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Carbonic anhydrase inhibitors: Design of spin-labeled sulfonamides incorporating TEMPO moieties as probes for cytosolic or transmembrane isozymes

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ABSTRACT

A series of spin-labeled sulfonamides incorporating TEMPO moieties were synthesized by a procedure involving the formation of a thiourea functionality between the benzenesulfonamide and free radical fragment of the molecules. The new compounds were tested as inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) and showed efficient inhibition of the physiologically relevant isozymes hCA II and hCA IX (hCA IX being predominantly found in tumors) and moderate to weak inhibitory activity against hCA I. Some derivatives were also selective for inhibiting the tumor-associated isoform over the cytosolic one CA II, and presented significant changes in their ESR signals when complexed to the enzyme active site, being interesting candidates for the investigation of hypoxic tumors overexpressing CA IX by ESR techniques, as well as for imaging/treatment purposes.

In previous work from this laboratory,[1,2] we reported that fluorescein-labeled sulfonamides act as very potent inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1).[3] These fluorescein derivatives are now in early phases of development for applications as diagnostic tools and therapeutic agents for tumors overexpressing CA isozymes IX and XII (among the 16 presently known in mammals).[3–5] With the aid of such fluorescent inhibitors it was possible to prove the involvement of CA IX in tumor acidification processes which lead to an excessive production of H^+ ions in the extracellular space where the active site of CA IX and XII are situated,[4,5] and the possibility to reverse this deleterious phenomenon by blocking the enzyme activity.[1–5] The success of this approach has encouraged us to now investigate alternative possibilities for the labeling of CAs present in tumors (i.e., CA IX and XII) or related isoforms, eventually present in the cytosol (CA I, II, VII, and XIII) or mitochondria (CA VA and VB) of eukaryotic cells.[3] Introduction of a spin label in the molecules of sulfonamides acting as well-known inhibitors of these enzymes[3] seemed to us a straightforward continuation of our previous work.[1,2] In this letter we report the synthesis, CA inhibitory activity against some physiologically relevant isoforms (CA I and II, cytosolic isozymes, and CA IX, transmembrane, tumor-associated isoform)[3] as well as the ESR properties for a series of benzenesulfonamides incorporating 2,2,6,6-tetramethylpiperidinyl-1-oxyl (TEMPO) and thioureido moieties.

Spin-labeled sulfonamides were in fact reported in the 70s,[6] when little was known on the binding of sulfonamides within the CA active site, as no X-ray crystallographic structures of any isoform alone or in complex with inhibitors were available at that time. Some of the compounds **A–E** reported earlier[6–8] incorporated either five-membered (pyrrolidine-*N*-oxide) or six-membered (piperidine-*N*-oxide) radical moieties in their molecule, together with the classical benzenesulfonamide warheads known to coordinate (in deprotonated form, as sulfonamidate anion) to the Zn(II) ion present in the enzyme active site and crucial for its catalytic activity (Chart 1).[3] These compounds were basically used for gaining insight on the topology of the CA active site and no detailed inhibition studies are available with them, also because only isoforms I and II were known in the period when they were reported.[6] Furthermore, the possibility of exploiting the ESR signals of these earlier compounds of types **A–E** for imaging/treatment purposes has never been taken into account. There is in fact a stringent need

