figure 1**), such as methazolamide **2**, ethoxzolamide **3** and dichlorophenamide **4** were and are still used for the treatment of edema due to congestive heart failure, and for drug-induced edema, in addition to their applications as antiglaucoma agents [4,14,56-60]. However, these systemic CAIs generally produce many undesired side effects due to the inhibition of CAs present in other organs than the kidneys. The structurally related compound to acetazolamide, benzolamide **5**, with an acidic pK_a of 3.2 for the secondary sulfonamide group is completely ionized at the physiological pH, as sulfonamidate anion [27,61]. Its renal effect on bicarbonate excretion is around 10 times as potent as that of acetazolamide, the drug being maximally active at doses of 1 mg/kg, and being actively and rapidly accumulated in the kidney, but its plasma half-life is of only 20 min. All these facts make benzolamide a renal specific CAI, but the compound remains an orphan drug and has not been developed for wide clinical use, due to its inappropriate pharmacokinetics, although some anecdotal reports indicate that it might be beneficial for patients suffering from chronic obstructive lung disease [27,61].

With all these limitations, acetazolamide, methazolamide, ethoxzolamide and dichlorophenamide are still used for the treatment of edema induced by drugs or congestive heart failure [62]. The same effects have been claimed in a recent patent for furosemide, another CAI (see later in the text) [63]. These inhibitors can cause, however, several undesired side effects, such as metabolic acidosis, nephrolithiasis, CNS symptoms and allergic reactions, which have limited their exploitation in therapy [1,14,62]. The loss of bicarbonate and sodium is considered self-limited on continued administration of CAIs, probably because the initial acidosis resulting from bicarbonate loss activates bicarbonate reabsorption via CA-independent mechanisms [14,25].

4. Second generation sulfonamide diuretics: thiazides, indapamide, chlorthalidone, furosemide, bumetanide and their congeners

The diuretics are widely employed drugs for controlling hypertension [64-66]. They belong to several classes of pharmacological agents, but here we will discuss only the derivatives incorporating a primary sulfonamide moiety in their molecule. Indeed, by using acetazolamide **1** as a lead, a large number of other quite successful sulfonamide diuretics were developed in the 1960s and 1970s, such as the

benzothiadiazines **6** (hydrochlorothiazide **6a**, hydroflumethiazide **6b** and the like), quinethazone **7**, metholazone **8**, chlorthalidone **9**, indapamide **10**, furosemide **11** and bumetanide **12** (**Figure 1**) [56-60]. Some of them are among the most widely clinically used diuretics [67-70], alone or in combination with other drugs such as β-adrenergic receptor antagonists [71], cholesterol-lowering agents [72], cardiac glycosides [73], inhibitors of neutral peptidase [74] and so on. (only to mention several recent patents in the field).

As mentioned above, these clinically used drugs **6 – 12** possess the primary SO_2NH_2 moiety in their molecule, acting as excellent zinc-binding groups for the metal ion present within the CA active site, and normally associated with potent CA inhibitory properties of the compounds incorporating it [1,42,44-46]. However, till recently, the CA inhibitory properties of these drugs were investigated only for one CA isozyme (i.e., CA II) that was presumed to be responsible for all the physiological effects of the sulfonamide drugs in the 1960s and 1970s, when these drugs were initially launched [56-60]. Only recently the CA inhibition with these diuretics, against all mammalian CA isoforms was thoroughly reinvestigated [56-60], offering several interesting findings, which can lead to important polypharmacological applications and drug repositioning of these agents [75-77].

CA inhibition data with all sulfonamide diuretics (first and second generation ones), of types **1 – 12** is shown in **Table 1**.

