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New selective carbonic anhydrase IX inhibitors: Synthesis and pharmacological evaluation of diarylpyrazole-benzenesulfonamides

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ABSTRACT

Carbonic anhydrase (CA) IX expression is increased upon hypoxia and has been proposed as a therapeutic target since it has been associated with poor prognosis, tumor progression and pH regulation. We report the synthesis and the pharmacological evaluation of a new class of human carbonic anhydrase (hCA) inhibitors, 4-(5-aryl-2-hydroxymethyl-pyrazol-1-yl)-benzenesulfonamides. A molecular modeling study was conducted in order to simulate the binding mode of this new family of enzyme inhibitors within the active site of hCA IX. Pharmacological studies revealed high hCA IX inhibitory potency in the parameters nanomolar range. This study showed that the position of sulfonamide group in *meta* of the 1-phenylpyrazole increase a selectivity hCA IX versus hCA II of our compounds. An in vitro antiproliferative screening has been performed on the breast cancer MDA-MB-231 cell using doxorubicin as cytotoxic agent and in presence of selected CA IX inhibitor. The results shown that the cytotoxic efficiency of doxorubicin in an hypoxic environment, expressed in IC_{50} value, is restored at 20% level with 1 μM CA IX inhibitor.

1. Introduction

Carbonic anhydrases (CAs) are a family of zinc metalloenzymes that catalyze the rapid reversible hydration of carbon dioxide to bicarbonate anion and proton ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$).[1] All human CAs (hCAs) belong to the α-class and differ widely in their cellular localizations: CA I, II, III, VII and XIII reside in cytosol, CA IV, IX, XII and XIV are associated with cell membrane, and CA V_A and CA V_B occur in mitochondria, whereas CA VI is secreted in saliva and milk.[2] CA isoenzymes play an important role in essential cellular processes such as pHe and pHi regulation, secretion of electrolytes, respiration and biosynthetic reactions that require CO_2 and HCO_3^- as substrates (e.g., lipogenesis, glucogenesis and ureagenesis).[3] CA IX is a dimeric transmembrane glycoprotein with an extracellular active site and a NH_2-terminal proteoglycan-like region and is almost exclusively associated with tumors. CA IX is implicated in cell adhesion as well as in acid–base balancing and intracellular communication.[4] High tumoral carbonic anhydrase IX (CA IX) expression has been associated with poor prognosis, tumor progression and aggressiveness.[5] CA IX is expressed in a limited number of normal tissues (mainly the gastrointestinal tract), whereas its overexpression is observed on the cell surface of a large number of solid tumors, and it is invariably associated with the hypoxic phenotype, mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1).[6] CA IX has been identified as a potentially important marker of hypoxia. Furthermore, CA IX overexpression is often associated with a poor responsiveness to the classical radio- and chemo-therapies.[6,3] Therapeutic inhibition of CA IX has recently been shown to decrease primary tumor growth and metastasis in preclinical breast tumor models.[5,7] The overall consequence of CA IX overexpression in tumors is a pH balance leading to acidification of the extracellular microenvironment of hypoxic tumor ($pH_e \approx 6.8$), in contrast to normal tissue ($pH_e \approx 7.4$).[8–11] This low pHe is associated with tumorigenic transformation, chromosomal rearrangements, extracellular matrix breakdown, migration, and invasion.[2,5] From a therapeutic point of view, acidic pH_e is also related to a chemoresistance by a decrease in uptake of weakly basic anticancer drugs as

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irinotecan, mitomycin, bleomycin and doxorubicin.[12,13] Doxorubicin is one of the most active chemotherapeutic agents for the treatment of advanced breast cancer and CA IX is described as being a predictive marker of doxorubicin resistance.[14]

The acidic extracellular environment can decrease the uptake of anthracyclins by cells, because these drugs are weak bases, which ionize at low pH. A recent study demonstrates that CA IX is correlated with worse outcome for early-stage breast cancer patients treated with doxorubicin.[15] Consequently, CA IX has been proposed as a potential therapeutic target.[5,7,14] A possible approach to target CA IX would be via inhibiting its enzymatic activity with specific pharmacological inhibitors. As all α-CAs, CA IX is inhibited by several main classes of inhibitors: inorganic anions, sulfonamides and their isosteres (sulfamates and sulfamides), phenols,[16] coumarins[17] and antibodies.[18]

Anions and sulfonamides coordinate to the metal ion within the enzyme cavity in a tetrahedral geometry[19] with three anchors. The best investigated class of inhibitors is the sulfonamide derivatives[20] which are characterized by a low nanomolar inhibition constant (K_i). Additional, a selective CA IX inhibition is required for a cancer therapeutic relevancy without side effects related to the inhibition of other CA isoforms, especially the ubiquitous CA II enzyme. Many low nanomolar sulfonamide CA IX inhibitors have been identified in the last several years; thus agents, that can inhibit CA IX activity, may have therapeutic value and offer opportunities for the treatment or prevention of various cancers. Unfortunately, most of these current inhibitors do not selectively target CA IX.

It has been shown that celecoxib, a COX-2 sulfonamide containing compound, was a strong inhibitor of hCA IX (K_i = 16 nM) as well as hCA II (K_i = 21 nM). Recently, our group described benzylanilinosulfonamides as hCA IX inhibitors (K_i = 1.8–27 nM).[21] Although very promising as hCA IX inhibitors, these compounds are not CA IX selective when compared to the inhibition of the ubiquitous hCA II (K_i = 17–49 nM). It was therefore proposed to develop aromatic molecules that can better fit into the active site of hCA IX to improve selectivity. Combining the structure of celecoxib and benzylanilinosulfonamides, series of 1,5-diarylpyroles have been designed with the aim to obtain selective hCA IX inhibitors. These compounds have an aromatic ring in position 5 and different amides in position 3, they show a nanomolar K_i (K_i = 10 nM) but are not selective for hCA IX versus hCA II (K_i ratio = 5.5).[5]

According to these results, we proposed a novel design by changing the heterocycle for a diarylpyrazole structure (Fig. 1). Various compounds were synthesized to determine structure–activity relationships. This work has permitted to identify a pharmacophore with three anchoring points that interact with the active site of the enzyme. The first is the central heterocycle ring, as scaffold that can rigidify the structure and so guide substituent. The second is the aryl sulfonamide that interacts in the anionic form (SO_2NH^-) with the zinc, and the last, is a moiety that will allow hydrogen bound to Trp 5 of the CA IX active site. Sulfonamides (SO_2NH_2), as deprotonated state (SO_2NH^-), bind in a tetrahedral geometry of the Zn(II) ion: the nitrogen atom of the sulfonamide moiety coordinated to Zn(II) and an extended network of hydrogen bonds, involving residues Thr199 and Glu106, also participating to the anchoring of the inhibitor molecule to the metal ion (Fig. 1).[18] The calculated and measured pK_a of our series of compounds (p$K_a \approx 9.3$)[22] were used to determine the percentage of anionic form in relation with the formation of the tetrahedral adduct. At physiological pH, our compounds are 99% as molecular form (SO_2NH_2) so these values measured in aqueous solution do not allow to explain the formation of the adduct and it suppose that the apparent pK_a would be acidic shifted in active site.

On the other hand a molecular modelisation study was performed on the compound having a 6-MeO-2-naphthyl and a primary alcohol at position 3 (Fig. 2). This docking show that 6-MeO-2-naphthyl fits perfectly into the hydrophobic pocket of CA IX active site, the sulfonamide interacts with zinc II active site and that hydrogen bound formed between the primary alcohol and Trp 5. In this study we designed novel derivatives, 1,5 diarylpyrazole, with different aromatic groups in 5 position (phenyl, biphenyl, 2-naphthyl, phenanthryl…), and substituting in the 3 position by donor or acceptor H bond (primary alcohol) (Fig. 1). Pharmacological evaluations are carried out using enzymatic activity and cell proliferation studies.

2. Results and discussion

2.1. Chemistry

The synthetic route to obtain the target derivatives **3a–3o**, **3a′** and **3j′** is outlined in Scheme 1. Treatment of suitable commercially available aryl ketones with sodium ethylate in absolute EtOH, followed by addition of diethyl oxalate (or malonate) gave with good yield (74–82%) the β-diketones **1a–o** according to a classical Claisen procedure. The cyclization of β-diketones **1a–o** with 4-aminosulfonylphenylhydrazine hydrochloride, in refluxing EtOH, gave a mixture of 1,5 and 1,3-diarylpyrazole ethyl esters with a 80:20 ratio in most cases. We were not able to achieve excellent regioisomeric ratio using modified pH reaction conditions as described by Singh et al.[23] In our case, complex mixtures were obtained, which complicated the purification. Separation of the regioisomers was also effected by silica gel chromatography and followed by a crystallization to afford the pure desired 1,5-diarylpyrazole carboxylic ethyl esters **2a–2o**. Two 1,3-diaryl compounds (**2a′** and **2j′**) were isolated by chromatography (the 1,5 or 1,3-diaryl structure was established by NOESY experiments). Primary alcohols **3a–3o**, **3a′** and **3j′** were obtained in excellent yields by reduction of the corresponding ethyl esters **2a–2o**, **2a′** and **2j′** with lithium aluminium hydride in dry THF.

In Scheme 2, the cyclization of β-diketones **1a–1j–1k** with 3-aminosulfonylphenyl hydrazine hydrochloride, in refluxing EtOH, gave a mixture of 1,5 and 1,3-diarylpyrazole ethyl esters with a 90:10 ratio in most of cases. Separation of the regioisomers was also performed by crystallization to afford the pure desired 1,5-diarylpyrazole carboxylic ethyl esters **4a–4j–4k**. The confirmation of the 1,5 structure was performed by NOESY experiments. 1,3-diaryl compounds (**4a′–4j′–4k′**) were not isolated. Primary alcohols **5a–5j–5k** were obtained in excellent yields by reduction of the corresponding ethyl esters **4a–4j–4k** with lithium aluminium hydride in dry THF.

The synthetic route to obtain the target derivatives **8a–8j–8k** is outlined in Scheme 3. Aryl ethyl dimethylaminoacrylates **6a–6j–6k** were obtained according to a Claisen condensation, by refluxing the required ethyl arylacetate and the *N*,*N*-dimethylformamide dimethylacetal. The cyclization of aryl ethyl dimethylaminoacrylate **6a–6j–6k** with 4-aminosulfonylphenyl hydrazine hydrochloride, in refluxing EtOH, gave compounds **7a–7j–7k**. Primary alcohols **8a–8j–8k** were obtained in excellent yields by reduction of corresponding ethyl esters **7a–7j–7k** with lithium aluminium hydride in dry THF.[24]

2.2. Carbonic anhydrase inhibition

The inhibitory potency of pyrazole derivatives **3a–o**, **3a′**, **3j′**, **5a–5j–5k, 8a, 8j, 8k,** (K_i, nM) against the three carbonic anhydrase isoforms, including physiologically ubiquitous isoforms hCA I, hCA II and transmembrane tumor-associated isoform hCA IX, was

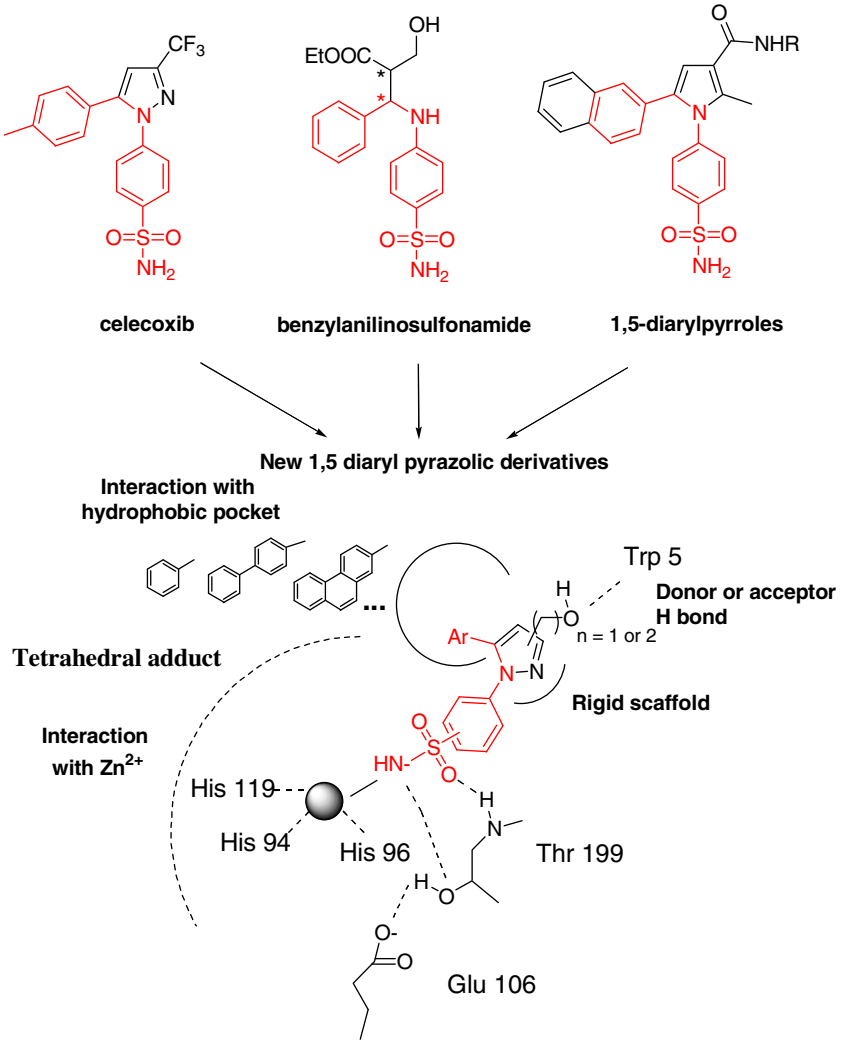


Figure 1. New developed (5-arylpyrazol-1-yl)-benzenesulfonamides from the celecoxib, benzylanilinosulfonamides and 1,5-diarylpyrroles described as hCA inhibitors.