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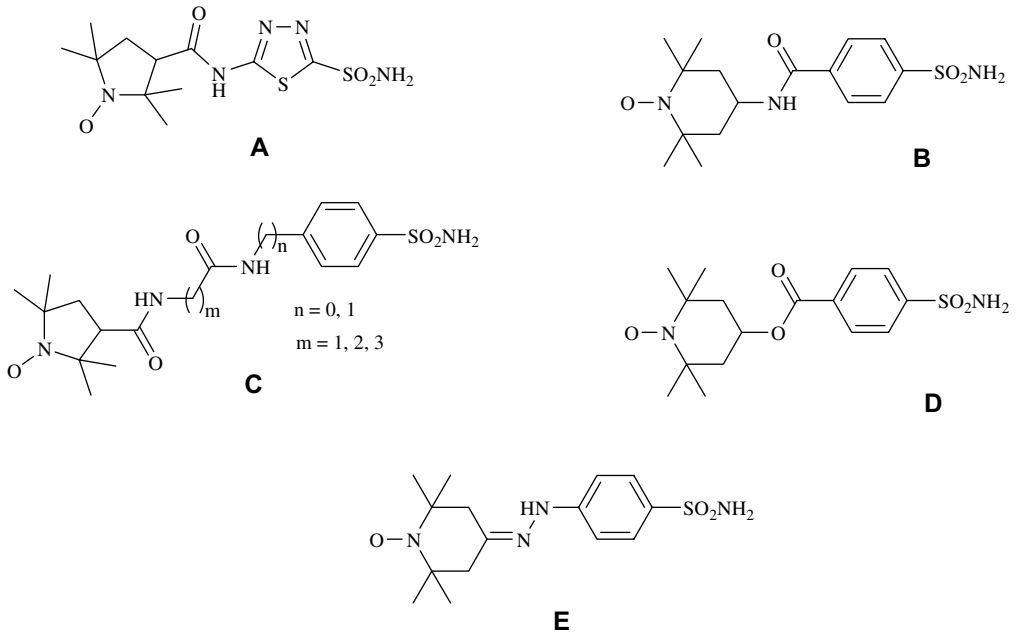


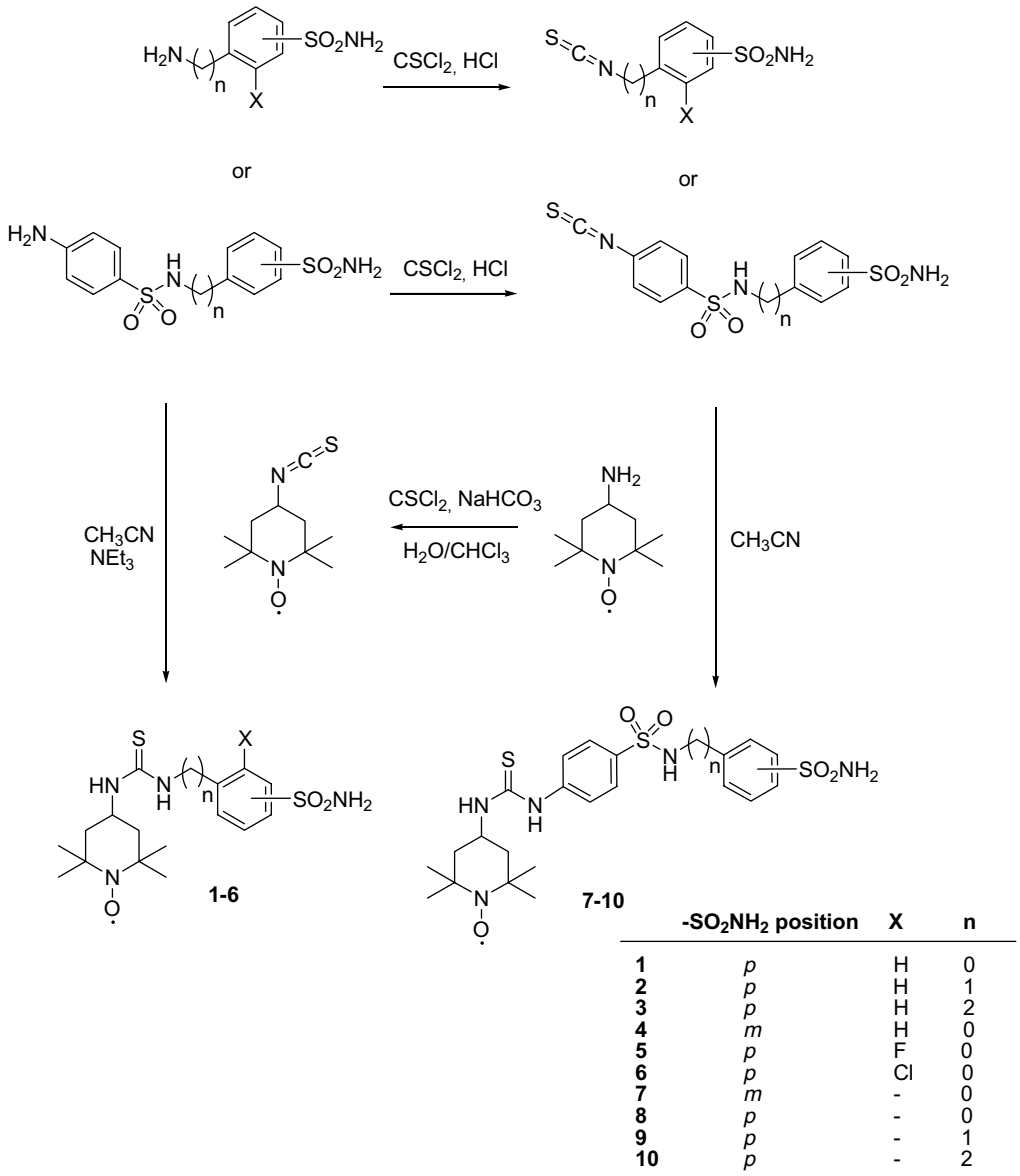
Chart 1. Spin-labeled sulfonamides **A–E** reported earlier.