Data of **Table 1** show that similar to the clinically used/orphan drug classical sulfonamide CAIs, that is, compounds **1 – 5**, the clinically used sulfonamide diuretics **6 – 12** act as inhibitors of all 13 investigated CA isozymes, but with an inhibition profile quite different from that of inhibitors investigated earlier, and particularly different from that of the first generation CAIs **1 – 5**. The following should be noted from these inhibition data of all catalytically active mammalian CA isoforms:

1) Hydrochlorothiazide **6a** acted as a medium potency inhibitor of isoforms hCA I, II, VB, IX and XII, with inhibition constants in the range of 290 – 603 nM, the compound being a weaker inhibitor of isoforms hCA VA, VI, VII, XIII and XIV (K_Is in the range of 3.655 – 5.010 μM) and an exceedingly weak one against hCA III (K_I of 0.79 mM). The best inhibited isoform was mCA XV, with a K_I of 135 nM;
2) Hydroflumethiazide **6b** showed an inhibition profile quite distinct from that of the closely structurally related diuretic **6a**, being a rather efficient inhibitor of the following isoforms hCA II, VB, VII, IX, XII, XIV and mCA XV, with inhibition constants in the range of 141 – 435 nM. It was a weaker inhibitor of hCA I, IV and VI (K_Is in the range of 2.84 – 8.25 μM) and showed very weak inhibition against isozymes hCA III, VA and XIII (K_Is of 10.2 – 870 μM). It is thus apparent that even small structural changes in the benzothiadiazine scaffold, such as the substitution of the chlorine atom in *ortho* to the sulfamoyl moiety by a trifluoromethyl group, such as in the pair **6a/6b**, has dramatic consequences for the CA inhibitory properties of the two compounds, basically against all investigated CA isozymes (**Table 1**).
3) Quinethazone **7** is the only diuretic among compounds **1 – 12** that is not approved for clinical use in Europe (being approved in US), and this derivative was not available for detailed investigations (its complete inhibition data are also not available in the literature). Some old literature data [14] showed it to be a very weak hCA I and a modest hCA II inhibitor, with inhibition constants in the range of 1.26 – 35 μM (**Table 1**).
4) Metholazone **8** showed very weak hCA I and III inhibitory properties (K_Is in the range of 54 – 610 μM), being a low micromolar inhibitor (thus, not a very efficient one) of hCA II, VI and XIV, with inhibition constants in the range of 1.714 – 5.432 μM. However, the drug was a medium potency inhibitor of isozymes hCA IV, VA, VB and IX (K_Is in the range of 216 – 750 nM) and a very efficient one against hCA VII, hCA XII, mCA XIII and mCA XV (K_Is in the range of 2.1 – 79 nM).
5) Chlorthalidone **9** also showed a very interesting inhibition profile, acting as a weak hCA III inhibitor (with a K_I of 11 μM, this compound is one of the most effective hCA III inhibitors ever detected among all known sulfonamides, [1], except trifluoromethanesulfonamide, which has a K_I of 0.9 μM), and a rather weak hCA VI and hCA XIV inhibitor (K_Is in the range of 1.347 – 4.95 μM). Chlorthalidone was a moderate hCA VA inhibitor (K_I of 917 nM) and an effective, or very effective inhibitor of the other mammalian CA isozymes. Thus, the ubiquitous hCA I and II, as well as hCA IV and mCA XV, showed inhibition constants in the range of 138 – 348 nM, but isoforms VB, VII, IX, XII and XIII were inhibited in the low nanomolar range (K_Is in the range of 2.8 – 23 nM). These results showing **9** to be such a strong CAI against many isoforms were rather unexpected, and considering the wide clinical use of the compound for the treatment of hypertension [78,79], at least two issues can be raised: first, is the inhibition of these CA isozymes responsible for some of the therapeutic effects of the drug (or for some side effects observed with it), and second, can these observations be useful for the design of CAIs with increased selectivity for some CA isoforms or for envisaging novel therapeutic applications of the drug (e.g., as an adjuvant to antitumor therapies, considering its strong inhibitory effects against the tumor-associated CA isozymes IX and XII)? [1,40] We shall try to reply to some of these questions after considering in detail the inhibition profile of the remaining three drugs.