evaluated. The results expressed as K_i (from IC_{50} values and the Cheng and Prussof calculations) are summarized in Tables 1 and 2 and reveal significant points. The hCA I/hCAIX and hCA II/hCA IX K_i ratios are representative of the isozyme selectivity towards hCA IX. The presence of an aryl group in the position 5 of the pyrazole ring, the isomer position, the 1-phenyl substitution by a sulfonamide in *meta* or *para* were systematically varied. CAs activities were carried out to determine the compounds inhibitory abilities to reduce the reaction of hydrogenocarbonate formation and to gain insight into structure–activity relationships.

As summarized in Table 1, we observed that all the reported compounds exhibit a weak inhibitory activity against hCAI with K_i values from 77 to 19,081 nM, independently of the nature of the aromatic group R1. These results, in agreement with literature, show that when compared to AZA, a large number of sulfonamides containing bulkier scaffolds show a weaker inhibition potential due to the presence of additional histidine residues (His 200 and His 67), which delimit a smaller active site in hCA I.[24]

We observed that most of compounds were considerably more potent against hCA II than against hCA I (K_i = 8.9–420 nM). On one hand, a general increase in inhibitory activities against hCA II is observed when the bulkiness of the aromatic group R1 at position 5 of the pyrazolic ring enhances. Indeed, compounds with a phenyl ring at R1 such **3a–3h**, **3o** (K_i = 117–420 nM) are less potent than compounds with bulkier groups such 1-naphthyl (**3i**: K_i = 46 nM), 2-naphthyl (**3j**: K_i = 9.6 nM) and 4-biphenyl (**3m**: K_i = 8.9 nM). On the other hand, compounds **3k**, **3l** and **3n** which possess respectively 6-OMe-2-naphthyl, 5-Br-6-OMe-2-naphthyl and 2-phenanthryl group are less active inhibitors with K_i values of 264, 388 and 10,232 nM towards hCA II.

As observed in Table 1, important inhibitory activities from 15 to 32 nM were found against hCAIX with compounds **3a–3h**, and **3o** possessing a phenyl group at R1. Substituents in *para*-position of the phenyl ring are well tolerated and the best result is obtained with a bromine in compound **3d** (K_i = 15 nM) compared to **3a** (K_i = 27 nM). Among compounds with bulkiest groups, the 2-naphthyl (**3j–3l**), and 4-biphenyl (**3m**) derivatives, gave the most potent inhibitors (K_i = 17–30 nM). Moreover, compounds **3i** and **3n** possessing 1-naphthyl and 2-phenanthryl group at R1 showed a large decrease in inhibition activity against hCA IX (K_i = 254 and 1857 nM).

Most of compounds except **3m** are strongly selective towards hCA IX versus hCA I. Interestingly, the six compounds **3a**, **3c**, **3d**, **3k**, **3l** and **3o** show an important selectivity profile for hCA IX versus hCA II. Compounds **3a** and **3k** exert the highest selectivity towards hCA IX versus hCA II (hCA II/hCA IX = 15) and hCA I (hCA I/hCA IX = 148) and represent the most promising inhibitors. Pharmacomodulations around these most promising inhibitors (**3a**, **3j**, **3k**) were investigated.

The regioisomeric 1,3-diarylpyrazole **3a**′ conducts to a loss of CA IX inhibition (K_i = 157 nM) and a poor selectivity. The regioiso-

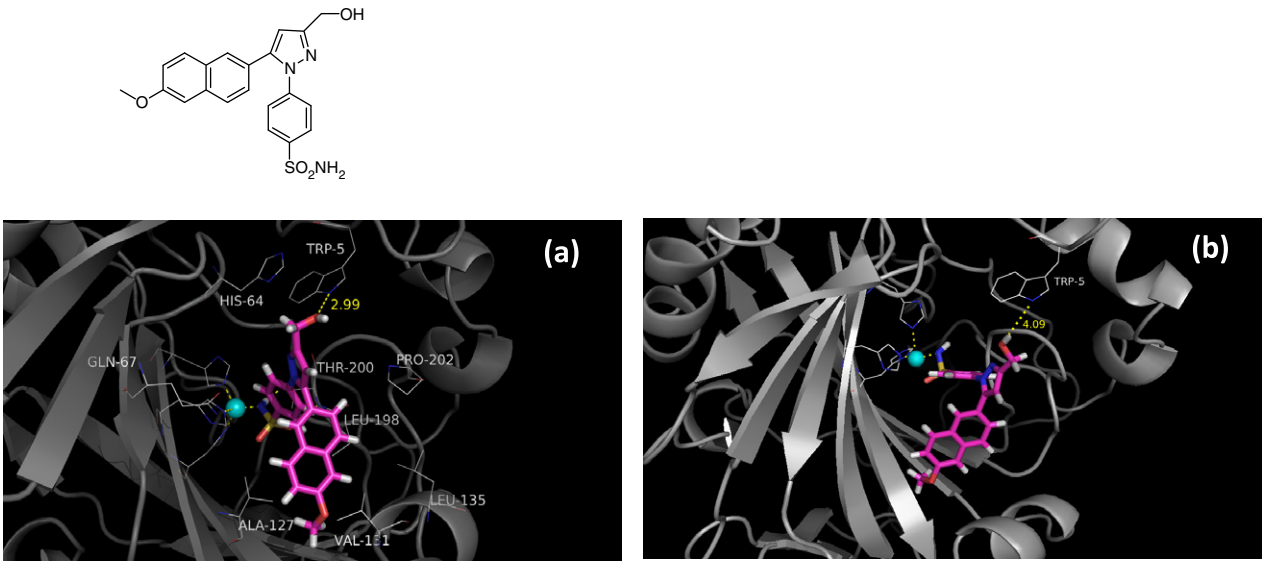
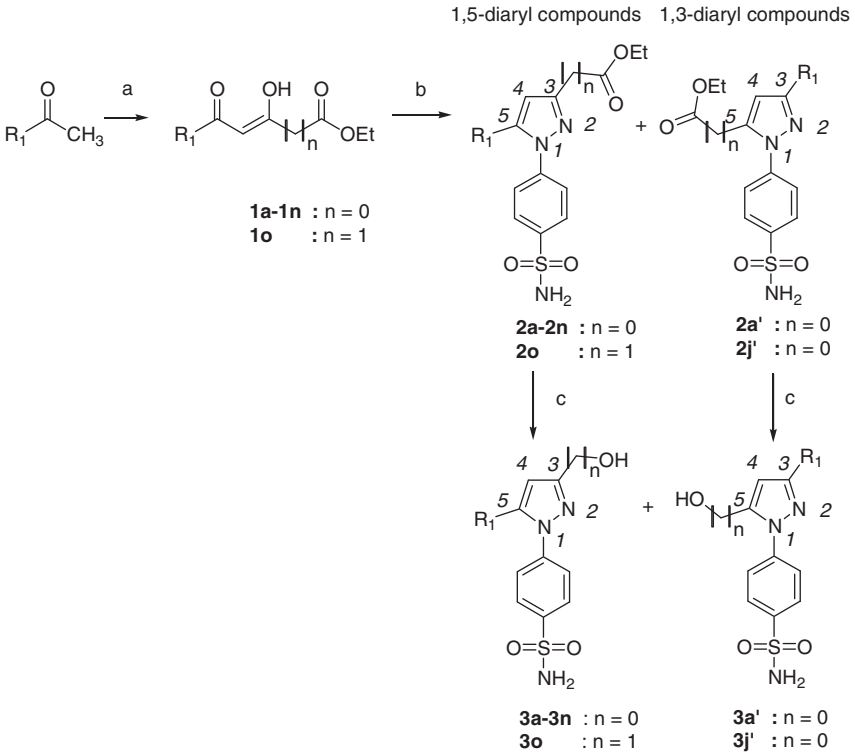


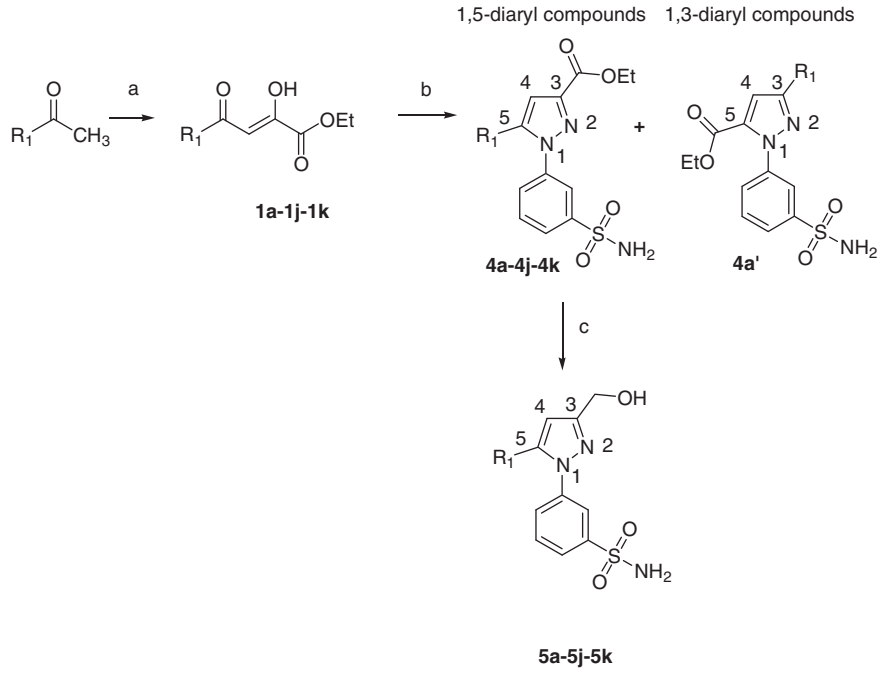
Figure 2. View of the 1-(4-aminosulfonylphenyl)-3-hydroxymethyl-5-(6-methoxynaphtalen-2-yl)-1*H*-pyrazole (**3k**), (a) inside the hCA IX active site, (b) inside the hCA II active site. The zinc ion is represented by a sphere. Pictures made using Pymol.