of CAIs which can be used for the selective labeling of CA isozymes involved in various pathological processes,[3,7,8] as exemplified among others also by the very recent report of Christianson's group of a ^{129}Xe-cryptophane-sulfonamide biosensor complexed to hCA II which can be used as a diagnostic tool for ^{129}Xe-magnetic resonance imaging (MRI).[8]

The design of the new CA inhibitors (CAIs) **1–10** reported here was approached by the classical tail strategy,[2,3] that is, maintaining the benzenesulfonamide head present in the spin-labeled compounds **A–E**[6] reported earlier, and by incorporation of a thiourea linker between this and the free radical TEMPO tail (Scheme 1), which was not present in any of the earlier derivatives. The benzenesulfonamide moiety was chosen due to its ability to tightly bind to the zinc ion within the enzyme active site, as shown by extensive X-ray crystallographic work from this and other groups.[1–3,8–11] Furthermore, the phenyl ring belonging to the benezenesulfonamide moiety may be substituted in positions 3 and 4 (with respect to the sulfamoyl group) with halogen atoms, as some of these derivatives were shown earlier to lead to potent CAIs.[1–3] The central thiourea linker motif was chosen because it was shown earlier that compounds incorporating it act as potent hCA I, II, and IX inhibitors.[12,13] We then employed the 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) moiety as the tail group. This free radical moiety is active in the ESR experiments, and possesses a simple spectrum.[14] In addition, it is stable, it induces a relatively good water solubility to the compounds incorporating it, and its spectral features (such as line width and intensity) can be regulated by tissue oxygen or redox status in in vivo experiments. This latter property also renders newly synthesized molecules containing this radical scaffold to be incorporated in spin probes that interact with biomolecules such as enzymes.[14,15]

The compounds **1–10** were prepared as shown in Scheme 1 by employing two alternative but similar reaction methods (depending on the stability of the isothiocyanate intermediates). The first method consisted in the reaction of aminosulfonamides with thiophosgene in acidic medium which leads to the corresponding isothiocyanate-sulfonamides.[12,13] Reaction of these intermediates with amino-TEMPO in acetonitrile led to thioureas **1** and **4–10**. As the precursor sulfonamide isothiocyanates could not be prepared in acceptable yields for the subsequent synthesis of compounds **2** and **3**, an alternative method was employed, that is, reaction of TEMPO isothiocyanate (obtained from amino-TEMPO and $CSCl_2$ similar to what is described above)[12,13] with the corresponding amines (Scheme 1).[16,17]

