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Carbonic anhydrase inhibitors. Diazenylbenzenesulfonamides are potent and selective inhibitors of the tumor-associated isozymes IX and XII over the cytosolic isoforms I and II

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ABSTRACT

A series of diazenylbenzenesulfonamides, azo-dye derivatives of sulfanilamide or metanilamide incorporating phenol and amine moieties, were tested for inhibition of the tumor-associated isozymes of carbonic anhydrase (CA, EC 4.2.1.1), CA IX and XII. These compounds showed moderate-low inhibitory activities against the cytosolic isoforms CA I and II (offtargets) and excellent, low nanomolar inhibitory activity against the transmembrane CA IX and XII (K_Is in the range of 3.5–63 nM against CA IX and 5.0–69.4 nM against CA XII, respectively). The selectivity ratio for inhibiting the tumor-associated CA IX over the offtarget CA II was in the range of 15–104 for these diazenylbenzenesulfonamides, making them among the most isoform-selective inhibitors targeting tumor-associated CAs (over the ubiquitous CA II). Since CA IX/XII were recently shown to be both therapeutic and diagnostic targets for hypoxic solid tumors overexpressing these proteins, such compounds held promise for the management of hypoxic tumors, which are largely non-responsible to classical chemo- and radio-therapy.

1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) constitute an ubiquitous family of metallo-enzymes found in prokaryotes and eukaryotes, which catalyze the reversible hydration of carbon dioxide to the bicarbonate ion and a proton ($CO_2 + H_2O \leftrightarrows HCO_3^- + H^+$).[1–5] These proteins are encoded by five evolutionarily unrelated gene families: the α-CAs (in vertebrates, bacteria, algae and cytoplasm of green plants), the β-CAs (predominantly in bacteria algae and chloroplasts), the γ-CAs (in archaea and some bacteria)[1–3] and the δ- and ξ-CAs (in marine diatoms).[2,3] There are no significant sequence homologies between representatives of the different CA families, but all members contain one Zn(II) or Cd(II) ion in their active site, which are critical for the catalytic activity of the enzymes.[1–3]

α-CA isozymes are widely distributed in many tissues and organs of vertebrates (including humans), as 16 different isoforms (only 15 are found in primates, which lack the CA XV isoform).[1] They play crucial roles in various physiological processes of such organisms, such as CO_2/HCO_3^- transport between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion, biosynthetic reactions (gluconeogenesis, lipogenesis and ureagenesis), bone resorption and tumorigenicity.[1,6–10] As a consequence, many of these CA isoforms are drug targets of interest for the design of various pharmacological agents, such as diuretics, antiglaucoma, antiobesity, antiepileptic or antitumor drugs/diagnostic agents.[1] Among all these applications, the potential use of CA inhibitors (CAIs) for the management of solid hypoxic tumors which overexpress two isoforms, CA IX and XII, became a hot topic, with many investigations aiming to develop potent and isoform-selective CAIs targeting these two isozymes.[1,6–11] Indeed, hypoxia, through the hypoxia inducible factor (HIF) cascade, leads to a strong overexpression of CA IX/XII in many tumors.[8] The overall consequence of these phenomena is a pH imbalance, with most hypoxic tumors having acidic pH values around 6–6.5, in contrast to normal tissue which have characteristic pH values around 7.4.[8–10] Constitutive expression of human CA IX (hCA IX) was recently shown to decrease extracellular pH (pH_e) also in Madin-Darby canine kidney (MDCK) epithelial cells (which normally do not express CA IX) by this group.[8] CA IX potent sulfonamide inhibitors were then shown to reduce the medium acidity by inhibiting the catalytic activity of the enzyme, and thus the generation of H^+ ions, binding specifically only to hypoxic cells expressing CA IX.[8] Deletion of the CA active site was demonstrated to reduce the medium acidity, but sulfonamide inhibitors did not bind to the active site of the mutant protein. Therefore, tumor cells decrease their pH_e both by production of lactic acid (due to the high glycolysis rates), and by CO_2 hydration catalyzed by the tumor-associated CA IX/XII, possessing extracellular catalytic domains.[8–10] A low pH_e has been associated with tumorigenic transformation, chromosomal rearrangements,

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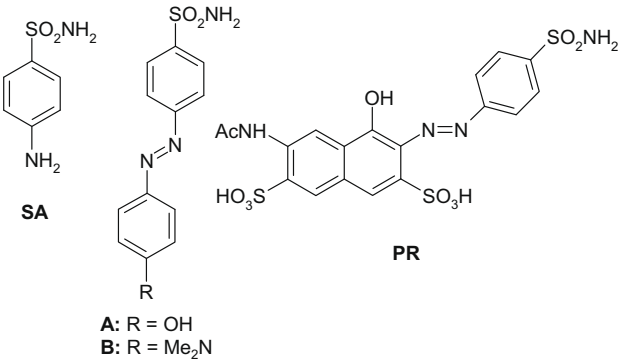
extracellular matrix breakdown, migration and invasion, induction of the expression of cell growth factors and protease activation.[10] CA IX also plays a role in providing bicarbonate to be used as a substrate for cell growth, whilst it is established that bicarbonate is required in the synthesis of pyrimidine nucleotides.[8–10] Thus, the proof-of-concept study mentioned above[8] showing the role of CA IX in tumor acidification, also established that sulfonamide inhibitors bind only to hypoxic cells overexpressing CA IX (and not to their normal counterparts) and that CA IX inhibition with such compounds reverts the tumor acidification processes, restoring a more physiologically normal pH_e. Dubois et al.[8c] also proved recently the possibility to use sulfonamide CA IX—specific inhibitors for imaging purposes in a xenograft tumor model in which CA IX is overexpressed.