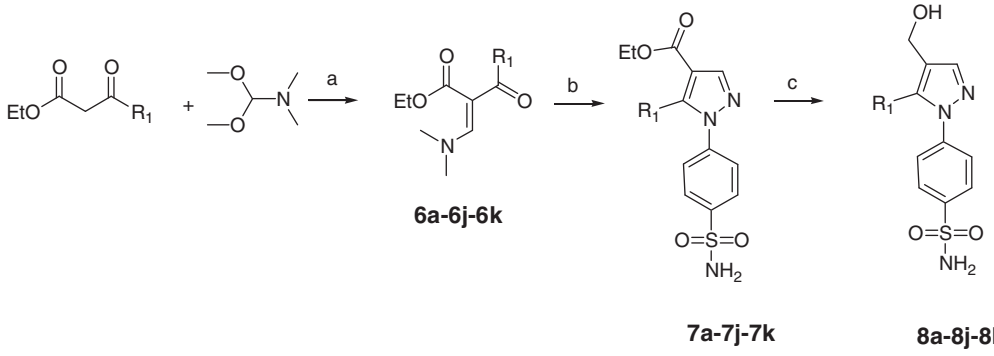
Scheme 1. [a]For R_1 see Table 1. [b]Reagents and conditions: (a) (i) EtONa, EtOH, 0 °C, 30 min; (ii) diethyl oxalate or malonate, reflux, 2 h; (b) 4-aminosulfonylphenylhydrazine hydrochloride, EtOH, reflux, 2 h; (c) $LiAlH_4$, anhydrous THF, 20 °C, 2 h.

meric 1,3-diarylpyrazole **3j'** does not affect the inhibition potential for hCA II (K_i = 9.1 nM) and hCA IX (K_i = 21 nM) but no selectivity is observed for hCA IX versus hCA II. Compounds with alcohol group in position 4 of pyrazole **(8a–8j–8k)** show good affinity but a decrease in selectivity for hCA IX versus hCA II (hCA II/hCA IX = 0.65). Compounds differing from sulfonamide position in the phenyl (**5a–5j–5k**) have an hCA IX activity of same magnitude order with K_i = 17–31 nM, but they are more selective towards hCA IX versus hCA II, with K_i hCA II/hCA IX = 0.6 (**3j**) against K_i hCA II/hCA IX = 26 for **5j**.

In our study, the influence of the substitution pattern of pyrazole scaffold was examined, as well as the contribution of the sulfonamide position on the 1-phenyl. The most promising compounds show a good affinity for hCA IX ($K_i \approx$ 15–30 nM). The position of primary alcohol group is a crucial factor. In fact, the position 3 of the alcohol appears to be more favorable for the forma-



Scheme 2. [a]For R_1 see Table 1. [b]Reagents and conditions: (a) (i) EtONa, EtOH, 0 °C, 30 min; (ii) diethyl oxalate or malonate, reflux, 2 h; (b) 3-aminosulfonylphenylhydrazine hydrochloride, EtOH, reflux, 2 h (c) $LiAlH_4$, anhydrous THF, 20 °C, 2 h (for R_1 see Table 1).



Scheme 3. Reagents and conditions: (a) reflux, 1 h; (b) 4-aminosulfonylphenylhydrazine hydrochloride, EtOH, reflux, 2 h; (c) $LiAlH_4$, anhydrous THF, 20 °C, 2 h (for R_1 see Table 1).

tion of H-bonded with the Trp 5 NH. The aryl group in position 5 of pyrazole can be straight but not too bulky (**3m**) and electronic effects of its substituents having no influence. The selectivity hCA IX versus hCA II is one of the main goals of this study. Finally, as observed in the Tables 1 and 2, all the hCA IX affinities are in the same order range from 20 to 70 nM except for two compounds (**3i** and **3a**′) whereas the variation of hCA II affinities are calculated from 10 to 780 nM. With **3a** chosen as reference compound, a greater hCA II affinity is observed for substituted aryl groups. Interestingly, the selectivity hCA IX versus hCA II is restored with substituted naphtyl groups of compounds **3k** and **3l**. Same conclusions should be made for compounds with sulfonamide group in *meta* position of in the 1-phenyl of pyrazole. In this series, the compound **5j** exhibit the greatest selectivity and a K_i value of 19 nM. A molecular modeling of **5j** inserted in the active site of hCA IX is described in Figure 3.

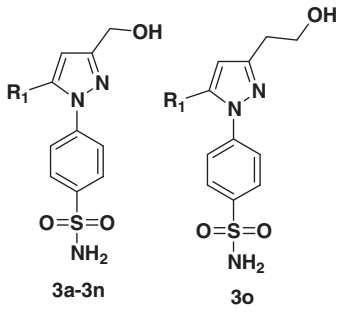
2.3. In vitro evaluation in normoxic and hypoxic conditions

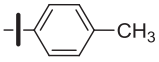
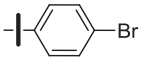
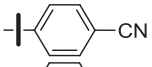
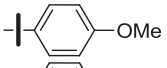
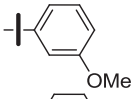
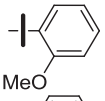
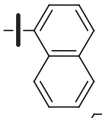
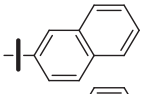
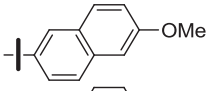
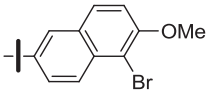
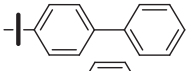
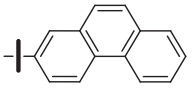
The most selective compounds described here as the most potent inhibitors of CA IX, that is, **3d**, **3k** and **5j**, have been selected for a preliminary in vitro study based on the proliferation inhibition of a breast cancer cell line, the MDA-MB-231 cells in normoxic or hypoxic conditions.

Anthracyclines, a group of chemotherapeutic agents, known to target the topoisomerase IIα enzyme, are the most common compounds used worldwide to treat breast cancer. Doxorubicin is the reference compound used in this study because this weak base is known to be more ionized in acidic extracellular environment and a reduced efficacy of the drug is related to the cytosolic membrane uptake decrease.[15] The IC_{50} of doxorubicin has been calculated from MDA-MB-231 cell proliferation in normoxic and in hypoxic conditions as reported in the Table 3. A clear reduced efficiency of doxorubicin is observed with a threefold increase of the IC_{50} value in normoxic versus in hypoxic conditions (0.12–0.37 μM).

The selected CA IX inhibitors have been first studied alone in both culture conditions and all of them revealed weak cytotoxic activities in both environments (IC_{50} >50 μM). As reported in the Table 3, the cytotoxic activity of doxorubicin was evaluated in the presence of 1 μM of CA IX inhibitor, dose for which the compounds present absolutely no cytotoxicity. In the normoxic condi-

Table 1
Inhibitory potency (K_i, nM), isozyme selectivity ratio data for the pyrazolic derivatives towards isozymes hCA I, hCA II and hCA IX (**3a–3o**)



Compounds	R1	K_i[a] (nM)			Ratio[b]	
		hCA I	hCA II	hCA IX	hCA I/hCA IX	hCA II/ hCA IX
3a		4007	420	27	148	15
3o		351	381	23	15	16.6
3b		87	43	13	6.7	3.3
3c		1509	176	22	69	8.0
3d		19,081	117	15	1272	7.8
3e		2644	122	27	98	4.5
3f		1789	136	20	89.5	6.8
3g		1710	237	26	66	9.1
3h		1168	153	32	36.5	4.8
3i		5932	46	254	23	0.2
3j		3204	9.6	17	188.5	0.6
3k		2786	264	19	147	13.9
3l		2046	388	30	68	12.9
3m		77	8.9	27	2.9	0.3
3n		>10,000	>10,000	1857	>5	>5

[a] Errors in the range 3–5% of reported values from three different assays. (AZA was chosen as the intern control: K_i for hCAI, II and IX are respectively 250, 12, and 25 nM).
[b] The K_i ratios are indicative of isozyme selectivity: a weak selective inhibitor is characterized by a low value ratio.

tion, the IC_{50} values are not significatively differents in the presence or not of the CA IX inhibitor. However in hypoxic conditions, for both compounds, **3d** and **5j**, a significant 20% reduction of the IC_{50} value is observed in comparison to that obtained for the doxorubicin alone. For compound **3k**, no variation of the doxorubicin efficiency is observed.

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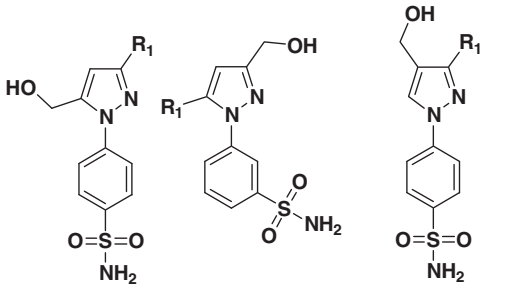
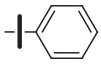
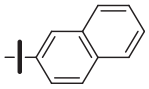
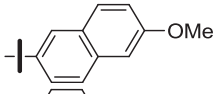
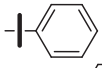
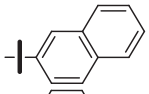
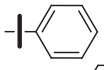
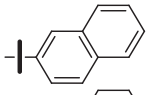
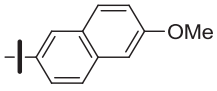
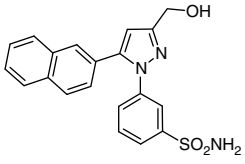
Table 2
Inhibitory potency (K_i, nM), isozyme selectivity ratio data for the pyrazolic derivatives towards isozymes hCA I, hCA II and hCA IX (**3a′, 3j′–5a, 5j, 5k, 8a, 8b, 8c**)



Compounds	R1	K_i[a] (nM)			Ratio[b]	
		hCA I	hCA II	hCA IX	hCA I/ hCA IX	hCA II/hCA IX
8a		134	38	66	2	0.6
8j		2740	36	49	56	0.7
8k		2105	31	48	68	0.65
3a′		1749	142	157	11	0.9
3j′		217	9.1	21	10	0.4
5a		7181	181	31	231	6
5j		1197	490	19	63	26
5k		2120	782	29	73	27

[a] Errors in the range 3–5% of reported values from three different assays. (AZA was chosen as the intern control: K_i for hCAI, II and IX are respectively 250, 12, and 25 nM).
[b] The K_i ratios are indicative of isozyme selectivity: a weak selective inhibitor is characterized by a low value ratio.



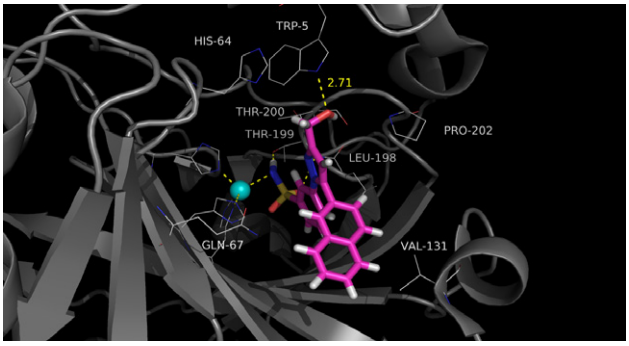


Figure 3. View of the 1-(3-aminosulfonylphenyl)-3-hydroxymethyl-5-(6-methoxynaphtyl)-1*H*-pyrazole (**5j**) inside the hCA IX active site. The zinc ion is represented by a sphere. Pictures made using Pymol.

3. Conclusions

In this investigation, we designed a series of novel 4-(5-aryl-2-hydroxymethyl-pyrazole-1-yl)-benzenesulfonamides with the aim to obtain selective hCA IX inhibitors. The R1-aryl which interacts with the hydrophobic pocket was a phenyl, a 2-naphthyl or a 6-methoxynaphthyl, while a hydroxymethyl substituted the pyrazole at the position 3. The derivatives **3a**, **3d**, **3k** were the most potent hCA IX inhibitors with a K_i value of 27, 17 and 19 nM, respectively (Table 1) and a good selectivity hCA IX versus hCA II.

Table 3
In vitro cell proliferation of breast cancer MDA-MB-231 cells[a]

Doxorubicin IC_{50} (μM), in MDA-MB-231 cells in normoxic conditions	
IC_{50} Doxorubicin without CA IX inhibitor	0.12 ± 0.02
IC_{50} Doxorubicin with **3d** (1 μM)	0.13 ± 0.01
IC_{50} Doxorubicin with **3k** (1 μM)	0.10 ± 0.03
IC_{50} Doxorubicin with **5j** (1 μM)	0.11 ± 0.02
Doxorubicin IC_{50} (μM), in MDA-MB-231 cells in hypoxic conditions	
IC_{50} Doxorubicin without CA IX inhibitor	0.37 ± 0.02
IC_{50} Doxorubicin with **3d** (1 μM)	0.30 ± 0.01
IC_{50} Doxorubicin with **3k** (1 μM)	0.38 ± 0.03
IC_{50} Doxorubicin with **5j** (1 μM)	0.29 ± 0.02

[a] Results are mean values ± SD of two independent experiment for $n = 6$.