The inhibition data of the new sulfonamides **1–10** and standard, clinically used inhibitors such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, and indisulam **IND** (Chart 2) against the cytosolic isozymes hCA I, hCA II, and the transmembrane tumor-associated isozyme hCA IX (h = human isoform) are presented in Table 1.[18] The following structure–activity relationships (SARs) should be noted: (i) against the ubiquitous, house-keeping, and physiologically relevant isoform hCA II the new sulfonamides **1–10** showed good inhibitory activity, with inhibition constants (K_i values) in the range of 12–165 nM. The derivatives with a *meta* substitution pattern on the benzenesulfonamide ring showed the least efficient inhibitory activity (K_is of 165 and 152 nM, respectively, for derivatives **4** and **7**). Compounds **1–3** showed a compact behavior of efficient CAIs, with K_is of 28–42 nM. An increased affinity for hCA II has been observed for the halogeno-substituted derivatives **5** and **6**, with the chlorine-substituted benzenesulfonamide **6** being a very efficient CAI, comparable to the clinically used compounds **AAZ, MZA**, and **EZA** (Table 1). The sulfanilyl-sulfonamides **8–10** were slightly less efficient hCA II inhibitors as compared to **6**, but they appreciably inhibited the enzyme with inhibition constants in the range of 20–47 nM. Thus, we evidenced various types of substitution patterns of the TEMPO-containing CAIs that lead to efficient, low nanomolar inhibitors of the physiologically relevant isozyme hCA II; (ii) the inhibition data of compounds **1–10** against the tumor-associated isozyme hCA IX (our target for imaging or treatment purposes)[1] showed them to possess excellent inhibitory activity, with K_is values in the range of 7–220 nM (Table 1). Similar to the hCA II inhibition discussed above, the *meta* substitution pattern present in derivatives **4** and **7** led to the least effective inhibitory properties (K_is of 132 and 220 nM, respectively) for the corresponding sulfonamides. The other compounds reported here were much better hCA IX inhibitors as compared to **4** and **7**, with inhibition constants in the range of 7–41 nM. SAR was rather sim-



	-SO_2NH_2 position	X	n
1	*p*	H	0
2	*p*	H	1
3	*p*	H	2
4	*m*	H	0
5	*p*	F	0
6	*p*	Cl	0
7	*m*	-	0
8	*p*	-	0
9	*p*	-	1
10	*p*	-	2

Scheme 1. Preparation of TEMPO-tailed sulfonamides **1–10**.

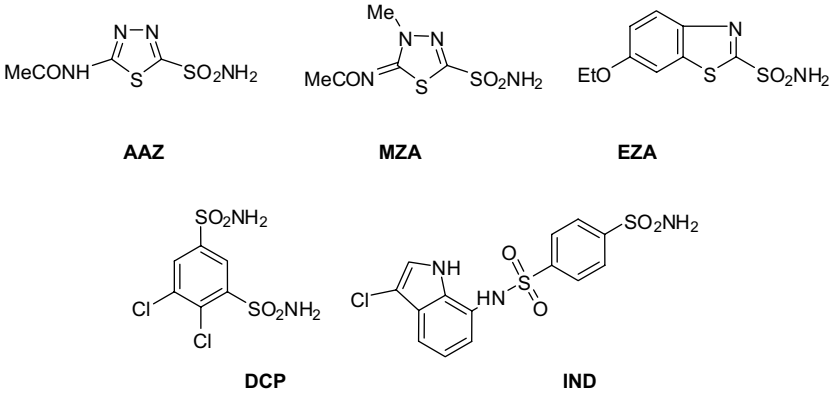


Chart 2. Structures of clinically used CAIs **AAZ-DCP** and of the antitumor sulfonamide in clinical development indisulam (**IND**).