Recently, Pouysségur and co-workers[9] showed that in hypoxic LS174Tr tumor cells expressing either CA IX or both CA IX and XII, in response to a CO_2 load, both enzymes contribute to extracellular acidification and to maintaining a more alkaline resting intracellular pH (pH_i), an action that preserves ATP levels and cell survival in a range of acidic outside pH (6.0–6.8) values, and low bicarbonate medium. In vivo experiments in cell cultures showed that silencing of CA IX alone leads to a 40% reduction in xenograft tumor volume in mice, with up-regulation of the second gene, the one encoding for CA XII. Silencing of both CA IX and CA XII gave an impressive 85% reduction of tumor growth.[9c] Thus, hypoxia-induced CA IX and CA XII are major tumor prosurvival pH-regulating enzymes, and their combined targeting (i.e., inhibition) has great potential for the design of anticancer drugs with a novel mechanism of action.[9] However, the in vivo proof-of-concept that sulfonamide CA IX inhibitors may indeed show antitumor effects, has been only very recently published by Neri and co-workers,[11] by using membrane-impermeant derivatives based on the acetazolamide **AAZ** scaffold to which either fluoresceincarboxylic acid or albumin-binding moieties were attached. This group demonstrated the strong tumor growth retardation in mice with xenografts of a renal clear cell carcinoma line (SK-RC-52) when the animals were treated for one month with some of these CAIs. Such preliminary data of our,[8,10] Pouyssegur's[9] and Neri and co-workers[11] groups show indeed the great promise of tumor CA IX/XII inhibition with sulfonamides or related agents for the development of alternative anticancer drugs. Furthermore such compounds may be also used for the imaging of hypoxic tumors.[8c] Here we investigate the inhibition of the tumor-associated isoforms CA IX and XII with a series of diazenylbenzenesulfonamides recently reported by our group,[12] which showed modest CA I and II inhibitory activity, in the search of tumor-isoforms selective inhibitors.

2. Results and discussion

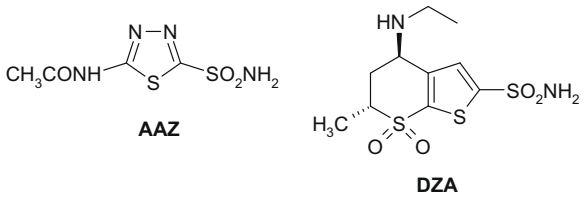
2.1. Chemistry

In a recent work[13] we have investigated the inhibition of the cytosolic isoforms CA II (ubiquitous)[1] and VII (present only in the brain)[1] with a series of simple benzenesulfonamides and two azo dyes obtained by the coupling of diazotized sulfanilamide **SA** with phenol or *N*,*N*-dimethylaniline, of types **A** and **B**. In fact, although very important from the drug design point of view, as the antibacterial sulfa drugs have been discovered considering prontosil **PR** as lead molecule,[14] the sulfonamide azo dyes (of which **PR** is a representative) were scarcely investigated for the inhibition of CAs.[15] One of the interesting findings of our recent study[13] was that the azo dyes **A** and **B** showed moderate-weak CA II inhibitory activity (in the range of 638–665 nM), but they were better inhibitors of the cytosolic isoform with restricted expression in the brain, CA VII. Thus, it appeared of interest to explore in more detail this class of sulfonamides, with weaker inhibitory properties against the ubiquitous (offtarget) isozymes CA I and II, which may show interesting inhibition of isoforms known to be medicinal chemistry targets (e.g., CA IX and XII).



We report here the preparation of a series of diazenylbenzenesulfonamides **1** and **2** structurally related to **A**, **B** and **PR**, obtained from sulfanilamide or metanilamide (Scheme 1). The chemistry for the preparation of these compounds is non-exceptional and involves diazotization of the aminosulfonamide followed by coupling with phenols or amines[13–16] (see Section 4). We have chosen various R moieties to be present in the molecules of the derivatives **1** and **2** (such as hydroxy, amino, methylamino, and dimethylamino, as well as the sulfonate ones from **1e, 1f, 2e, 2f** which may induce enhanced water solubility to these compounds, as sodium salts). The isomers **23** and **24** also differ by the *para*- or *meta*-bulky substituent (with respect to the sulfamoyl moiety). Finally, as sulfonates have not been investigated earlier as CA inhibitors, we also included in the study the intermediates **3** and **3** which have been obtained in order to prepare the azo dyes **1e, 1f, 2e** and **2f**.

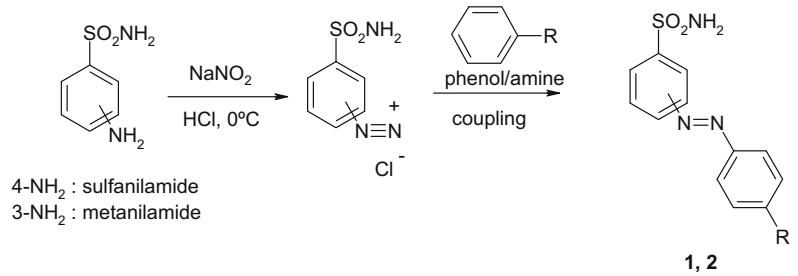
We decided to investigate whether the physiologically relevant CA isoforms associated with tumors, that is, CA IX and XII, are inhibited by these derivatives, a class of compounds not investigated earlier for its interaction with the extracellular, tumor-associated isoforms.