The modulation of the position of sulfonamide group in *meta* of the 1-phenylpyrazole preserved a good affinity for hCA IX and for the derivatives **5j** and **5k** increase twofold selectivity hCA IX versus hCA II. Our compounds present theoretical log P (*Molinspiration Cheminformatics 2012*) calculated between 1.8 and 3.9 which suggest that all the compounds cross over the cellular membrane. This property reinforces the interest to possess selective inhibition of the hCA IX membrane isoform versus the hCA II cytosolic isoform. An in vitro antiproliferative screening has been performed on the breast cancer MDA-MB-231 cell with compounds **3d**, **3k** and **5j**. Results have shown that 1 μM **3d** or **5j** restore 20% level the cytotoxic efficiency of doxorubicin in a hypoxic environment. The absence of effect of these compounds in normoxia is consistent with the fact that CA IX is overexpressed only in hypoxia and proved their specificity of action in vitro.

4. Experimental section

4.1. Chemistry

All commercial reagents and solvents were used without further purification. Tetrahydrofuran (THF) was distilled from sodium/benzophenone prior to use. All reactions were monitored by analytical thin-layer chromatography (TLC) on 0.2 mm, Polygram SIL G/UV254 plates (Macherey-Nagel); compounds were visualized by UV (254 and 366 nm) and/or with iodine. Flash chromatography (FC) was performed with silica gel Kieselgel Si 60 0.015–0.040 mm (Macherey-Nagel). Melting points (mp) were determined with a Büchi 535 capillary melting point apparatus and remain uncorrected. The structures of each compound were supported by IR (neat, FT-Brücker Vector 22 instrument) and by ^{1}H NMR at 300 MHz on a Brucker DPX-300 spectrometer. Chemical shifts (δ) are reported in ppm downfield from tetramethylsilane (TMS), J values are in hertz, and the splitting patterns are designed as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br s, broad singlet. The purity of compounds was checked using LC–MS system Thermo Electron Surveyor MSQ. The mass spectra were operated in the atmospheric pressure chemical ionization mode ($APCI^+$). Elemental analyses (C, H, N) were performed on a ThermoFinnigan Flash EA 112 elemental analyser.

4.1.1. 2-Hydroxy-4-oxo-4-phenyl-2-but-2-enoic acid ethyl ester[25] (1a)

Ethanol (200 mL) was converted to sodium ethoxide by portion wise addition of sodium (6.26 g; 0.27 mol) before a solution of diethyl oxalate (37 mL; 0.27 mol) and acetophenone (16.36 g; 0.14 mol) in ethanol (100 mL) was added dropwise at 50 °C. The mixture was heated at reflux for 3 h. After cooling, the precipitate formed was filtered and then washed with absolute ethanol. The residue was taken up in water (20 mL) and acidified with 1 N HCl solution (5 mL). The precipitate formed was filtered and washed with petroleum ether to give **1a** as yellow crystal (yield: 62%), mp 72–74 °C. ^{1}H NMR ($CDCl_3$) δ: 15.30 (br s, 1H), 7.96 (m, 2H), 7.58 (m, 1H), 7.47 (m, 2H), 7.06 (s, 1H), 4.35 (q, J = 7.2 Hz, 2H), 1.28 (t, J = 7.2 Hz, 3H). IR (cm^{-1}: neat): 1735, 1600. LC–MS ($APCI^+$) m/z: 221 $(M+H)^+$.

4.1.2. 2-Hydroxy-4-oxo-4-pyridin-3-yl-but-2-enoic acid ethyl ester sodium salt (1b)

Ethanol (30 mL) was converted to sodium ethoxide by portion wise addition of sodium (0.92 g, 40 mmol). Then a solution of diethyl oxalate (5.45 mL; 40 mmol) and 3-acetopyridine (2 mL; 20 mmol) in ethanol was added dropwise at 50 °C. The mixture was heated at reflux for 3 h. After cooling, the precipitate formed is filtered and then washed with petroleum ether to give **1b** as yellow crystal (yield: 78%), mp > 250 °C. ^{1}H NMR (DMSO-d_6) δ: 8.86 (m, 1H), 8.57 (m, 1H), 8.50 (m, 1H), 8.04 (m, 1H), 6.21 (s, 1/2H), 5.27 (s, 1/2H), 4.07 (m, 2H), 1.22 (m, 3H). IR (cm^{-1}: neat): 1706, 1631. LC–MS ($APCI^+$) m/z: 222 $(M+H)^+$.

4.1.3. 2-Hydroxy-4-oxo-4-*p*-tolyl-but-2-enoic acid ethyl ester (1c)

The title compound was prepared from *p*-methylacetophenone and diethyl oxalate using the same procedure as for **1a** to give **1c** as orange oil (yield: 98%) ^{1}H NMR (DMSO-d_6) δ: 15.20 (br s, 1H), 7.89 (d, J = 8,2 Hz, 2H), 7.29 (d, J = 9.0 Hz, 2H), 6.99 (s, 1H), 4.39 (q, J = 7.1 Hz, 2H), 2.43 (s, 3H), 1.40 (t, J = 7.1 Hz, 3H). IR (cm^{-1}: neat): 1732, 1607. LC–MS ($APCI^+$) m/z: 235 $(M+H)^+$.

4.1.4. 2-Hydroxy-4-oxo-4-*p*-bromophenyl-but-2-enoic acid ethyl ester (1d)

The title compound was prepared from *p*-bromoacetophenone and diethyl oxalate using the same procedure as for **1a** to give **1d** as orange oil (yield: 97%). ^{1}H NMR (DMSO-d_6) δ: 15.40 (br s, 1H), 7.89 (m, 3H), 7.55 (m, 2H), 4.42 (q, J = 7.1 Hz, 2H), 1.47 (t, J = 7.2 Hz, 3H). IR (cm^{-1}: neat): 1732, 1607. LC–MS ($APCI^+$) m/z: 299 [$(M+H)^+$ for ^{79}Br] and 301 [(M+H) for ^{81}Br].

4.1.5. 2-Hydroxy-4-oxo-4-*p*-cyanophenyl-but-2-enoic acid ethyl ester sodium salt (1e)

The title compound was prepared from 4-cyanoacetophenone and diethyl oxalate using the same procedure as for **1b** to give **1e** as beige crystal (yield: 98%), mp 203–205 °C ^{1}H NMR (DMSO-d_6) δ: 7.83 (m, 4H), 6.46 (s, 2/3H), 5.30 (s, 1/3H), 4.07 (q, J = 7.0 Hz, 2H), 1.23 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 2450, 1714, 1626. LC–MS ($APCI^+$) m/z: 246 $(M+H)^+$.

4.1.6. 2-Hydroxy-4-oxo-4-(4-methoxyphenyl)-but-2-enoic acid ethyl ester (1f)

The title compound was prepared from *p*-methoxyacetophenone and diethyl oxalate using the same procedure as for **1a** to give **1f** as orange crystal (yield: 63%), mp 57–59 °C ^{1}H NMR (DMSO-d_6) δ: 15.2 (br s, 1H), 8.07 (d, J = 9.0 Hz, 2H), 7.10 (d, J = 9.0 Hz, 2H), 7.08 (s, 1H), 4.29 (q, J = 7.0 Hz, 2H), 3.85 (s, 3H), 1.29 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 1720. LC–MS ($APCI^+$) m/z: 251 $(M+H)^+$.

4.1.7. 2-Hydroxy-4-oxo-4-(3-methoxyphenyl)-but-2-enoic acid ethyl ester sodium salt (1g)

The title compound was prepared from *m*-methoxyacetophenone and diethyl oxalate using the same procedure as for **1b** to give **1g** as yellow crystal (yield: 73%), mp 60–62 °C ^{1}H NMR (DMSO-d_6) δ: 7.26 (m, 3H), 6.90 (m, 1H), 6.25 (s, 2/3H), 5.26 (s, 1/3H), 4.01 (m, 2H), 3.76 (s, 3H), 1.26 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 1690, 1626. LC–MS ($APCI^+$) m/z: 251 $(M+H)^+$.

4.1.8. 2-Hydroxy-4-oxo-4-(2-methoxyphenyl)-but-2-enoic acid ethyl ester (1h)

The title compound was prepared from *o*-methoxyacetophenone and diethyl oxalate using the same procedure as for **1a** to give **1h** as yellow crystal (yield: 79%), mp 79–81 °C ^{1}H NMR (DMSO-d_6) δ: 15.30 (br s, 1H), 7.91 (dd, J = 7.9 Hz J = 1.7 Hz, 1H), 7.53 (dt, J = 7.9 Hz J = 1.7 Hz, 1H), 7.31 (s, 1H), 7.04 (m, 2H), 4.40 (q, J = 7.1 Hz, 2H), 3.96 (s, 3H), 1.41 (t, J = 7.1 Hz, 3H). IR (cm^{-1}: neat): 1741. LC–MS (APCI$^+$) m/z: 251 (M+H)$^+$.

4.1.9. 2-Hydroxy-4-oxo-4-(1-naphthyl)-but-2-enoic acid ethyl ester (1i)

The title compound was prepared from 1-acetylnaphtalene and diethyl oxalate using the same procedure as for **1a** to give **1i** as yellow crystal (yield: 85%), mp 60–62 °C ^{1}H NMR (DMSO-d_6) δ: 15.22 (br s, 1H), 8.61 (d, J = 8.4 Hz, 1H), 8.06 (d, J = 8.0 Hz, 1H), 7.97 (m, 2H), 7.68 (m, 3H), 7.3 (s, 1H), 4.42 (q, J = 7.3 Hz, 2H), 1.43 (t, J = 7.3 Hz, 3H). IR (cm^{-1}: neat): 1728, 1610. LC–MS (APCI$^+$) m/z: 271 (M+H)$^+$.

4.1.10. 2-Hydroxy-4-oxo-4-(2-naphthyl)-but-2-enoic acid ethyl ester (1j)

The title compound was prepared from 2-acetylnaphtalene and diethyl oxalate using the same procedure as for **1a** to give **1j** as yellow crystal (yield: 74%), mp 54–56 °C ^{1}H NMR (DMSO-d_6) δ: 15.21 (br s, 1H), 8.61 (d, J = 8.4 Hz, 1H), 8.06 (d, J = 8.0 Hz, 1H), 7.97 (m, 2H), 7.68 (m, 3H), 7.3 (s, 1H), 4.42 (q, J = 7.3 Hz, 2H), 1.43 (t, J = 7.3 Hz, 3H). IR (cm^{-1}: neat): 1728, 1610. LC–MS (APCI$^+$) m/z: 271 (M+H)$^+$.

4.1.11. 2-Hydroxy-4-oxo-4-(6-methoxy-2-naphthyl)-but-2-enoic acid ethyl ester (1k)

The title compound was prepared from 2-acetyl-6-methoxynaphtalene and diethyl oxalate using the same procedure as for to **1a** to give **1k** as yellow crystal (yield: 48%), mp 51–53 °C ^{1}H NMR (DMSO-d_6) δ: 15.02 (br s, 1H), 8.86 (m, 1H), 7.91 (m, 2H), 7.23 (m, 2H), 7.88 (m, 2H), 4.49 (q, J = 7.2 Hz, 2H), 3.98 (s, 3H), 1.45 (t, J = 7.2 Hz, 3H). IR (cm^{-1}: neat): 1728, 1610. LC–MS (APCI$^+$) m/z: 301 (M+H)$^+$.

4.1.12. 2-Hydroxy-4-oxo-4-(5-bromo-6-methoxy-2-naphthyl)-but-2-enoic acid ethyl ester sodium salt (1l)

The title compound was prepared from 2-acetyl-1-bromo-6-methoxynaphtalene and diethyl oxalate using the same procedure as for **1b** to give **1l** as yellow crystal (yield: 97%), mp >250 °C ^{1}H NMR (DMSO-d_6) δ: 8.32 (m, 1H), 8.05 (m, 3H), 7.52 (m, 1H), 6.42 (s, 2/3H), 5.46 (s, 1/3H), 4.09 (m, 2H), 3.99 (s, 3H), 1.24 (m, 3H). IR (cm^{-1}: neat): 1722, 1615. LC–MS (APCI$^+$) m/z: 379 [(M+H)$^+$ for ^{79}Br] and 381 [(M+H) for ^{81}Br].

4.1.13. 2-Hydroxy-4-oxo-4-(4-biphenylyl)-but-2-enoic acid ethyl ester (1m)

The title compound was prepared from 4-acetyl-biphenyl and diethyl oxalate using the same procedure as for **1a** to give **1m** as yellow crystal (yield: 78%), mp 109–111 °C ^{1}H NMR (DMSO-d_6) δ: 15.40 (br s, 1H), 8.10 (d, J = 8.8 Hz, 2H), 7.76 (d, J = 8.8 Hz, 2H), 7.64 (m, 2H), 7.55 (m, 3H), 7.15 (s, 1H), 4.46 (q, J = 7.2 Hz, 2H), 1.47 (t, J = 7.2 Hz, 3H). IR (cm^{-1}: neat): 1734, 1604. LC–MS (APCI$^+$) m/z: 297 (M+H)$^+$.