ilar to what is discussed above for hCA II, except that the best inhibitor was in this case **9**, which with its elongated molecule may probably better fill the hCA IX active site which is presumed to be larger than that of hCA II;[20] (iii) against the slow cytosolic iso-

Table 1
CA inhibition data of compounds **1**–**10** and standard CAIs (**AAZ**–**IND**) against human isoforms hCA I, II (cytosolic), and IX (transmembrane, tumor-associated)

Compound	K_i^* (nM)			Selectivity ratio
	hCA I[a]	hCA II[a]	hCA IX[b]	$K_{IhCAII}/K_{IhCA\ IX}$
AAZ	900	12	25	0.48
MZA	780	14	27	0.52
EZA	25	8	34	0.23
DCP	1200	38	50	0.76
IND	31	15	24	0.62
1	179	41	41	1.00
2	204	42	35	1.20
3	182	28	39	0.72
4	2070	165	132	1.25
5	233	37	22	1.68
6	128	12	14	0.86
7	784	152	220	1.55
8	365	47	30	1.57
9	89	20	7	2.86
10	170	33	41	0.80

[a] Human, recombinant isozymes.
[b] Catalytic domain of human, cloned isoform.[13]
* Errors in the range of 5–10% of the data shown, from three different assays, by a CO_2 hydration stopped-flow assay.[18]

form hCA I, whose physiological function is less well defined as compared to those of isoforms II and IX discussed above,[1–3] sulfonamides **1–10** showed inhibition constants in the range of 89–2070 nM. Derivatives **4**, **7**, and **8** were the least effective hCA I inhibitors (K_is of 365–2070 nM), while the remaining compounds possessed medium efficacy as CAIs against this isoform (K_i 89–233 nM). The reduced affinity of some of the first such molecules against hCA I is probably due to the fact that they possess a bulky moiety *meta* to the sulfamoyl zinc binding group of the benzenesulfonamide warhead, a trend we have observed for other classes of compounds bearing this substitution pattern.[19] The inhibition constants of compounds **1**, **2**, and **3** (K_is of 179–204 nM) were also very similar (as for the inhibition of the other two isozymes discussed here). This demonstrates that the presence/absence of a CH_2 or CH_2CH_2 spacer between the benzenesulfonamide scaffold and thiourea moiety does not lead to important differences in the inhibitory properties of these derivatives. However, for the structurally similar, more elongated compounds of types **8**–**10**, with an internal secondary sulfonamide group, compound **9** showed a better inhibition constant (K_i 89 nM) as compared to its shorter or longer congener, suggesting the importance of the overall length of the inhibitor for an optimal interaction with the enzyme active site (in this case the CH_2 linker present in **9** led to the best such interactions).[9,11] Derivatives **1**–**10** showed an overall inhibition profile against hCA I similar to that of the clinically used sulfonamides **AAZ**, **EZA**, and **IND** (Table 1); (iv) among all the free radical inhibitors synthesized here, compounds **2**, **5**, **8**, and **9** showed good selectivity for the inhibition of the tumor-associated isoform hCA IX over the cytosolic one hCA II, with the best selectivity ratio of 2.9 achieved for compound **9** (Table 1 and Fig. 1). All these compounds were obviously much better hCA IX inhibitors than hCA I inhibitors (Table 1). These are not at all trivial results, since few compounds reported in the literature selectively inhibit the tumor-associated isoform over the cytosolic CAs. As also seen from data of Figure 1, all the clinically used compounds **AAZ-IND** show selectivity ratios for the inhibition of CA IX over CA II less than 1 (thus being better CA II than CA IX inhibitors), whereas many of the new nitroxylated CAIs reported here (e.g., **2, 4, 5, 7, 8**, and **9**) have a selectivity ratio >1. This means that these compounds will preferentially bind CA IX (already overexpressed in hypoxic tumors)[1,2] and much less CA II, which is a house-keeping enzyme, necessary for many physiological processes of the cell.[1–3]