6) Indapamide **10** acted as an inefficient CA I and III inhibitor (K_Is in the range of 51.9 – 230 μM), was a weak inhibitor of isoforms CA II, VA, VI, and XIV (K_Is in the range of 890 – 4950 nM) but showed significant inhibitory activity against CA IV and VB as well as mCA XV (K_Is in the range of 213 – 274 nM). Indapamide was an excellent inhibitor of CA VII, IX, XII and mCA XIII, with inhibition constants in the low nanomolar range (K_Is in of 0.23 – 36 nM). These data are indeed remarkable, also considering the wide use of the drug as a diuretic and its beneficial effects in patients with type 2 diabetes mellitus, as recently reported in several important clinical trials [67-69].
7) Furosemide **11** acted as a very weak hCA III inhibitor (K_I of 3200 μM), but showed moderate inhibitory activity against many isoforms, such as CA IV, VA, VB, VI, VII, IX, XII and mCA XIII/XV, with K_Is in the range of 176 – 564 nM. The compound was on the other hand a much better inhibitor of hCA I, II and XIV, with K_Is in the range of 52 – 65 nM. Furosemide is probably one of the most investigated diuretics, and a rather large number of patents deal with improving the bioavailability [80], combining it with other agents [81], or synthesizing some of its analogs in which the COOH moiety was transformed to esters or amides for improving the penetration through membranes [82].
8) Bumetanide **12** was an extremely weak hCA III inhibitor (K_I of 3400 μM), similar to furosemide **11** with which it is structurally related. However, bumetanide was also a weak inhibitor of hCA I, II and XIII (K_Is in the range of 2570 – 6980 nM), probably due to the quite bulky phenoxy moiety in *ortho* to the sulfamoyl zinc-binding group. The compound showed better inhibitory activity against isoforms CA IV, VA, XIV and mCA XV (K_Is in the range of 250 – 700 nM) but excellent inhibition of the tumor-associated isoforms CA IX and XII (K_Is in the range of 21.1 – 25.8 nM, that is,, the same order of magnitude as acetazolamide **1**, methazolamide **2** or ethoxzolamide **3**). Thus, it may be observed that bumetanide was an effective inhibitor of only the tumor-associated isoforms CA IX and XII, thus discriminating between these novel drug targets [40] and other CA isoforms that should not be inhibited by a cancer-specific CAI.

But what is important is the fact that the revisitation of these old diuretics may have relevance for the drug design of CAIs with diverse pharmacological applications [56-60]. There are at least several aspects that need to be considered here regarding the polypharmacology of these diuretic sulfonamides. First, these widely used drugs were considered to be inactive as CAIs, due to the fact that they were launched in a period when only CA II was well known (and considered as responsible of all physiological effects of CAIs) [14]. However, the latest research in the field [56-60] led to the observation reported here, that many of them do inhibit substantially several CA isoforms involved in crucial physiological and pathological processes. In contrast to the classical CAIs of type **1** – **5** (generally low nanomolar CA II inhibitors), all compounds **6** – **12** (except furosemide **11**) are much weaker inhibitors of this isozyme, usually in the micromolar range. Only furosemide **11** is a good CA II inhibitor among these diuretics, with a K_I of 65 nM, whereas all others showed K_Is in the range of 138 – 6980 nM (**Table 1**). Again with the exception of furosemide **11**, the diuretics **6** – **12** have low affinity for CA I, the other isoform known when these drugs have been discovered [14]. Data of **Table 1** show that many of the drugs **6** – **12** appreciably inhibit CAs discovered after their introduction in clinical use, with some low nanomolar (or even sub-nanomolar) inhibitors against many of them. Examples of such situations are: metholazone **8** against CA VII, XII and XIII; chlorthalidone **9** against CA VB, VII, IX, XII and XIII; indapamide **10** against CA VII, IX, XII and XIII; furosemide **11** against CA I, II and XIV and bumetanide **12** against CA IX and XII among others (**Table 1**). As already mentioned above, bumetanide **12** is a tumor-specific (targeting CA IX and XII) CAI, of equal potency to acetazolamide **1**, but without the promiscuity of acetazolamide, which is a potent CAI against most mammalian isozymes. Indeed, bumetanide is a weak inhibitor of all other isoforms except CA IX and XII, which are overexpressed in tumors [40]. Indapamide **10** and chlorthalidone **9** are also strong inhibitors of the tumor-associated CAs, but they are also effective in inhibiting CA VII and XIII (**Table 1**). It is thus clear, that these old drugs may indeed have newer applications in therapy or as experimental agents, in situations which require the selective inhibition of some CA isozymes, and which cannot be obtained with the clinically used compounds of types **1** – **4**. As far as we know, for the moment, no clinical trials are being conducted to understand in detail the polypharmacological aspects related to CA inhibition of these diuretics. This would be favorable, as it would help the discovery of new agents (it is worth mentioning that no diuretic possessing sulfonamide moieties has been discovered for the last 40 years).