4.1.14. 2-Hydroxy-4-oxo-4-(2-phenantryl)-but-2-enoic acid ethyl ester (1n)

The title compound was prepared from 2-acetyl-phenanthrene and diethyl oxalate using the same procedure as for **1a** to give **1n** as yellow crystal (yield: 82%). ^{1}H NMR (DMSO-d_6) δ: 15.40 (br s, 1H), 8.77 (d, J = 8.8 Hz, 1H), 8.70 (m, 1H), 8.55 (d, J = 1.8 Hz, 1H), 8.22 (dd, J = 8.8 Hz J = 1.8 Hz, 1H), 7.93 (m, 1H), 7.84 (m, 2H), 7.77 (m, 2H), 7.27 (s, 1H), 4.46 (q, J = 7.2 Hz, 2H), 1.47 (t, J = 7.2 Hz, 3H). IR (cm^{-1}: neat): 1742, 1633. LC–MS (APCI$^+$) m/z: 321 (M+H)$^+$.

4.1.15. 3-Hydroxy-5-oxo-5-phenyl-pent-3-enoic acid ethyl ester[26] (1o)

A solution of lithium diisopropylamidure was prepared by addition of diisopropylamine (5 g, 0.05 mol) in *n*-butyllithium (31 mL, 0.05 mol) at 0 °C. After addition of tetramethylethylenediamine (2 mL) in ether (50 mL), a mixture of ethyl acetoacetate (2.6 g, 0.02 mol) and ethyl benzoate (3.75 g, 0.025 mol) diluted in ether was added under an inert atmosphere. After 17 h of reaction, dilute acetic acid (6 g, 0.1 mol) in ether (75 mL) was added to the reaction medium before water (50 mL) was added at 0 °C. The ether phase was dried over $MgSO_4$. After evaporation of the solvent, the residue was purified by chromatography of silica gel (heptane–ethyl acetate 70:10) to give the yellow oil (yield: 86%). ^{1}H NMR ($CDCl_3$) δ: 12.00 (br s, 1H), 7.85 (m, 2H), 7.45 (m, 3H), 6.30 (m, 1H), 4.40 (m, 2H), 3.55 (m, 2H), 1.2 (m, 3H). IR (cm^{-1}: neat): 1739, 1629. LC–MS (APCI$^+$) m/z: 235 (M+H)$^+$.

4.1.16. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-phenyl-1*H*-pyrazole (2a) and 1-(4-aminosulfonylphenyl)-5-ethoxycarbonyl-3-phenyl-1*H*-pyrazole (2a′)

The diketoester **1a** (4 mmol, 0.9 g) was dissolved in ethanol (25 mL) and a solution of 4-aminosulfonylphenylhydrazine hydrochloride (4 mmol, 0.9 g) in ethanol was introduced. The mixture was refluxed for 4 h. After evaporation under reduced pressure, the two isomers were separated by chromatography on silica gel (dichloromethane–ethyl acetate 70:30) to give titles compounds **2a** as yellow crystals (yield: 68%) and **2a′** as yellow crystals (yield: 9%).

Compound ***2a***: mp 195–197 °C, ^{1}H NMR (DMSO-d_6) δ: 7.87 (dd, J = 8.8 Hz, J = 2.0 Hz, 2H), 7.45 (dd, J = 8.8 Hz, J = 2.0 Hz, 2H), 7.33 (m, 3H), 7.20 (m, 2H), 7.04 (s, 1H), 5.26 (s, 2H), 4.45 (q, J = 7.1 Hz, 2H), 1.43 (t, J = 7.1 H, 3H). IR (cm^{-1}: neat): 1723, 1310, 1150. LC–MS (APCI$^+$) m/z: 372 (M+H)$^+$.

Compound ***2a′***: mp 219–221 °C, ^{1}H NMR (DMSO-d_6) δ: 7.90 (m, 2H), 7.65 (m, 4H), 7.50 (m, 3H), 7.30 (m, 2H), 7.10 (s, 1H), 4.45 (q, J = 7.0 Hz, 2H), 1.30 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 1722, 1335, 1164. LC–MS (APCI$^+$) m/z: 372 (M+H)$^+$.

4.1.17. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-(pyridin-3-yl)-1*H*-pyrazole (2b)

The diketoester **1b** (4 mmol, 0.9 g) was dissolved in ethanol (25 mL), then 4-aminosulfonylphenylhydrazine hydrochloride (4 mmol, 0.9 g) was introduced. The mixture was refluxed for 4 h. After cooling to 20 °C the precipitate was filtered under pression and washed with petroleum ether to give the title compound **2b** as yellow crystals (yield: 89%), mp 137–139 °C. ^{1}H NMR (DMSO-d_6) δ: 8.67 (s, 1H), 8.51 (d, J = 4.7 Hz, 1H), 7.93 (s, 1H), 7.80 (d, J = 8.4 Hz, 1H), 7.58 (d, J = 9.0 Hz, 2H), 7.38 (dd, J = 7.8 Hz J = 4.7 Hz, 1H), 7.18 (d, J = 8.7 Hz, 2H), 7.10 (s, 2H), 4.31 (q, J = 7.0 Hz, 2H), 1.29 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 1702, 1310, 1151. LC–MS (APCI$^+$) m/z: 373 (M+H)$^+$.

4.1.18. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-*p*-tolyl-1*H*-pyrazole (2c)

The title compound was prepared from diketoester **1c** and 4-aminosulfonylphenyl hydrazine hydrochloride using the same procedure as for **2b** to give **2c** as white crystal (yield: 78%), mp 224–226 °C. ^{1}H NMR (DMSO-d_6) δ: 7.86 (d, J = 8.2 Hz, 2H), 7.51 (d, J = 8.2 Hz, 2H), 7.50 (m, 3H), 7.18 (m, 4H), 4.34 (q, J = 7.3 Hz, 2H), 2.31 (s, 3H), 1.29 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 1714, 1330, 1159. LC–MS (APCI$^+$) m/z: 386 (M+H)$^+$.

4.1.19. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-(4-bromophenyl)-1*H*-pyrazole (2d)

The title compound was prepared from diketoester **1d** and 4-aminosulfonylphenyl hydrazine hydrochloride using the same procedure as for **2b** to give **2d** as white crystal (yield: 60%), mp 199–201 °C. ^{1}H NMR (DMSO-d_6) δ: 7.88 (d, J = 6.8 Hz, 2H), 7.63 (d, J = 8.7 Hz, 2H), 7.52 (m, 4H), 7.26 (d, J = 8.7 Hz, 2H), 7.22 (s, 1H), 4.35 (q, J = 7.1 Hz, 2H), 1.32 (t, J = 7.1 Hz, 3H). IR (cm^{-1}: neat): 1725, 1327, 1158. LC–MS (APCI$^+$) m/z: 450 [(M+H)$^+$ for ^{79}Br] and 452 [(M+H)$^+$ ^{81}Br].

4.1.20. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-*p*-cyanophenyl-1*H*-pyrazole (2e)

The title compound was prepared from diketoester **1e** and 4-aminosulfonylphenylhydrazine hydrochloride using the same procedure as for **2b** to give **2e** as white crystal (yield: 77%), mp 226–228 °C. ^{1}H NMR (DMSO-d_6) δ: 7.88 (m, 4H), 7.50 (m, 6H), 7.22 (s, 1H), 4.35 (q, J = 7.1 Hz, 2H), 1.32 (t, J = 7.1 Hz, 3H). IR (cm^{-1}: neat): 1712, 1335, 1155. LC–MS (APCI$^+$) m/z: 397 (M+H)$^+$.

4.1.21. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-(4-methoxyphenyl)-1*H*-pyrazole (2f)

The title compound was prepared from diketoester **1f** and 4-aminosulfonylphenylhydrazine hydrochloride using the same procedure as for **2b** to give **2f** as white crystal (yield: 74%), mp 205–207 °C. ^{1}H NMR (DMSO-d_6) δ: 7.86 (d, J = 8.4 Hz, 2H), 7.52 (d, J = 8.4 Hz, 2H), 7.51 (s, 2H), 7.22 (d, J = 8.7 Hz, 2H), 7.07 (s, 1H), 6.95 (d, J = 8.7 Hz, 2H), 4.33 (q, J = 7.0 Hz, 2H), 3.76 (s, 3H), 1.31 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 1711, 1339, 1161. LC–MS (APCI$^+$) m/z: 402 (M+H)$^+$.

4.1.22. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-(3-methoxyphenyl)-1*H*-pyrazole (2g)

The title compound was prepared from diketoester **1g** and 4-aminosulfonylphenylhydrazine hydrochloride using the same procedure as for **2b** to give **2g** as beige crystal (yield: 74%), mp 185–187 °C. ^{1}H NMR (DMSO-d_6) δ: 7.87 (d, J = 8.4 Hz, 2H), 7.54 (s, 2H), 7.53 (d, J = 8.4 Hz, 2H), 7.28 (m, 1H), 6.98 (s, 1H), 6.94 (d, J = 6.4 Hz, 1H), 6.88 (s, 1H), 6.78 (d, J = 7.8 Hz, 1H), 4.35 (q, J = 7.1 Hz, 2H), 3.68 (s, 3H), 1.31 (t, J = 7.1 Hz, 3H). IR (cm^{-1}: neat): 1712, 1356, 1169. LC–MS (APCI$^+$) m/z: 402 (M+H)$^+$.

4.1.23. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-(2-methoxyphenyl)-1*H*-pyrazole (2h)

The title compound was prepared from diketoester **1h** and 4-aminosulfonylphenylhydrazine hydrochloride using the same procedure as for **2b** to give **2h** as yellow crystal (yield: 65%), mp 157–159 °C. ^{1}H NMR (DMSO-d_6) δ: 7.79 (d, J = 8.4 Hz, 2H), 7.40 (m, 6H), 7.02 (m, 3H), 4.33 (q, J = 7.0 Hz, 2H), 3.32 (s, 3H), 1.31 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 1699, 1349. LC–MS (APCI$^+$) m/z: 402 (M+H)$^+$.

4.1.24. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-(1-naphthyl)-1*H*-pyrazole (2i)

The title compound was prepared from diketoester **1i** and 4-aminosulfonylphenylhydrazine hydrochloride using the same procedure as for **2b** to give **2i** as yellow crystal (yield: 45%), mp 103–105 °C. ^{1}H NMR (DMSO-d_6) δ: 7.97 (m, 2H), 7.64 (d, J = 8.5 Hz, 2H), 7.38 (m, 9H), 7.19 (s, 1H), 4.38 (q, J = 7.0 Hz, 2H), 1.34 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 1719, 1340, 1164. LC–MS (APCI$^+$) m/z: 422 (M+H)$^+$.

4.1.25. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-(2-naphthyl)-1*H*-pyrazole (2j) and 1-(4-aminosulfonylphenyl)-5-ethoxycarbonyl-3-(2-naphthyl)-1*H*-pyrazole (2j′)

Title compounds were prepared from diketoester **1j** and 4-aminosulfonylphenylhydrazine hydrochloride using the same procedure as for **2a** to give **2j** as yellow crystal (yield: 77%) and **2j′** as white crystal (yield: 8%).

*Compound **2j**:* mp 204–206 °C ^{1}H NMR (DMSO-d_6) δ: 8.04 (s, 1H), 7.83 (m, 5H), 7.50 (m, 6H), 7.29 (m, 2H), 4.38 (q, J = 7.0 Hz, 2H), 1.35 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 1714, 1338, 1169. LC–MS (APCI$^+$) m/z: 422 (M+H)$^+$.

*Compound **2j′**:* mp 198–201 °C ^{1}H NMR (DMSO-d_6) δ: 8.00 (s, 1H), 7.58 (m, 5H), 7.53 (m, 5H), 7.30 (m, 3H), 4.48 (q, J = 7.0 Hz, 2H), 1.54 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 1722, 1335, 1166. LC–MS (APCI$^+$) m/z: 422 (M+H)$^+$.

4.1.26. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-(6-methoxy-2-naphthyl)-1*H*-pyrazole (2k)

The title compound was prepared from diketoester (**1k**) and 4-aminosulfonylphenylhydrazine hydrochloride using the same procedure as for **2b** to give **2k** as white crystal (yield: 79%), mp 99–101 °C. ^{1}H NMR (DMSO-d_6) δ: 7.71 (m, 2H), 7.66 (m, 3H), 7.54 (m, 2H), 7.19 (m, 2H), 7.12 (m, 4H), 4.47 (q, J = 7.0 Hz, 2H), 3.94 (s, 3H), 1.42 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 1726, 1342, 1164. LC–MS (APCI$^+$) m/z: 452 (M+H)$^+$.