2.2. Carbonic anhydrase inhibition

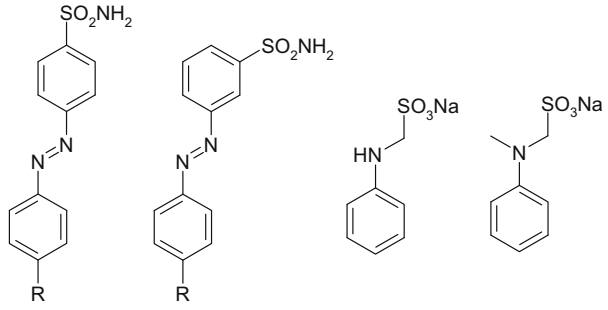
The azo-sulfonamides **1a–1f** and **2a–2f**, as well as the sulfonates **3** and **4** were assayed as inhibitors of the tumor-associated isoforms hCA IX and XII (Table 1) by means of a stopped-flow assay.[17] Standard sulfonamide drugs, such as acetazolamide **AAZ** and dorzolamide **DZA** were also included in the assay for comparison reasons. The following structure–activity relationship (SAR) has been evidenced from data of Table 1:

(i) Unlike the cytosolic isoforms hCA I and II, which were weakly inhibited by the sulfonamides **1** and **2**,[12] the transmembrane, tumor-associated hCA IX was strongly inhibited by all these derivatives, with inhibition constants in the range of 3.5–63 nM. For the *para*-substituted azo dyes **1**, all substitution patterns at the second phenyl ring were highly beneficial for the inhibitory activity, with the phenol **1a**, amines **1b–1d** and sulfonates **1e, 1f**



Scheme 1. Preparation of diazenylbenzenesulfonamides **1** and **2** by diazotization of sulfanilamide/metanilamide followed by coupling with phenols/amines.

Table 1
Inhibition of hCA isoforms I, II (cytosolic), and IX and XII (transmembrane, tumor-associated) with sulfonamides **1** and **2**, the sulfonates **3** and **4**, and standard sulfonamide inhibitors (**AAZ** and **DZA**), by a stopped-flow CO_2 hydrase assay[17]



1 **2** **3** **4**

No.	R	K_I (nM)[a]			Selectivity ratio	
		hCA I[b]	hCA II[b]	hCA IX[c]	hCA XII[c]	K_I (CA II)/K_I (CA IX)
1a	OH	393	665	6.4	5.0	103.9
1b	NH_2	95	106	6.0	7.3	17.6
1c	NHMe	170	93	4.9	69.4	18.9
1d	NMe_2	621	638	6.6	7.9	96.6
1e	$NHCH_2SO_3Na$	106	105	5.4	6.1	19.4
1f	$N(Me)CH_2SO_3Na$	96	104	3.5	6.3	29.7
2a	OH	95	106	6.1	7.3	17.3
2b	NH_2	96	88	5.9	7.3	14.9
2d	NMe_2	113	105	6.4	7.9	16.4
2e	$NHCH_2SO_3Na$	34,488	107	57	7.2	1.8
2f	$N(Me)CH_2SO_3Na$	2435	109	63	7.4	1.7
3	—	25,240	58,300	5100	6250	11.4
4	—	56,030	63,600	5850	5925	10.8
AAZ**	—	250	12	25	5.7	0.48
DZA**	—	50,000	9	52	3.5	0.17

Data for hCA I and II inhibition from Ref. 12.
[a] Mean value from at least 3 different measurements.[17] Errors were in the range of ±5% of the obtained value (data not shown).
[b] Human cloned full length enzyme.
[c] Catalytic domain of human recombinant enzyme.

acting as low nanomolar inhibitors (K_Is of 3.5–6.6 nM). The sulfonates **1e** and **1f** showed good water solubility (as sodium salts), which constitutes a favorable feature for a compound that should target an extracellular CA such as CA IX (and XII). In addition, due to the permanent negative charge present in their molecules, these compounds are also membrane-impermeant, being thus restricted to the extracellular space with no interaction with cytosolic or mitochondrial CAs in vivo.[18] This may lead to a favorable inhibition profile for these compounds in vivo, and thus potentially less side effects due to undesired inhibition of other isozymes than the extracellular ones.[19] For the *meta*-substituted azo dyes **2**, the inhibitory profile was very good for the phenol **2a** and amines **2b**, **2c** (K_Is of 6.1–6.4 nM) whereas the sulfonates **2e** and **2f** showed only moderate inhibitory activity, with inhibition constants of 57–63 nM. Similar inhibition was in fact observed with the clinically used drugs **AAZ** and **DZA** (K_Is of 25–52 nM). The simple sulfonates **3** and **4** were even less effective CA IX inhibitors, with inhibition constants of 5.10–5.85 μM. However this is the first time that compounds possessing the sulfonate moiety as zinc-binding group are investigated for their interaction with CA IX. It may be observed that CA IX has around 10-times higher affinity for these sulfonates compared to CA II (Table 1).