A second important aspect of the findings mentioned above is whether some recent observations of clinical trials in which such diuretic agents have been employed can be explained, taking into consideration the CA inhibition, and how this is reflected in drug design processes. Thus, a relevant question arising when these diuretics are used is how the inhibitory activities of compounds of type **6** – **12** are reflected by the pharmacological effects (or side effects profile) of these drugs? It has recently been observed that indapamide **10** in combination with an ACE inhibitor (as diuretics) is highly beneficial for the treatment of patients with hypertension and type 2 diabetes [68]. Treatment with indapamide was also shown to lead to a significant decrease of plasma adiponectin concentration in patients with essential hypertension [69]. Adiponectin is considered to participate in the pathogenesis of carbohydrate metabolism disturbances often found in patients treated

with other thiazide-type diuretics [68,69]. On the other hand, classical sulfonamide CAIs such as acetazolamide **1**, methazolamide **2** ethoxzolamide **3**, and other compounds possessing such properties, are known to induce vasodilation in a variety of tissues and organs, including the kidneys, eye vasculature, brain vessels and so on [83-85]. However, the exact mechanisms by which they produce this beneficial effect for many pathologies (e.g., hypertension, glaucoma, diabetic retinopathy, etc.), or the isoforms involved in it, are unknown for the moment [86-89]. An attractive theory to explain this was recently proposed by Tsikas' group [90]. It is well known that the kidney also plays an important role in nitrite/NO homeostasis in the vasculature and that NO is one of the main molecules involved in vasodilation [90,91]. The involvement of renal CAs in endogenous nitrite reabsorption in the proximal tubule was thus investigated [90]. The potent and pan-CA inhibitor acetazolamide **1** was administered orally to six healthy volunteers in the dosage of 5 mg/kg, and nitrite was measured in spot urine samples before and after administration. Acetazolamide abruptly increased the nitrite excretion in urine, suggesting that renal CAs are involved in nitrite reabsorption in humans. Additional *in vitro* experiments supported the hypothesis that nitrite reacts with CO_2, analogous to the reaction of peroxynitrite ($ONOO^-$) with CO_2, leading to the formation of the acid-labile nitrito carbonate [$ONOC(O)O^-$] [90]. This reaction is hypothesized to be catalyzed by CAs and that nitrito carbonate represents the nitrite form that is actively transported into the kidney [90]. The significance of nitrite reabsorption in the kidney and the underlying mechanisms, notably a direct involvement of CAs in the reaction between nitrite and CO_2, remain to be elucidated at this moment [90].

In line with these studies, a recent report also showed that indapamide **10** has a protective role against ischemia-induced injury and dysfunction of the blood–brain barrier, probably due to its vasodilating effects [92]. An organ-protective effect of indapamide in animal models of renal failure has also been reported, showing the drug to be beneficial in preventing damage to the capillary structures, the endothelium, and in reducing the hypertrophy of superficial glomeruli among others [93]. All these effects are probably mediated by inhibition of CAs present in blood vessels or in the kidneys, but no specific pharmacological or biochemical studies are available so far, except for these clinical observations mentioned here and the Tsikas paper discussed above [90]. The lesson we learn from all these data is that probably many of the recently reported beneficial clinical properties of indapamide **10** are indeed due to its diuretic effects, but in conjunction with its strong inhibition of some CA isozymes (such as CA IV, VB, VII, IX, XII and/or XIII) present in kidneys and blood vessels. This hypothesis may explain both the blood pressure lowering effects as well as organ-protective activity of the drug. For medicinal chemists this means that it is probably possible to design sulfonamide CAIs possessing an inhibition profile similar to **10**, but with a stronger activity against the target isoform(s) involved in these pathologies. It is thus not unexpected that a large number of recent patents claim the combination therapy of hypertension and ensuing cardiovascular comorbidities by using combination of these diuretic agents (prevalently indapamide, chlorthalidone and hydrochlorothiazide) with various other agents, such as renin-angiotensin inhibitors, neutral endopeptidase inhibitors, dronedarone, calcium channel blockers, adrenergic agonists and so on [94-102].