4.1.27. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-(5-bromo-6-methoxy-2-naphthyl)-1*H*-pyrazole (2l)

The title compound was prepared from diketoester **1l** and 4-aminosulfonylphenylhydrazine hydrochloride using the same procedure as for **2b** to give **2l** as white crystal (yield: 92%), mp 245–247 °C. ^{1}H NMR (DMSO-d_6) δ: 8.02 (m, 3H), 7.86 (d, J = 8.6 Hz, 2H), 7.58 (m, 3H), 7.51 (s, 2H), 7.38 (d, J = 8.6 Hz, 1H), 7.28 (s, 1H), 4.37 (q, J = 7.1 Hz, 2H), 3.34 (s, 3H), 1.34 (t, J = 7.1 Hz, 3H). IR (cm^{-1}: neat): 1720, 1342, 1165. LC–MS (APCI$^+$) m/z: 530 [(M+H)$^+$ ^{79}Br] and 532 [(M+H)$^+$ ^{81}Br].

4.1.28. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-(4-biphenylyl)-1*H*-pyrazole (2m)

The title compound was prepared from diketoester **1m** and 4-aminosulfonyphenylhydrazine hydrochloride using the same procedure as for **2b** to give **2m** as white crystal (yield: 60%), mp 214–216 °C. ^{1}H NMR (DMSO-d_6) δ: 7.92 (d, J = 8.8 Hz, 2H), 7.78 (m, 4H), 7.57 (d, J = 8.8 Hz, 2H), 7.53 (s, 2H), 7.44 (m, 2H), 7.36 (m, 3H), 7.22 (s, 1H), 4.38 (q, J = 7.0 Hz, 2H), 1.33 (t, J = 7.0 Hz, 3H). IR (cm^{-1}: neat): 1717, 1327, 1160. LC–MS (APCI$^+$) m/z: 448 (M+H)$^+$.

4.1.29. 1-(4-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-(3-phenantryl)-1*H*-pyrazole (2n)

The title compound was prepared from diketoester **1n** and 4-aminosulfonylphenylhydrazine hydrochloride using the same procedure as for **2b** to give **2n** as white crystal (yield: 36%), mp >250 °C. ^{1}H NMR (DMSO-d_6) δ: 8.12 (d, J = 1.8 Hz, 1H), 8.04 (m. 1H), 7.95 (m, 4H), 7.75 (m, 2H), 7.57 (d, J = 8.8 Hz, 2H), 7.60 (d, J = 8.8 Hz, 2H), 7.52 (s, 2H), 7.44 (dd, J = 7.0 Hz, J = 1.8 Hz, 1H), 7.34 (s, 1H), 4.37 (q, J = 7.2 Hz, 2H), 1.33 (t, J = 7.2 Hz, 3H). IR (cm^{-1}: neat): 1714, 1338, 1169. LC–MS (APCI$^+$) m/z: 472 (M+H)$^+$.

4.1.30. 1-(4-Aminosulfonylphenyl)-3-ethylacetate-5-phenyl-1*H*-pyrazole (2o)

The diketoester **1o** (0.11 g, 0.49 mmol) was dissolved in ethanol (15 mL) and a solution of 4-aminosulfonylphenylhydrazine hydrochloride (0.1 g, 0.49 mmol) in ethanol was introduced. The mixture was refluxed for 3 h. After cooling, the reaction mixture was concentrated under reduced pressure and the residue was taken up into ethylacetate and washed with water (20 mL), and brine (2 × 20 mL). The organic layer was dried over $MgSO_4$ and then concentrated under reduced pressure. The residue was purified by chromatography of silica gel (dichloromethane–ethyl acetate 70:30) to give a yellow crystal (25%), mp 233–235 °C.

^{1}H NMR (DMSO-d_6) δ: 7.85 (d, J = 8.7 Hz, 2H), 7.46–7.23 (m, 9H), 6.55 (s, 1H), 4.24 (q, J = 7,3 Hz, 2H), 3.8 (s, 2H), 1.32 (t, J = 7,3 Hz, 3H). IR (cm^{-1}: neat): 1736, 1343, 1164. LC–MS (APCI$^+$) m/z: 386 (M+H)$^+$.

4.1.31. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-phenyl-1*H*-pyrazole (3a)

The ethyl ester (4.8 mmol, 1.4 g) was dissolved in tetrahydrofuran (20 mL) and then a suspension of $LiAlH_4$ (9.6 mmol, 0.37 g) in tetrahydrofuran (5 mL) was slowly added. After 3 h of reaction at 20 °C, an aqueous solution of HCl 1 N was added dropwise until a neutral pH. The reaction mixture was concentrated under reduced pressure the residue was taken into ethyl acetate and washed with water and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was recrystallized in acetonitrile. The alcohol **(3a)** give a white crystal (yield: 84%), mp 186–188 °C ^{1}H NMR (DMSO-d_6) δ: 7.79 (d, J = 8.4 Hz, 2H), 7.45 (m, 7H), 7.25 (m, 2H), 6.64 (s, 1H), 5.27 (t, J = 5.8 Hz, 1H), 4.76 (s, 2H). IR (cm^{-1}: neat): 3297, 1593. LC–MS (APCI$^+$) m/z: 330 (M+H)$^+$. Anal. Calcd for $C_{16}H_{15}N_3O_3S$: C, 58.35; H, 4.59; N, 12.76. Found: C, 58.42; H, 4.68; N, 12.86.

4.1.32. 1-(4-Aminosulfonylphenyl)-5-hydroxymethyl-3-phenyl-1*H*-pyrazole (3a′)

The title compound was prepared from ester **2a′** using the same procedure as for **3a** to give **3a′** as yellow crystal (yield: 64%), mp 200–202 °C. ^{1}H NMR (DMSO-d_6) δ: 7.8 (d, J = 8.7 Hz, 2H), 7.44 (m, 7H), 7.24 (m, 2H), 6.64 (s, 1H), 5.27 (t, J = 5.8 Hz, 1H), 4.53 (d, J = 5.8 Hz, 2H). IR (cm^{-1}: neat): 3336, 1336, 1167. LC–MS (APCI$^+$) m/z: 330 (M+H)$^{+\cdot}$ Anal. Calcd for $C_{16}H_{15}N_3O_3S$: C, 58.35; H, 4.59; N, 12.76. Found: C, 58.32; H, 4.74; N, 12.90.

4.1.33. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-pyridinyl-1*H*-pyrazole (3b)

The title compound was prepared from ester **2b** using the same procedure as for **3a** to give **3b** as yellow crystal (yield: 57%), mp 154–156 °C ^{1}H NMR (DMSO-d_6) δ: 8.55 (s, 1H), 8.46 (s, 1H), 7.82 (d, J = 8.4 Hz, 2H), 7.66 (d, J = 8.1 Hz, 1H), 7.45 (m, 5H), 6.77 (s, 1H), 5.29 (t, J = 5.8 Hz, 1H), 4.54 (d, J = 5.8 Hz, 2H). IR (cm^{-1}: neat): 1333, 1158. LC–MS (APCI$^+$) m/z: 331 (M+H)$^+$. Anal. Calcd for $C_{15}H_{14}N_4O_3S$: C, 54.54; H, 4.27; N, 16.96. Found: C, 54.45; H, 4.45; N, 16.90.

4.1.34. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-*p*-tolyl-1*H*-pyrazole (3c)

The title compound was prepared from ester **2c** using the same procedure as for **3a** to give **3c** as white crystal (yield: 73%), mp 166–168 °C. ^{1}H NMR (DMSO-d_6) δ: 7.80 (d, J = 8.7 Hz, 2H), 7.44 (m, 3H), 7.39 (d, J = 8.7 Hz, 2H), 7.20 (d, J = 8.0 Hz, 1H), 7.13 (d, J = 8.0 Hz, 2H), 6.58 (s, 1H), 5.24 (t, J = 5.8 Hz, 1H), 4.51 (d, J = 5.8 Hz, 2H) 2.31 (s, 3H). IR (cm^{-1}: neat): 1327, 1162. LC–MS (APCI$^+$) m/z: 344 (M+H)$^+$. Anal. Calcd for $C_{17}H_{17}N_3O_3S$: C, 59.46; H, 4.99; N, 12.24. Found: C, 59.69; H, 5.08; N, 12.58.

4.1.35. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-*p*-bromophenyl-1*H*-pyrazole (3d)

The title compound was prepared from ester **2d** using the same procedure as for **3a** to give **3d** as white crystal (yield: 62%), mp 203–205 °C ^{1}H NMR (DMSO-d_6) δ: 7.81 (d, J = 6.8 Hz, 2H), 7.62 (d, J = 8.7 Hz, 2H), 7.44 (m, 4H), 7.22 (d, J = 8.7 Hz, 2H), 6.67 (s, 1H), 5.27 (t, J = 6.2 Hz, 1H), 4.53 (d, J = 6.2 Hz, 2H). IR (cm^{-1}: neat): 1396, 1159. LC–MS (APCI$^+$) m/z: 408 [(M+H)$^+$ ^{79}Br] and 410 [(M+H)$^+$ ^{81}Br]. Anal. Calcd for $C_{16}H_{14}BrN_3O_3S$: C,47.07; H, 3.46; N, 10.29. Found: C, 47.40; H, 3.52; N, 10.34.

4.1.36. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-*p*-cyanophenyl-1*H*-pyrazole (3e)

The title compound was prepared from ester **2e** according using the same procedure as for **3a** to give **3e** as white crystal (yield: 10%), mp 201–203 °C. ^{1}H NMR (DMSO-d_6) δ: 7.90 (d, J = 8.5 Hz, 2H), 7.80 (d, J = 8.5 Hz, 2H), 7.45 (s, 2H), 7.40 (m, 4H), 6.80 (s, 1H), 5.30 (t, J = 5.8 Hz, 1H), 4.50 (d, J = 5.8 Hz, 2H). IR (cm^{-1}: neat): 2235, 1344, 1165. LC–MS (APCI$^+$) m/z: 355 (M+H)$^+$. Anal. Calcd for $C_{17}H_{14}N_4O_3S$: C, 57.62; H, 3.98; N, 15.81. Found: C, 57.69; H, 4.31; N, 15.98.

4.1.37. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-(4-methoxyphenyl)-1*H*-pyrazole (3f)

The title compound was prepared from ester **2f** using the same procedure as for **3a** to give **3f** as white crystal (yield: 32%), mp 114–116 °C. ^{1}H NMR (DMSO-d_6) δ: 7.80 (d, J = 8.7 Hz, 2H), 7.44 (s, 2H), 7.41 (d, J = 8.7 Hz, 2H), 7.17 (d, J = 8.7 Hz, 2H), 6.96 (d, J = 8.7 Hz, 2H), 6.55 (s, 1H), 5.22 (t, J = 5.8 Hz, 1H), 4.50 (d, J = 5.8 Hz, 2H), 3.76 (s, 3H). IR (cm^{--1}: neat): 1336, 1161. LC–MS (APCI$^+$) m/z: 360 (M+H)$^+$. Anal. Calcd for $C_{17}H_{17}N_3O_4S$: C, 56.81; H, 4.77; N, 11.69. Found: C, 56.98; H, 4.63; N, 11.78.

4.1.38. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-(3-methoxyphenyl)-1*H*-pyrazole (3g)

The title compound was prepared from ester **2g** using the same procedure as for **3a** to give **3g** as white crystal (yield: 59%), mp 177–179 °C ^{1}H NMR (DMSO-d_6) δ: 7.81 (d, J = 8.7 Hz, 2H), 7.46 (s, 2H), 7.42 (d, J = 8.7 Hz, 2H), 7.30 (t, J = 7.9 Hz, 1H), 6.97 (d, J = 8.1 Hz, 1H), 6.83 (s, 1H), 6.77 (d, J = 7.7 Hz, 1H), 6.66 (s, 1H), 5.27 (t, J = 5.7 Hz, 1H), 4.52 (d, J = 5.7 Hz, 2H), 3.69 (s, 3H). IR (cm^{-1}: neat): 1164. LC–MS (APCI$^+$) m/z: 360 (M+H)$^+$. Anal. Calcd for $C_{17}H_{17}N_3O_4S$: C, 56.81; H, 4.77; N, 11.69. Found: C, 56.68; H, 4.87; N, 11.83.