The ESR spectra of the nitroxide derivatives reported here were recorded both in the absence and in the presence of enzyme (Fig. 2A and B). hCA II was used as model enzyme for this study owing to both the good inhibitory activity of most compounds synthesized in this work, and to its ease of handling and low cost as compared to hCA IX, the actual diagnostic target for which these compounds were designed. ESR experiments were crucial for assessing the mobility rates of TEMPO-CAIs in solution and upon binding to the enzyme. Figure 2A and B shows the ESR spectra of compounds **1** and **6**, both free in solution and complexed to the enzyme, respectively. The signals of the spin-labeled CAIs dissolved in buffer were superposable, and this was in agreement with the fact that the two compounds **1** and **6** only differ by the presence of a chlorine atom which is placed far from the free radical piperidin-*N*-oxide moiety. Both radicals underwent rapid motion in buf-

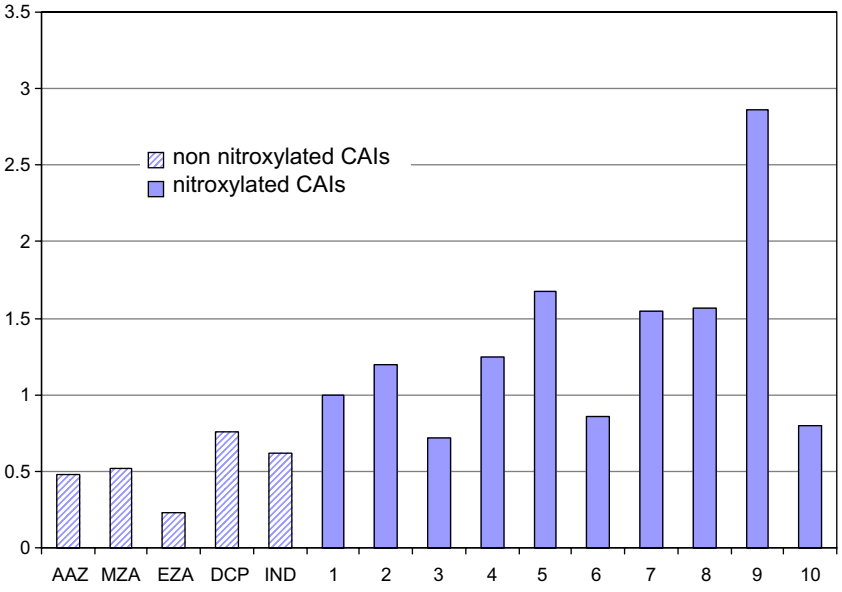


Figure 1. Histogram representing the enhanced selectivity ratio for the inhibition of the tumor-associated isozyme hCA IX over the cytosolic, house-keeping isoform hCA II with some of the TEMPO-labeled, nitroxylated sulfonamides **1–10** over the classical, clinically used CAIs **AAZ-IND** (non-nitroxylated CAIs).