(ii) The second tumor-associated isoform, CA XII, was also strongly inhibited by sulfonamides **1** and **2**, but with a slightly different inhibition profile compared to CA IX (Table 1). Thus, for the *para*-subseries, the phenol **1a**, the primary amine **1b** and the tertiary amine **1d** showed similar, potent inhibitory properties, with

K_Is of 5.0–7.3 nM, whereas the secondary amine **1c** was a much weaker inhibitor (K_I of 69.4 nM). This constitutes a nice example of how a very small structural difference in the molecule of the CAI leads to a strong (roughly a 10-times fold) decrease of inhibitory power (if one compares the secondary amine **1c** with the primary or tertiary ones **1b** and **1d**, respectively). It is rather difficult to rationalize this behavior without X-ray crystal structures of these adducts with the enzyme. The sulfonamides incorporating sulfonate tails **1e** and **1f** on the other hand, showed again excellent CA XII inhibitory properties, with K_Is of 6.1–6.3 nM (Table 1). For the *meta*-substituted azo dyes of type **2**, all compounds showed a compact behavior of highly effective inhibitors, with K_Is of 7.2–7.9 nM (but we were unable to prepare the secondary amine corresponding to **1c**). Thus, all substitution patterns are in this case highly beneficial for obtaining metanilamide-derived azo dyes with strong CA XII inhibitory activity. In fact the sulfonamides **1** and **2** reported here showed, similarly to **AAZ** and **DZA**, a very potent CA XII inhibition. As for CA IX discussed earlier, the simple sulfonates **3** and **4** were less effective CA XII inhibitors, with K_Is of 5.92–6.25 μM. Again, as for CA IX, this is the first report regarding the interaction of CA XII with this type of compounds.

(iii) A very important aspect when designing CAIs is related to their selectivity for the target isoform,[20] due to the fact that at least 13 of the 16 mammalian CA isozymes show catalytic activity for the CO_2 hydration reaction, and affinity for sulfonamide inhibitors.[1,20] Thus, obtaining compounds with a good inhibition profile for the target isoform, in this case the tumor-associated ones CA IX and CA XII, but mainly with CA II-sparing (or CA II less avid) properties, is highly desirable. In fact CA II inhibition leads to a range of unpleasant side effects due to the wide distribution of this isoform all over the mammalian body and due to its high affinity for most sulfonamides, such as the clinically used ones, which generally show low nanomolar activity against CA II[1,20] (see Table 1 for **AAZ** and **DZA** inhibition data). It may be observed from data of Table 1 that the selectivity ratio for inhibiting CA IX over CA II for **AAZ** and **DZA** is in the range of 0.17–0.48, meaning that these compounds act as much better CA II than CA IX inhibitors. However, the new sulfonamides investigated here of types **1** and **2** showed an excellent selectivity ratio for inhibiting the tumor-associated isoforms CA IX/XII over the cytosolic ones CA I/II, as presented in detail for the CA IX versus CA II selectivity ratios from Table 1. Indeed, except for **2e** and **2f** which were only weakly CA IX-selective (selectivity ratios of 1.7–1.8) inhibitors, all other sulfonamides **1** and **2** have this parameter in the range of 14.9–103.9, making them among the most isoform-selective CAIs reported to date for targeting the tumor-associated isozymes CA IX and XII. Highly CA IX-elective compounds were the phenol **1a** and the dimethylamino-aniline derivative **1d**, with selectivity ratios of 96.6–103.9. Overall, the *para*-substituted azo dyes **1** were more isoform-selective inhibitors of the tumor-associated isozymes, compared to the corresponding metanilamide azo dyes **2**.

3. Conclusions

A series of diazenylbenzenesulfonamides, azo dye derivatives of sulfanilamide or metanilamide incorporating phenol and amine moieties, were tested for inhibition of the tumor-associated isozymes of carbonic anhydrase, CA IX and XII. These compounds showed moderate-low inhibitory activities against the cytosolic isoforms CA I and II (offtargets) and excellent, low nanomolar inhibitory activity against the transmembrane CA IX and XII (K_Is in the range of 3.5–63 nM against CA IX and 5.0–69.4 nM against CA XII, respectively). The selectivity ratio for inhibiting the tumor-associated CA IX over the offtarget CA II was in the range of 15–104 for these diazenylbenzenesulfonamides, making them among the most isoform-selective inhibitors targeting tumor-associated CAs (over the ubiquitous CA II). Since CA IX/XII were recently shown to be both therapeutic and diagnostic targets for hypoxic solid tumors overexpressing these proteins, such compounds held promise for the management of hypoxic tumors, which are largely non-responsible to classical chemo- and radio-therapy.

4. Experimental

4.1. Chemistry

4.1.1. General experimental details

^{1}H, ^{13}C, DEPT, NOESY, COSY, HMQC and HMBC spectra were recorded using a Bruker Advance III 400 MHz spectrometer. The chemical shifts are reported in parts per million (ppm) and the coupling constants (*J*) are expressed in hertz (Hz). For all new compounds DEPT, COSY, HMQC and HMBC were routinely used to definitely assign the signals of ^{1}H and ^{13}C. Infrared spectra were recorded on a Perkin–Elmer Spectrum R XI spectrometer as solids on KBr plates. Melting points (mp) were measured in open capillary tubes, unless otherwise stated, using a Büchi Melting Point B-540 melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was carried out on Merck Silica Gel 60 F_{254} aluminum backed plates. Elution of the plates was carried out using ethyl acetate/*n*-hexane or MeOH/DCM systems. Visualization was achieved with UV light at 254 nm, by dipping into a 0.5% aqueous potassium permanganate solution, by Hanessian's stain solution and heating with a hot air gun or by exposure to iodine. Flash column chromatography was carried out using silica gel (obtained from Aldrich Chemical Co.) as the adsorbent. The crude product was introduced into the column as a solution in the same elution solvent system, alternatively as a powder obtained by mixing the crude product with the same weight of silica gel in acetone and then removing the solvent in vacuo at room temperature, or dissolved into a minimum amount of DCM or carbon tetrachloride. All moisture or air sensitive reactions were carried out in oven-dried glassware under a positive pressure of nitrogen or argon using standard syringe/septa techniques. All the inert gases used (nitrogen and argon) were passed through jacket columns fitted with activated silica gel containing cobalt(II) chloride adsorbed as humidity indicator. All other solvents and chemicals were used as supplied from Aldrich Chemical Co., Acros, Fisher, Alfa Aesar or Lancaster Synthesis. Sulfonamides used for the diazotization, amines, phenols and **AAZ**–**DZA** are commercially available from Sigma–Aldrich (Milan, Italy), and were used without further purification. All CA isozymes were recombinant ones produced and purified in our laboratory as described earlier.[7,8]