4.1.39. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-(2-methoxyphenyl)-1*H*-pyrazole (3h)

The title compound was prepared from ester **2h** using the same procedure as for **3a** to give **3h** as white crystal (yield: 82%), mp 201–203 °C. ^{1}H NMR (DMSO-d_6) δ: 7.72 (d, J = 8.7 Hz, 2H), 7.41 (t, J = 7.9 Hz, 2H), 7.29 (m, 4H), 7.03 (t, J = 7.3 Hz, 1H), 6.97 (d, J = 8.4 Hz, 1H), 6.49 (s, 1H), 5.22 (t, J = 5.8 Hz, 1H), 4.51 (d, J = 5.8 Hz, 2H), 3.33 (s, 3H). IR (cm^{-1}: neat): 1327, 1162. LC–MS (APCI$^+$) m/z: 343 (M+H)$^+$. Anal. Calcd for $C_{17}H_{17}N_3O_4S$: C, 56.81; H, 4.77; N, 11.69. Found: C, 56.67; H, 4.78; N, 11.54.

4.1.40. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-(1-naphthyl)-1*H*-pyrazole (3i)

The title compound was prepared from ester **2i** using the same procedure as for **3a** to give **3i** as yellow crystal (yield: 96%), mp 190–192 °C. ^{1}H NMR (DMSO-d_6) δ: 8.82 (m, 2H), 8.05 (d, J = 1.4 Hz, 2H), 8.03 (m, 1H), 7.91 (m, 4H), 7.73 (m, 2H), 6.49 (m, 4H), 6.80 (s, 1H), 5.32 (t, J = 5.7 Hz, 1H). IR (cm^{-1}: neat): 3320, 1339, 1160. LC–MS (APCI$^+$) m/z: 380 (M+H)$^+$. Anal. Calcd for $C_{20}H_{17}N_3O_3S$: C, 63.31; H, 4.52; N, 11.07. Found: C, 63.65; H, 4.59; N, 11.48.

4.1.41. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-(2-naphthyl)-1*H*-pyrazole (3j)

The title compound was prepared from ester **2j** using the same procedure as for **3a** to give **3j** as yellow crystal (yield: 23%), mp 226–228 °C. ^{1}H NMR (DMSO-d_6) δ: 8.40 (s, 1H), 7.90 (m, 8H), 7.60 (m, 4H), 7.20 (s, 1H), 5.80 (t, J = 5.0 Hz, 1H), 4.80 (d, J = 6.3 Hz, 2H). IR (cm^{-1}: neat): 3336, 1336, 1167. LC–MS (APCI$^+$) m/z: 380 (M+H)$^+$. Anal. Calcd for $C_{20}H_{17}N_3O_3S$: C, 63.31; H, 4.52; N, 11.07. Found: C, 63.45; H, 4.65; N, 11.28.

4.1.42. 1-(4-Aminosulfonylphenyl)-5-hydroxymethyl-3-(2-naphthyl)-1*H*-pyrazole (3j′)

The title compound was prepared from ester **2j′** using the same procedure as for **3a** to give **3j′** as white crystal (yield: 23%), mp 150–152 °C. ^{1}H NMR (DMSO-d_6) δ: 8.40 (s, 1H), 8.10 (m, 8H), 7.60 (m, 4H), 7.20 (s, 1H), 5.80 (t, J = 5.0 Hz, 1H), 4.80 (d, J = 6.3 Hz, 2H). IR (cm^{-1}: neat): 3336, 1336, 1167. LC–MSLC–MS (APCI$^+$) m/z: 380 (M+H)$^+$. Anal. Calcd for $C_{20}H_{17}N_3O_3S$: C, 63.31; H, 4.52; N, 11.07. Found: C, 63.23; H, 4.48; N, 11.38.

4.1.43. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-(6-methoxynaphtalen-2-yl)-1*H*-pyrazole (3k)

The title compound was prepared from ester **2k** using the same procedure as for **3a** to give **3k** as white crystal (yield: 48%), mp 191–193 °C. ^{1}H NMR (DMSO-d_6) δ: 8.02 (m, 4H), 7.82 (m, 2H), 7.65 (m, 1H), 7.41 (m, 2H), 7.37 (s, 2H), 7.34 (m, 1H), 6.74 (s, 1H), 5.29 (t, J = 5.8 Hz, 1H), 4.59 (d, J = 5.8 Hz, 2H), 4.12 (s, 3H). IR (cm^{-1}: neat): 1327, 1155. LC–MS (APCI$^+$) m/z: 410 (M+H)$^+$. Anal. Calcd for $C_{21}H_{19}N_3O_4S$: C, 61.60; H, 4.68; N, 10.26. Found: C, 61.65; H, 4.37; N, 10.29.

4.1.44. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-(5-bromo-6-methoxynaphtalen-2yl)-1*H*-pyrazole (3l)

The title compound was prepared from ester **2l** using the same procedure as for **3a** to give **3l** as white crystal (yield: 61%), mp >250 °C. ^{1}H NMR (DMSO-d_6) δ: 7.99 (m, 3H), 7.78 (d, J = 8.7 Hz, 2H), 7.57 (d, J = 8.5 Hz, 1H), 7.43 (d, J = 8.7 Hz, 2H), 7.41 (s, 2H), 7.34 (d, J = 8.7 Hz, 1H), 6.74 (s, 1H), 5.29 (t, J = 5.8 Hz, 1H), 4.55 (d, J = 5.8 Hz, 2H), 4.00 (s, 3H). IR (cm^{-1}: neat): 1342, 1165. LC–MS (APCI$^+$) m/z: 488 [(M+H)$^+$ ^{79}Br] and 490 [(M+H)$^+$ ^{81}Br)]. Anal. Calcd for $C_{21}H_{18}BrN_3O_4S$: C, 51.65; H, 3.72; N, 16.36. Found: C, 51.43; H, 3.87; N, 16.67.

4.1.45. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-biphenylyl-1*H*-pyrazole (3m)

The title compound was prepared from ester **2m** using the same procedure as for **3a** to give **3m** as white crystal (yield: 86%), mp 224–226 °C. ^{1}H NMR (DMSO-d_6) δ: 7.85 (d, J = 8.4 Hz, 2H), 7.75 (m, 4H), 7.52 (m, 6H), 7.42 (m, 3H), 6.70 (s, 1H), 5.30 (m, 1H), 4.54 (m, 2H). IR (cm^{-1}: neat): 3320, 1339, 1160. LC–MS (APCI$^+$) m/z: 406 (M+H)$^+$. Anal. Calcd for $C_{22}H_{19}N_3O_3S$: C, 65.17; H, 4.72; N, 10.36. Found: C, 65.45; H, 4.65; N, 10.40.

4.1.46. 1-(4-Aminosulfonylphenyl)-3-hydroxymethyl-5-(2-phenantryl)-1*H*-pyrazole (3n)

The title compound was prepared from ester **2n** using the same procedure as for **3a** to give **3n** as white crystal (yield: 85%), mp >250 °C. ^{1}H NMR (DMSO-d_6) δ: 8.82 (m, 2H), 8.05 (d, J = 1.4 Hz, 1H), 8.03 (m, 1H), 7.91 (m, 4H), 7.73 (m, 2H), 6.49 (m, 5H), 6.80 (s, 1H), 5.32 (t, J = 5.7 Hz, 1H), 5.30 (d, J = 5.7 Hz, 2H). IR (cm^{-1}: neat): 3312, 1335, 1158. LC–MS (APCI$^+$) m/z: 430 (M+H)$^+$. Anal. Calcd for $C_{24}H_{19}N_3O_3S$: C, 67.12; H, 4.46; N, 9.78. Found: C, 67.36; H, 4.56; N, 9.67.

4.1.47. 1-(4-Aminosulfonylphenyl)-3-hydroxyethyl-5-phenyl-1*H*-pyrazole (3o)

The title compound was prepared from ester **2o** using the same procedure as for **3a** to give **3o** as white crystal (yield: 85%), mp 196–198 °C. ^{1}H NMR (DMSO-d_6) δ: 7.80 (m, 2H), 7.42 (m, 7H), 7.25 (m, 2H), 6.56 (s, 1H), 4.74 (br s, 1H), 3.74 (m, 2H), 2.81 (m, 2H). IR (cm^{-1}: neat): 1736, 1343, 1164. LC–MS (APCI$^+$) m/z: 344 (M+H)$^+$. Anal. Calcd for $C_{17}H_{17}N_3O_3S$: C, 59.46; H, 4.99; N, 12.24. Found: C, 59.34; H, 4.76; N, 12.60.

4.1.48. 1-(3-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-phenyl-1*H*-pyrazole (4a)

The title compound was prepared from diketoester **1a** and 3-aminosulfonylphenylhydrazine hydrochloride using the same procedure as for **2a** to give **4a** as yellow crystal (yield: 62%), mp 187–189 °C. ^{1}H NMR (DMSO-d_6) δ: 7.92 (m, 1H), 7.67 (m, 3H), 7.50 (s, 2H), 7.24 (m, 2H), 7.17 (m, 2H), 7.08 (m, 1H), 6.29 (s, 1H), 3.12 (q, J = 7.3 Hz, 2H), 1.26 (t, J = 7.3 Hz, 3H). IR (cm^{-1}: neat): 1714, 1330, 1160. LC–MS (APCI$^+$) m/z: 372 (M+H)$^+$.

4.1.49. 1-(3-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-naphthyl-1*H*-pyrazole (4j)

The title compound was prepared from diketoester **1j** and 3-aminosulfonylphenylhydrazine hydrochloride using the same procedure as for **2a** to give **4j** as yellow crystal yellow crystal (yield: 59%), mp 197–199 °C. ^{1}H NMR (DMSO-d_6) δ: 7.99 (m, 3H), 7.85 (m, 3H), 7.59 (m, 1H), 7.43 (m, 3H), 7.37 (m, 1H), 7.30 (s, 2H), 6.68 (s, 1H), 4.23 (q, J = 9.0 Hz, 2H), 1.84 (t, J = 9.0 Hz, 3H). IR (cm^{-1}: neat): 1718, 1280, 1164. LC–MS (APCI$^+$) m/z: 422 (M+H)$^+$.

4.1.50. 1-(3-Aminosulfonylphenyl)-3-ethoxycarbonyl-5-(6-methoxy-2-naphtalenyl)-1*H*-pyrazole (4k)

The title compound was prepared from diketoester **1k** and 3-aminosulfonylphenylhydrazine hydrochloride using the same procedure as for **2a** to give **4k** as yellow crystal (yield: 53%), mp 204–206 °C. ^{1}H NMR (DMSO-d_6) δ: 7.92 (m, 1H), 7.90 (m, 1H), 7.80 (m, 1H), 7.77 (m, 1H), 7.54 (m, 2H), 7.32 (m, 2H), 7.21 (s, 2H), 7.18 (m, 2H), 6.74 (s, 1H), 4,37 (q, J = 9.1 Hz, 2H), 3.94 (s, 3H), 1.33 (t, J = 9.1 Hz, 3H). IR (cm^{-1}: neat): 1754, 1287, 1162. LC–MS (APCI$^+$) m/z: 452 (M+H)$^+$.

4.1.51. 1-(3-Aminosulfonylphenyl)-3-hydroxymethyl-5-phenyl-1*H*-pyrazole (5a)

The ethyl ester (0.53 mmol, 200 mg) was dissolved in tetrahydrofuran (10 mL) and then a suspension of $LiAlH_4$ (2.70 mmol, 102 mg) in tetrahydrofuran (5 mL) was slowly added. After 4 h of reaction at 20 °C, an aqueous solution of HCl 1 N was added dropwise until a neutral pH. The reaction mixture was concentrated under reduced pressure and the residue was taken up into ethyl acetate and washed with water and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was recrystallized in acetonitrile to give **5a** as white crystal (yield: 59%), mp 198–200 °C. ^{1}H NMR (DMSO-d_6) δ: 7.89 (m, 1H), 7.74 (m, 1H), 7.58 (m, 2H), 7.51 (m, 1H), 7.47 (s, 2H), 7.38 (m, 2H), 7.23 (m, 2H), 6.63 (s, 1H), 5.19 (t, J = 9.1 Hz, 1H), 4.52 (d, J = 9.1 Hz, 2H). IR (cm^{-1}: neat): 1289, 1164. LC–MS (APCI$^+$) m/z: 330 (M+H)$^+$. Anal. Calcd for $C_{16}H_{15}N_3O_3S$: C, 58.35; H, 4.59; N, 12.76. Found: C, 58.23; H, 4.75; N, 12.98.