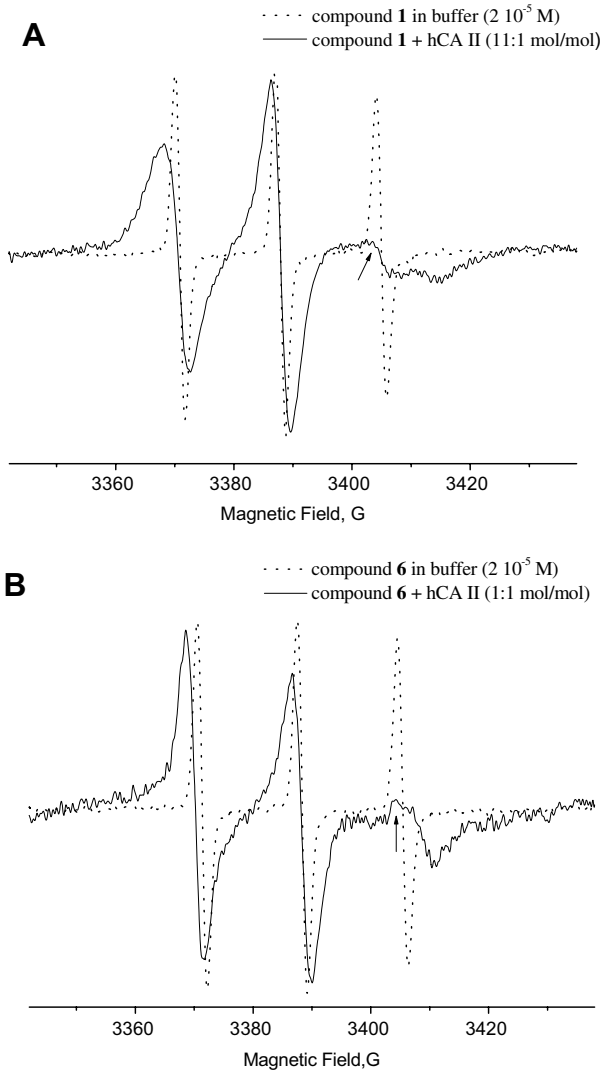


Figure 2. ESR spectra of compounds **1** (A), and **6** (B) in solution without enzyme (dotted line) and in the presence of hCA II at a molar ratio of 1:1 (continuous line).

fer solution (in the absence of enzyme). The measured hyperfine coupling constant was typical of a polar medium, being $\langle A_N \rangle$ = 17.1. When in the presence of hCA II, the ESR spectrum of compounds **1** and **6** significantly changed to a slow motion signal, indicating that binding within the enzyme active site has efficiently occurred. It should be noted that a very small fraction of labeled compounds did not interact with the enzyme. This fraction was found to be of the order of 0.1–3%, as measured from double integration of the different ESR peaks. The exact amount of non-interacting TEMPO-CAIs (whose corresponding peak is indicated by an arrow in Figure 2A and B) depended both on the structure of the nitroxide derivative and the molar ratio with respect to hCA II. However, as mentioned above, in all cases the ESR line shape of TEMPO-CAIs in the presence of hCA II was completely dominated by the spectrum of the bound molecules, which showed the anisotropic features characteristic to restricted motional conditions.[6–8] A similar behavior was found for the vast majority of compounds **1–10** (data not shown) proving that these free radicals were able to interact strongly with the amino acid residues lining the cavity of the hCA II enzyme, and making them promising molecules for the investigation of various CA isozymes by means of ESR techniques. Both imaging as well as therapeutic applications of such molecules can be envisaged,[3,21,22] considering also their potential as bioreductive drugs.

In conclusion, we report here a novel series of spin-labeled sulfonamides incorporating TEMPO moieties which have been synthesized by a procedure involving the formation of a thiourea functionality between the benzenesulfonamide and free radical fragment of the molecules. These new sulfonamides showed moderate–weak inhibitory activity against isozyme hCA I, but were in general much more efficient inhibitors of hCA II and hCA IX, the last one being predominantly found in tumors. Some of these compounds showed significant changes in their ESR signals when complexed within the enzyme active site, making them potential candidates of interest for the investigation of hypoxic tumors overexpressing CA IX by ESR techniques for imaging and diagnostic purposes.