4.1.2. General procedure for preparation of diazonium chloride solutions of 4-aminobenzenesulfonamide and 3-aminobenzenesulfonamide[13]

Aminobenenzenesulfonamide (sulfanilamide or metanilamide) (0.20 g, 1.0 equiv) was dissolved in a freshly prepared 40% solution of concentrated hydrochloric acid in deionised water (3.0 ml) and then cooled down to −5 °C. Then a 2.3 M aqueous solution of $NaNO_2$ (1.2 equiv) was added dropwise and the mixture was kept stirring at the same temperature until a persistent pale yellow solution, for sulfanilamide, or pale orange solution, for metanilamide, was formed (5–10 min). The solution was used freshly prepared prior to use.

4.1.3. Diazo coupling of the diazonium chloride of 4-aminobenzenesulfonamide 3 with *N,N*-dimethylaminobenzene[13]

A solution of diazotized sulfanilamide/metanilamide (prepared as above) was added dropwise to a solution of *N,N*-dimethylaminobenzene (0.14 g, 0.15 ml, 1.0 equiv) in a saturated aqueous solution of AcONa (3.0 ml) at −5 °C. The solution turned bright orange immediately and a precipitate was formed. The mixture was stirred at the

same temperature for 15 min, then warmed to rt and the pH adjusted to 7. The solid was collected by filtration, washed with a minimum amount of H_2O, dried under vacuo and purified by silica gel column chromatography eluting with 5% MeOH in DCM to give **1d** as an orange solid in 79% yield. 4-(4′-Dimethylaminophenyl)diazenylbenzenesulfonamide **1d**: mp 260–261 °C silica gel TLC R_f 0.38 (MeOH/DCM 5%); ν_{max} (KBr) cm^{-1}, 1604 (aromatic), 1520 (N=N), 1370 (SO_2–N); δ_H (400 MHz, DMSO-d_6) 3.13 (6H, s, 2 × CH_3), 6.90 (2H, d, J 9.2, 2 × 3′-H), 7.48 (2H, s, SO_2NH_2, exchange with D_2O), 7.87 (2H, d, J 9.2, 2 × 2′-H), 7.93 (2H, d, J 7.8, 2 × 2-H), 7.99 (2H, d, J 7.8, 2 × 3-H); δ_C (100 MHz, DMSO-d_6) 155.9 (ipso), 154.8 (ipso), 145.0 (ipso), 144.0 (ipso), 128.5 (C-2), 127.0 (C-3), 123.6, 113.2, 30.8 (2 × CH_3).

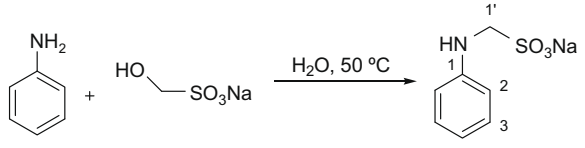
3-(4′-Dimethylaminophenyl)diazenylbenzenesulfonamide **2d**: mp 198–200 °C; silica gel TLC R_f 0.35 (MeOH/DCM 5%); ν_{max} (KBr) cm^{-1} 1600 (aromatic), 1519 (N=N), 1367 (SO_2–N); δ_H (400 MHz, DMSO-d_6) 3.13 (6H, s, 2 × CH_3), 6.90 (2H, d, J 9.4, 2 × 3′-H), 7.50 (2H, s, SO_2NH_2, exchange with D_2O), 7.76 (1H, t, J 7.7, 4-H), 7.88 (2H, d, J 9.4, 2 × 2′-H), 7.90 (1H, ddd, J 7.7 2.0 1.2, 5-H), 8.02 (1H, ddd, J 7.7 2.0 1.2, 6-H), 8.21 (1H, dd, J 3.6 2.0, 2-H); δ_C (100 MHz, DMSO-d_6) 155.0 (C-3), 154.3 (C-4′), 146.3 (C-1′), 144.0 (C-1), 132.3 (C-4), 129.0, 128.0, 127.2 (C-2′), 119.0 (C-2), 113.5 (C-3′), 41.8 (2 × CH_3).