It is interesting to note that some recent patents also claim the possibility to treat hypertension and hyperuricemia by combining diuretics of types 6 – 12 with inhibitors of the organic acid transporter isoform 4 (OA4), such as 2-(5-bromo-4-(cyclopropylnaphthalen-1-yl)4*H*-1,2,4-triazol-3-ylthio) acetic acid [103], or that furosemide may act as a submicromolar inhibitor of tautomerase from the parasite *Ancylostoma ceylanicum* [104]. Undoubtedly, this class of 'old' drugs may still be able to afford interesting opportunities for developing novel therapies based on such repositioning or polypharmacological approaches.

5. Expert opinion

CAs are present in a multitude of isoforms in mammals, with 13 such catalytically active enzymes distributed in many tissues. In kidneys CA II, IV, VB, IX (kidney tumors), XII, and XIV are present in rather large amounts. These enzymes play a fundamental role in the kidney physiology for the acid–base balance homeostasis (by secreting and excreting protons, due to the CO_2 hydration reaction catalysed by these enzymes, in the bicarbonate reabsorption processes and in the renal NH_4^+ output). These important functions are well localized in the different segments of the nephron: bicarbonate reabsorption occurs in the proximal tubule, whereas urinary acidification and NH_4^+ output occur in the distal tubule and collecting duct, and made renal CAs were thus investigated as drug targets, with the report of the first CAI, acetazolamide, in the 1950s. This was followed by the development of the thiazide and high-ceiling diuretics that show such an effect also independently of their CA inhibitory action [14]. However, the presence of free SO_2NH_2 moieties in the molecules of the benzothiadiazines **6** (hydrochlorothiazide **6a**, hydroflumethiazide **6b** and the like), quinethazone **7**, metholazone **8**, chlorthalidone **9**, indapamide **10**, furosemide **11** and bumetanide **12** induce strong CA inhibitory activities to these drugs, which have been only recently re-evaluated against all the mammalian active CA isoforms, that is,, CA I – CA XV [56-60]. These widely used drugs were in fact launched in a period when only isoform CA II was known and considered physiologically/pharmacologically relevant and thus no inhibition data against other CA isoforms were available till recently. The new studies showed intriguing evidences regarding the CA inhibitory properties of these drugs. Although acting as moderate-weak inhibitors of CA II and CA I, all these drugs considerably inhibited other CA isozymes known nowadays to be involved in critical physiological processes. Some low

nanomolar (or even sub-nanomolar) inhibitors against such isoforms were thus detected, such as metholazone against CA VII, XII and XIII; chlorthalidone against CA VB, VII, IX, XII and XIII; indapamide against CA VII, IX, XII and XIII; furosemide against CA I, II and XIV and bumetanide against CA IX and XII. The X-ray crystal structure of the adducts of CA II with indapamide and chlorthalidone were also resolved [56-60], explaining at molecular level the features associated with this phenomenon, which may be useful for the drug design of novel classes of CAIs.

It has also been proposed, based on these new data [56-60], that the recently observed beneficial effect of indapamide/chlorthalidone for the treatment of patients with hypertension and type 2 diabetes may be due to the potent inhibition of the two drugs against CA isoforms present in kidneys and blood vessels, which would explain both the blood pressure lowering effects as well as organ-protective activity of the drug. Indeed, in a very important paper, Tsikas' group explained that inhibition of the renal CAs leads to a large increase in nitrite excretion in urine, suggesting that renal CAs are involved in nitrite reabsorption in humans [90]. Additional *in vitro* experiments supported the hypothesis that nitrite reacts with CO_2, analogous to the reaction of peroxynitrite ($ONOO^-$) with CO_2, leading to the formation of the acid-labile nitrito carbonate [$ONOC(O)O^-$] [90]. Undoubtedly, such features are probably also common to the other diuretic sulfonamides that potently inhibit some renal CA isoforms, as discussed above. It is thus not unexpected that a large number of recent patents propose a repositioning of these old drugs for novel biomedical applications, alone or in combination with other agents. Important lessons for the drug design of novel CAIs, based on the findings discussed in the review for the diuretic sulfonamides, can also be drawn. In fact, by using such drugs as lead molecules, a multitude of compounds possessing interesting polypharmacological features may be obtained.

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Declaration of interest

The authors declare conflict of interest, being authors on many patents dealing with various classes of CA inhibitors (most of which cited in the review). They were not paid for writing this review.

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