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16. *Synthesis of thioureido-sulfonamides **1**, **4**–**10***. The isothiocyanate sulfonamides[13] (1 mmol) were dissolved in 25 mL of MeCN and a solution of amino-TEMPO in MeCN (1 mmol) was added dropwise. The reaction was monitored by TLC and when amino-TEMPO was no longer present, the solution was filtered and the precipitated solid washed with Et_2O. The product was purified by flash chromatography; yields were in the range of 51–92%. *4-({[(1-Oxyl-2,2,6,6-tetramethylpiperidin-4-yl)amino]carbonothioyl} amino)benzene sulfonamide* (**1**): MS:ESI$^+$ *m*/*z* 408 (M+Na)$^+$, 793 (2M+Na)$^+$; *3-({[(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)amino]carbonothioyl}amino)benzene sulfonamide* (**4**); MS:ESI$^+$ *m*/*z* 386 (M+H)$^+$, 408 (M+Na)$^+$; ESI$^-$ *m*/*z* 384 (M−H)$^-$, 769 (2M−H)$^-$: *3-fluoro-4-({[(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)amino]carbonothioyl} amino)benzene sulfonamide* (**5**); MS:ESI$^+$ *m*/*z* 404 (M+H)$^+$, 426 (M+Na)$^+$; ESI$^-$ *m*/*z* 402 (M−H)$^-$, 805 (2M−H)$^-$: *3-chloro-4-({[(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)amino]-carbonothioyl} amino)benzene sulfonamide* (**6**); MS:ESI$^+$ *m*/*z* 420 (M+H)$^+$, 442 (M+Na)$^+$: *N-[3-(aminosulfonyl)phenyl]-4-{[(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)amino]carbono thioyl} amino)benzene sulfonamide* (**7**); MS:ESI$^+$ *m*/*z* 543 (M+H)$^+$; ESI$^-$ *m*/*z* 541 (M−H)$^-$; *N-[4-(aminosulfonyl)phenyl]-4-{[(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)amino]carbono thioyl}amino)benzene sulfonamide* (**8**); MS:ESI$^+$ *m*/*z* 543 (M+H)$^+$; *N-[4-(aminosulfonyl)benzyl]-4-{[(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)amino]carbono thioyl}amino)benzene sulfonamide* (**9**); MS:ESI$^+$ *m*/*z* 577 (M+Na)$^+$; *N-{2-[4-(aminosulfonyl)phenyl]ethyl} -4-{[(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)amino]carbono thioyl} amino)benzene sulfonamide* (**10**): MS:ESI$^+$ *m*/*z* 569 (M+H)$^+$.
17. The TEMPO isothiocyanate was prepared cf. Zakrzewski, J.; Hupko, J.; Kryczka, K. *Monatsh. Chem.* **2003**, *134*, 843. In a 50 mL round-bottomed flask, thiophosgene (0.43 mL, 5.50 mmol) was added in a mixture of 3 mL of $NaHCO_3$ 4.7 M and 2.5 mL of CH_2Cl_2. The solution was stirred vigorously at room temperature and then amino-TEMPO (0.8 g, 4.74 mmol) was added dropwise. The aqueous layer was extracted with CH_2Cl_2, the organic layers collected, and dried over anhydrous Na_2SO_4 and evaporated. The obtained oil was purified by flash-chromatography and an orange solid was obtained (0.64 g, 63%). MS:ESI$^+$ *m*/*z* 214 (M+H)$^+$; ESI$^-$ *m*/*z* 212 (M−H)$^-$: *Synthesis of thioureido-sulfonamides **2** and **3***. One equivalent of amino-sulfonamides in 20 mL of MeCN was treated with a solution of TEMPO isothiocyanate (213 mg, 1 mmol) in MeCN, and the mixture was stirred at room temperature for 48 h. The reaction mixture was worked up as described above.[16] *4-[({[(1-Oxyl-2,2,6,6-tetramethylpiperidin-4-yl)amino]carbono thioyl} amino)methyl]benzene sulfonamide* (**2**): MS:ESI$^+$ *m*/*z* 422 (M+Na)$^+$; ESI$^-$ *m*/*z* 398 (M−H)$^-$; *4-[({[(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)amino]carbono thioyl} amino)ethyl]benzene sulfonamide* (**3**): MS:ESI$^+$ *m*/*z* 414 (M+H)$^+$, 426 (M+Na)$^+$..
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