4.1.4. Diazo coupling of the diazonium chloride of 3/4-aminobenzenesulfonamide with phenol[13]

A solution of diazotized sulfanilamide/metanilamide (prepared as above) was added dropwise to a solution of phenol (0.11 g, 0.10 ml, 1.0 equiv) in a 10 M aqueous solution of NaOH (3.0 ml) at −5 °C. The solution turned orange immediately and a precipitate was readily formed. The mixture was stirred at the same temperature for 15 min, then warmed to rt and the pH adjusted to 7. The solid was collected by filtration, washed with a minimum amount of H_2O, dried under vacuo and purified crystallization from H_2O/EtOH to give the title compound as an orange solid in 52% yield. 4-(4′-Hydroxyphenyl)diazenylbenzenesulfonamide **1a**: mp 259–261 °C; silica gel TLC R_f 0.60 (MeOH/DCM 10%); ν_{max} (KBr) cm^{-1}, 3349 (O–H), 1601 (aromatic), 1503 (N=N), 1399 (SO_2–N); δ_H (400 MHz, DMSO-d_6) 7.00 (2H, d, J 8.8, 2 × 3′-H), 7.53 (2H, s, SO_2NH_2, exchange with D_2O), 7.89 (2H, d, J 8.8, 2 × 2′-H), 7.99 (2H, d, J 8.8, 2 × 2-H), 8.00 (2H, d, J 8.8, 2 × 3-H), 10.50 (1H, s, OH, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 154.6 (ipso), 153.9 (ipso), 146.2 (ipso), 145.0 (ipso), 127.9 (C-2), 126.3 (C-3), 123.4, 117.

3-(4′-Hydroxyphenyl)diazenylbenzenesulfonamide **1b**: mp 231–232 °C with decomposition; silica gel TLC R_f 0.56 (MeOH/DCM 10%); ν_{max} (KBr) cm^{-1}, 3372 (O–H), 1609 (aromatic), 1505 (N=N), 1320 (SO_2–N); δ_H (400 MHz, DMSO-d_6) 7.02 (2H, d, J 8.8, 2 × 3′-H), 7.53 (2H, s, SO_2NH_2, exchange with D_2O), 7.81 (1H, appt, J 8.0, 4-H), 7.89 (1H, d, J 8.8, 2′-H), 7.98 (1H, ddd, J 8.0 2.0 1.2, 5-H), 8.08 (1H, ddd, J 8.0 2.0 1.2, 6-H), 8.25 (1H, appt, J 1.6, 2-H), 10.47, (1H, s, OH, exchange with D_2O); δ_C (100 MHz, DMSO-d_6) 162.8 (C-4′), 153.5 (C-3), 146.7 (C-1′), 146.4 (C-1), 132.1, 128.6, 128.3, 126.9 (C-3′), 119.2 (C-2), 117.6 (C-2′).

4.1.5. Treatment of aniline with formaldehyde sodium bisulfite adduct (preparation of 3)



3

Formaldehyde sodium bisulfite adduct (1.34 g, 1.0 equiv) was dissolved in H_2O (1.8 ml) at 40 °C and then aniline (1.0 g, 0.98 ml, 1.0 equiv) was added dropwise. A white precipitate was readily formed and the mixture was stirred at 50 °C for 5 h. Then the reaction was cooled down to rt and the solid was collected by filtration, triturated with diethyl ether and dried under vacuo to give **3** as a white solid in 80%yield. Sodium phenylaminomethanesulfonate **3**: mp > 370 °C; ν_{max} (KBr) cm^{-1}, 3468 (N–H), 1600 (aromatic), 1421 (SO_2–O); δ_H (400 MHz, DMSO-d_6) 3.86 (2H, d, J 6.6, 1′-H_2), 5.88 (1H, t, J 6.6, NH, exchange with D_2O), 6.50 (1H, tt, J 7.2 1.2, 4-H), 6.72 (2H, dd, J 8.6 1.2, 2-H), 7.05 (2H, dd, J 8.6 7.2, 3-H); δ_C (100 MHz, DMSO-d_6) 149.0 (C-1), 129.4 (C-3), 116.6 (C-4), 113.4 (C-2), 61.5 (C-1′).

4.1.6. Diazo coupling of the diazonium chloride of 4-aminobenzenesulfonamide with 3[13]

A solution of diazonium salt of sulfanilamide (prepared as above) was added dropwise to a suspension of **3** (0.24 g, 1.0 equiv) in a saturated aqueous solution of AcONa (1.5 ml) at −5 °C. The reaction turned orange immediately and was stirred at the same temperature for 15 min, then warmed to rt and the pH adjusted to 7. The solid was collected by filtration, dried under vacuo and purified by silica gel column chromatography eluting with 20% MeOH in DCM to give **1e** as an orange solid in 75% yield. Sodium 4-(4′-*N*-methylensulfophenyl)diazenyl)benzenesulfonamide **1e**: mp 340 °C decomposition; silica gel TLC R_f 0.13 (MeOH/DCM 20%); ν_{max} (KBr) cm^{-1} 3427 (N–H), 1604 (aromatic), 1515 (N=N), 1318 (SO_2–N); δ_H (400 MHz, DMSO-d_6) 4.03 (2H, d, J 6.8, 5′-H_2), 6.90 (2H, d, J 9.2, 2 × 3′-H), 7.42 (1H, t, J 6.8, NH, exchange with D_2O), 7.45 (2H, s, SO_2NH_2, exchange with D_2O), 7.75 (2H, d, J 9.2, 2 × 2′-H), 7.91 (2H, d, J 8.8, 2 × 2-H), 7.97 (2H, d, J 8.8, 2 × 3-H); δ_C (100 MHz, DMSO-d_6) 155.2, 153.4, 144.7, 144.0, 127.8, 126.2, 122.8, 113.5, 60.6 (C-5′).

Sodium 3-(4′-(*N*-methylensulfophenyl)diazenylbenzenesulfonamide **2e** was prepared in a similar manner; mp 236–237 °C; silica gel TLC R_f 0.12 (MeOH/DCM 20%); ν_{max} (KBr) cm^{-1} 3427 (N–H), 1604 (aromatic), 1527 (N=N), 1326 (SO_2–N); δ_H (400 MHz, DMSO-d_6) 4.04 (2H, d, J 6.8, 5′-H_2), 6.90 (2H, d, J 9.2, 2 × 3′-H), 7.39 (1H, t, J 6.8, NH, exchange with D_2O), 7.49 (2H, s,SO_2NH_2, exchange with D_2O), 7.74 (1H, appt, J 7.6, 5-H), 7.75 (2H, d, J 9.2, 2 × 2′-H), 7.86 (1H, ddd, J 7.6 2.0 1.2, 4-H), 8.00 (1H, ddd, J 7.6 2.0 1.2, 6-H), 8.20 (1H, appt, J 1.6, 2-H); δ_C (100 MHz, DMSO-d_6) 153.4 (C-3), 153.3 (C-4′), 146.1 (C-4), 143.7 (C-2), 130.9 (7C-1′), 127.0, 126.6, 126.1, 118.3 (C-2), 113.5 (C-3′), 60.6 (C-5′).

4.1.7. Treatment of *N*-methylaniline with formaldehyde sodium bisulfite adduct (preparation of 4)

Formaldehyde sodium bisulfite adduct (1.34 g, 1.0 equiv) was dissolved in H_2O (1.8 ml) at 40 °C and then *N*-methylaniline (1.07 g, 1.09 ml, 1.0 equiv) was added dropwise. The solution was stirred at 50 °C for 4 h. Then the reaction was cooled down to rt and the solid formed was collected by filtration, triturated with diethyl ether and dried under vacuo to give **4** as a white solid in 60% yield. Sodium *N*-methylphenylaminomethanesulfonate **4**: mp > 370 °C; ν_{max} (KBr) cm^{-1}, 1603 (aromatic), 1369 (SO_2–O); δ_H (400 MHz, DMSO-d_6) 3.02 (3H, s, CH_3), 4.08 (2H, s, 1′-H_2), 6.62 (1H, t, J 7.2, 4-H), 6.84 (2H, d, J 8.8, 2 × 2-H), 7.14 (2H, dd, J 8.8 7.2, 2 × 3-H); δ_C (100 MHz, DMSO-d_6) 149.7 (C-1), 129.4 (C-3), 116.8 (C-4), 113.2 (C-2), 69.6 (C-1′), 39.3 (CH_3).

4.1.8. Diazo coupling of the diazonium chloride of 4-aminobenzenesulfonamide with 4

A solution of **3** (prepared as above) was added dropwise to a suspension of **4** (0.26 g, 1.0 equiv) in a saturated aqueous solution of AcONa (1.0 ml) at −5 °C. The reaction turned orange immediately and was stirred at the same temperature for 15 min, then warmed to rt and the pH adjusted to 7. The solid was collected by filtration, dried under vacuo and purified by silica gel column chromatography eluting with 20% MeOH in DCM to give **1f** as an orange solid in 76% yield.

Sodium 4-(4′-*N*-methylphenylaminomethanesulfonate)diazenylbenzenesulfonamide **1f**: mp 257–258 °C; silica gel TLC R_f 0.29 (MeOH/DCM 20%); ν_{max} (KBr) cm^{-1}, 1604 (aromatic), 1514 (N=N), 1376 (SO_2–N); δ_H (400 MHz, DMSO-d_6) 3.19 (3H, s, CH_3), 4.30 (2H, s, 5′-H_2), 7.03 (2H, d, *J* 9.6, 2 × 3′-H), 7.43 (2H, s, SO_2NH_2, exchange with D_2O), 7.82 (2H, d, *J* 9.6, 2 × 2′-H), 7.94 (2H, d, *J* 8.8, 2 × 2-H), 7.98 (2H, d, *J* 8.8, 2 × 3-H); δ_C (100 MHz, DMSO-d_6) 155.1 (C-4), 153.1 (C-4′), 145.0 (ipso), 143.9 (ipso), 127.8 (C-2′), 125.8, 123.0, 113.4 (C-3′), 68.7 (C-5′), 40.0 (*C*H_3).

Sodium 3-(4′-*N*-methylphenylaminomethanesulfonate)diazenylbenzenesulfonamide **2f** was prepared in a similar manner: mp 330 °C with decomposition; silica gel TLC R_f 0.22 (MeOH/DCM 20%); ν_{max} (KBr) cm^{-1}, 1601 (aromatic), 1515 (N=N), 1377 (SO_2–N); δ_H (400 MHz, DMSO-d_6) 3.19 (3H, s, CH_3), 4.28 (2H, s, 5′-H_2), 7.03 (2H, d, *J* 9.2, 2 × 3′-H), 7.49 (2H, s, SO_2NH_2, exchange with D_2O), 7.53 (1H, appt, *J* 7.6, 5-H), 7.82 (2H, d, *J* 9.2, 2 × 2′-H), 7.88 (1H, ddd, *J* 7.6 2.0 1.2, 4-H), 8.025 (1H, ddd, *J* 7.6 2.0 1.2, 6-H), 8.22 (1H, appt, *J* 1.6, 2-H); δ_C (100 MHz, DMSO-d_6) 153.4 (C-3), 153.0 (C-4′), 146.2 (C-1′), 143.7 (C-1), 131.0 (C-1′), 127.1, 126.9, 125.6 (C-2′), 118.4 (C-2), 113.4 (C-3′), 68.7 (C-5′), 40.0 (*C*H_3).

4.1.9. Treatment of sodium 4-(4′-*N*-methylensulfophenyl)diazenylbenzenesulfonamide 1e with NaOH[13]

Sodium 4-(4′-*N*-methylensulfophenyl)diazenylbenzenesulfonamide **1e** (0.03 g, 1.0 equiv) was dissolved in a 10% aqueous solution of NaOH (2.0 ml) and the reaction mixture was stirred overnight at rt, the pH adjusted to 7 and extracted with ethyl acetate (3 × 10 ml). The combined organic layers were washed with brine (2 × 10 ml), dried over Na_2SO_4, filtered and concentrated in vacuo to give a yellow residue that was purified by silica gel column chromatography eluting with 10% MeOH in DCM to give **1b** as an orange solid in 62% yield.

4-(4′-Aminophenyl)diazenylbenzenesulfonamide **1b**: mp 233–235 °C (lit[5] 234–236 °C); silica gel TLC R_f 0.41 (MeOH/DCM 10%); ν_{max} (KBr) cm^{-1}3342 (N–H), 1602 (aromatic), 1505 (N=N), 1301 (SO_2–N); δ_H (400 MHz, DMSO-d_6) 6.35 (2H, s, NH_2 exchange with D_2O), 6.72 (2H, d, *J* 8.8, 2 × 3′-H), 7.45 (2H, s, SO_2NH_2, exchange with D_2O), 7.74 (2H, d, *J* 8.8, 2 × 2′-H), 7.90 (2H, d, *J* 8.8, 2 × 2-H), 7.97 (2H, d, *J* 8.8, 2 × 3-H); δ_C (100 MHz, DMSO-d_6) 155.1 (C-4), 154.7 (C-4′), 144.8 (Ipso), 143.7 (Ipso), 127.8 (C-2), 126.8, 122.8, 114.4 (C-3′).

3-(4′-Aminophenyl)diazenylbenzenesulfonamide **2b** was prepared in a similar manner from **2e**: mp 197–199 °C; silica gel TLC R_f 0.37 (MeOH/DCM 10%); ν_{max} (KBr) cm^{-1} 3350 (N–H), 1601 (aromatic), 1504 (N=N), 1325 (SO_2–N); δ_H (400 MHz, DMSO-d_6) 6.31 (2H, s, NH_2, exchange with D_2O), 6.73 (2H, d, *J* 9.2, 2 × 3′-H), 7.50 (2H, s, SO_2NH_2, exchange with D_2O), 7.74 (1H, appt, *J* 7.6, 5-H), 7.75 (2H, d, *J* 9.2, 2 × 2′-H), 7.86 (1H, ddd, *J* 7.6 2.0 1.2, 4-H), 7.99 (1H, ddd, *J* 7.6 2.0 1.2, 6-H), 8.20 (1H, appt, *J* 1.6, 2-H); δ_C (100 MHz, DMSO-d_6) 154.7 (C-3), 153.5 (C-4′), 146.3 (C-1′), 143.7 (C-1), 131.1, 127.1, 126.9, 126.8, 118.4, 114.5 (C-3′).

4.1.10. Treatment of sodium 4-(4′-*N*-methyl-*N*-methylensulfophenyl)diazenylbenzenesulfonamide 1f with NaOH

Sodium 4-(4′-*N*-methylensulfophenyl)diazenylbenzenesulfonamide **1f** (0.03 g, 1.0 equiv) was dissolved in a 10% aqueous solution of NaOH (2.0 ml) and the reaction mixture was stirred O.N. at rt, the pH adjusted to 7 and extracted with ethyl acetate (3 × 10 ml). The combined organic layers were washed with brine (2 × 10 ml), dried over Na_2SO_4, filtered and concentrated in vacuo to give a yellow residue that was purified by silica gel column chromatography eluting with 10% MeOH in DCM to give **1c** as a yellow solid in 52% yield. 4-(4′-*N*-Methylphenyl)diazenylbenzenesulfonamide **1c**: mp 213–214 °C; silica gel TLC R_f 0.38 (MeOH/DCM 10%); ν_{max} (KBr) cm^{-1} (3410 N–H), 1606 (aromatic), 1529 (N=N), 1390 (SO_2–N); δ_H (400 MHz, DMSO-d_6) 2.84 (3H, d, *J* 5.4, CH_3), 6.72 (2H, d, *J* 8.8, 2 × 3′-H), 6.95 (1H, br q, *J* 5.4, N*H*, exchange with D_2O), 7.47 (2H, s, SO_2NH_2, exchange with D_2O), 7.81 (2H, d, *J* 8.8, 2′-H), 7.90 (2H, d, *J* 8.8, 2 × 2-H), 7.97 (2H, d, *J* 8.8, 2 × 3-H); δ_C (100 MHz, DMSO-d_6) 154.8 (C-4), 154.2 (C-4′), 144.3 (Ipso), 143.8 (Ipso), 127.5 (C-2), 124.9, 124.0, 114.2 (C-3′), 39.3 (*C*H_3).

4.2. CA inhibition assay

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic/inhibition of various CA isozymes as reported by Khalifah.[17] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.4) as buffer, 0.1 M Na_2SO_4 or $NaClO_4$ (for maintaining constant the ionic strength; these anions are not inhibitory in the used concentration),[8] following the CA-catalyzed CO_2 hydration reaction for a period of 5–10 s. Saturated CO_2 solutions in water at 25 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO–water 1:1, v/v) and dilutions up to 0.01 nM done with the assay buffer mentioned above. At least 7 different inhibitor concentrations have been used for measuring the inhibition constant. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[8] and represent the mean from at least three different determinations. All CA isozymes used here were recombinant proteins obtained as reported earlier by our group.[7,8]

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