4.1.52. 1-(3-Aminosulfonylphenyl)-3-hydroxymethyl-5-naphthyl-1*H*-pyrazole (5j)

The title compound was prepared from ester **4j** using the same procedure as for **5a** to give **5j** as white crystal (yield: 67%), mp 175–177 °C. ^{1}H NMR (DMSO-d_6) δ: 7.95 (m, 3H), 7.89 (m, 3H), 7.66 (m, 1H), 7.55 (m, 3H), 7.49 (m, 1H), 7.10 (s, 2H), 6.75 (s, 1H), 5.30 (t, J = 7.1 Hz, 1H), 4.57 (d, J = 7.1 Hz, 2H). IR (cm^{-1}: neat): 1280, 1164. LC–MS (APCI$^+$) m/z: 380 (M+H)$^+$. Anal. Calcd for $C_{20}H_{17}N_3O_3S$: C, 63.31; H, 4.52; N, 11.07. Found: C, 63.23; H, 4.42; N, 11.01.

4.1.53. 1-(3-Aminosulfonylphenyl)-3-hydroxymethyl-5-(6-methoxy-2-naphthalenyl)-1*H*-pyrazole (5k)

The title compound was prepared from ester **4k** using the same procedure as for **5a** to give **5k** as white crystal (yield: 56%), mp 182–184 °C. ^{1}H NMR (DMSO-d_6) δ: 7.86 (m, 1H), 7.75 (m, 1H), 7.61 (s, 2H), 7.58 (m, 1H), 7.47 (m, 3H), 7.32 (m, 1H), 7.17 (m,

3H), 6.71 (s, 1H), 5.28 (t, J = 9.0 Hz, 1H), 4.59 (d, J = 7.2 Hz, 2H), 3.87 (s, 3H). IR (cm^{-1}: neat): 1287, 1162. LC–MS ($APCI^+$) m/z: 410 $(M+H)^+$. Anal. Calcd for $C_{21}H_{19}N_3O_4S$: C, 61.60; H, 4.68; N, 10.26. Found: C, 61.45; H, 4.57; N, 10.34.

4.1.54. Ethyl 2-benzoyl-3-dimethylaminoacrylate (6a)

The ethyl benzoylacetate (20 mmol, 3.46 mL) and *N*,*N*-dimethylformamide dimethyl acetal was refluxed for 1 h. After evaporation under reduced pressure, the residue was purified by chromatography on silica gel (diethyl ether) to give title compound **6a** as yellow crystals (yield: 40%), mp 154–156 °C. ^1H NMR (DMSO-d_6) δ: 7.72 (m, 2H), 7.44 (m, 1H), 7.39 (m, 2H), 7.36 (s, 1H), 3.94 (q, J = 7.0 Hz, 2H), 2.97 (s, 6H), 0.85 (t, J = 6.9 Hz, 3H). IR (cm^{-1}: neat): 1681, 1586. LC–MS ($APCI^+$) m/z: 248 $(M+H)^+$.

4.1.55. Ethyl 2-naphtoyl-3-dimethylaminoacrylate (6j)

The title compound was prepared from ethyl naphtoylacetate using the same procedure as for **6a** to give **6j** as yellow crystal (yield: 58%), mp 145–147 °C. ^1H NMR (DMSO-d_6) δ: 8.10 (m, 2H), 7.90 (m, 1H), 7.78 (m, 2H), 7.35 (m, 2H), 7.29 (s, 1H), 3.80 (q, J = 6.9 Hz, 2H), 3.10 (s, 6H), 0.90 (t, J = 6.9 Hz, 3H). IR (cm^{-1}: neat): 1687, 1562. LC–MS ($APCI^+$) m/z: 298 $(M+H)^+$.

4.1.56. Ethyl 2-methoxynaphtoyl-3-dimethylaminoacrylate (6k)

The title compound was prepared from ethyl methoxynaphtoylacetate using the same procedure as for **6a** to give **6k** as orange crystal (yield: 85%), mp 150–152 °C. ^1H NMR (DMSO-d_6) δ: 8.20 (s, 1H), 7.95 (s, 1H), 7.72 (m, 1H), 7.60 (m, 2H), 7.25 (m, 1H), 7.20 (m, 1H), 3.92 (q, J = 6.9 Hz, 2H), 3.31 (s, 3H), 2.75 (s, 6H), 0.78 (t, J = 6.9 Hz, 3H). IR (cm^{-1}: neat): 1692, 1572. LC–MS ($APCI^+$) m/z: 328 $(M+H)^+$.

4.1.57. 1-(4-Aminosulfonylphenyl)-4-ethoxycarbonyl-5-phenyl-1*H*-pyrazole (7a)

The ester **6a** (0.8 mmol, 0.92 g) was dissolved in ethanol (10 mL), then 4-aminosulfonylphenylhydrazine hydrochloride (0.8 mmol, 0.19 g) was introduced. The mixture was refluxed for 2 h. After cooling to 20 °C the precipitate was filtered and washed with petroleum ether to give the title compound **7a** as yellow crystals (yield: 73%), mp 178–180 °C. ^1H NMR (DMSO-d_6) δ: 7.33 (m, 4H), 8.24 (s, 2H), 7.75 (m, 3H), 7.44 (s, 1H), 7.31 (m, 2H), 4.10 (q, J = 6.5 Hz, 2H), 1.10 (t, J = 6.5 Hz, 3H). IR (cm^{-1}: neat): 1702, 1310, 1151. LC–MS ($APCI^+$) m/z: 372 $(M+H)^+$.

4.1.58. 1-(4-Aminosulfonylphenyl)-4-ethoxycarbonyl-5-naphthyl-1*H*-pyrazole (7j)

The title compound was prepared from ester **6j** using the same procedure as for **7a** to give **7j** as orange crystal (yield: 74%), mp 145–147 °C. ^1H NMR (DMSO-d_6) δ: 8.29 (m, 1H), 7.93 (d, J = 8.5 Hz, 2H), 7.90 (s, 1H), 7.82 (m, 2H), 7.62 (d, J = 8.5 Hz, 2H), 7.30 (m, 2H), 7.29 (s, 2H), 7.20 (m, 2H), 4.10 (q, J = 5.5 Hz, 2H), 1.00 (t, J = 5.5 Hz, 3H). IR (cm^{-1}: neat): 1734, 1345, 1158. LC–MS ($APCI^+$) m/z: 422 $(M+H)^+$.

4.1.59. 1-(4-Aminosulfonylphenyl)-4-ethoxycarbonyl-5-(6-methoxy-2-naphtalenyl)-1*H*-pyrazole (7k)

The title compound was prepared from ester **6k** using the same procedure as for **7a** to give **7k** as orange crystal (yield: 83%), mp 175–177 °C. ^1H NMR (DMSO-d_6) δ: 8.37 (s, 1H), 8.02 (s, 1H), 7.98 (d, J = 8.6 Hz, 2H), 7.92 (m, 2H), 7.65 (s, 2H), 7.44 (d, J = 8.6 Hz, 2H), 7.33 (m, 1H), 7.19 (m, 2H), 4.00 (q, J = 7.3 Hz, 2H), 3.55 (s, 3H), 1.00 (t, J = 7.3 Hz, 3H). IR (cm^{-1}: neat): 1754, 1352, 1171. LC–MS ($APCI^+$) m/z: 452 $(M+H)^+$.

4.1.60. 1-(4-Aminosulfonylphenyl)-4-hydroxymethyl-5-phenyl-1*H*-pyrazole (8a)

Ethyl ester **7a** (1.3 mmol, 0.500 g) was dissolved in tetrahydrofuran (25 mL) and then a suspension of $LiAlH_4$ (3.9 mmol, 0.15 g) in tetrahydrofuran (5 mL) was slowly added. After 2 h of reaction at 20 °C, an aqueous solution of HCl 1 N was added dropwise until a neutral pH. The reaction mixture was concentrated under reduced pressure, the residue was taken into ethyl acetate and washed with water and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was recrystallized in acetonitrile. The alcohol (**8a**) give a white crystal (yield: 29%), mp 150–152 °C. ^1H NMR (DMSO-d_6) δ: 7.85 (s, 2H), 7.75 (d, J = 8.4 Hz, 2H), 7.65 (s, 1H), 7.34 (d, J = 8.4 Hz, 2H), 7.26 (m, 5H), 5.10 (br s, 1H), 4.35 (m, 2H). IR (cm^{-1}: neat): 3267, 1598. LC–MS ($APCI^+$) m/z: 330 $(M+H)^+$. Anal. Calcd for $C_{16}H_{15}N_3O_3S$: C, 58.35; H, 4.59; N, 12.76. Found: C, 58.45; H, 4.82; N, 12.58.

4.1.61. 1-(4-Aminosulfonylphenyl)-4-hydroxymethyl-5-(2-naphthyl)-1*H*-pyrazole (8j)

The title compound was prepared from ester **7j** using the same procedure as for **8a** to give **8j** as white crystal (yield: 69%), mp 210–212 °C. ^1H NMR (DMSO-d_6) δ: 7.89 (m, 1H), 7.88 (d, J = 7.2 Hz, 2H), 7.79 (s, 1H), 7.75 (m, 2H), 7.56 (d, J = 7.2 Hz, 2H), 7.40 (m, 2H), 7.53 (s, 2H), 7.19 (m, 2H), 4.36 (br s, 1H), 3.88 (m, 2H). IR (cm^{-1}: neat): 3245, 1603. LC–MS ($APCI^+$) m/z: 380 $(M+H)^+$. Anal. Calcd for $C_{20}H_{17}N_3O_3S$: C, 63.31; H, 4.52; N, 11.07. Found: C, 63.03; H, 4.69; N, 11.26.

4.1.62. 1-(4-Aminosulfonylphenyl)-4-hydroxymethyl-5-(6-methoxy-2-naphtalenyl)-1*H*-pyrazole (8k)

The title compound was prepared from ester **7k** using the same procedure as for **8a** to give **5a** as white crystal (yield: 63%), mp 221–223 °C. ^1H NMR (DMSO-d_6) δ: 7.99 (s, 1H), 7.94 (m, 2H), 7.92 (d, J = 8.6 Hz, 2H), 7.89 (s, 1H), 7.42 (s, 2H), 7.37 (d, J = 8.6 Hz, 2H), 7.24 (m, 1H), 7.21 (m, 2H), 5.05 (br s, 1H), 4.40 (m, 2H), 3.40 (s, 3H). IR (cm^{-1}: neat): 3345, 1599. LC–MS ($APCI^+$) m/z: 410 $(M+H)^+$. Anal. Calcd for $C_{21}H_{19}N_3O_4S$: C, 61.60; H, 4.68; N, 10.26. Found: C, 61.89; H, 4.98; N, 10.69.

4.2. Enzymatic evaluation

The CA catalyzed CO_2 hydration activity was followed by an SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (to maintain constant the ionic strength). Saturated CO_2 solutions in water at 25 °C were used as substrate. Stock solutions of inhibitor were prepared at a concentration of 10 mM (in DMSO/water 1:1, v/v) and dilutions up to 0.01 nM were done with the assay buffer mentioned above. At least seven different inhibitor concentrations were used for measuring the inhibition constant. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior 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. K_Is were obtained from Lineweaver–Burk plots, as reported earlier.[27]

4.3. In vitro anticancer screening

4.3.1. Cell culture

MDA-MB-231 breast cancer cells were kindly provided by Professor X. Le Bourhis (Inserm U908, Lille, France). Cell lines were maintained as monolayer culture in a humidified incubator at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% fetal calf serum (FCS), 1000 U/ml penicillin and 100 μg/ml streptomycin. All the products for cell culture were from Life Technologies.

4.3.2. Cell growth inhibition assay

Growth inhibition was assessed using MTS assay with CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega). Cells (2×10^3) were seeded into a 96-well plate in DMEM with 10% FCS and buffered with 22.3 mM bicarbonate,[28] and transfered to hypoxia (1% O_2 and 5% CO_2 balanced with N_2) generated in a H35 hypoxystation (Don Withley). Twenty-four hours later, CA IX inhibitors (1 μM) were added and cells were treated with increasing concentrations of Doxorubicin (Sigma–Aldrich) and incubated for 72 h at 37 °C under hypoxia. MTS reagent was then added to each well and the plate was incubated for 1 h at 37 °C. Absorbance was measured at 492 nm with a microplate reader. Three independent experiments were performed in six replicate wells for each drug concentration. The IC_{50} (inhibition concentration 50) value was defined as the concentration of Doxorubicin needed for 50% reduction in absorbance and calculated from the survival curves by SoftMax Pro software (Molecular Devices).

4.4. Molecular modeling and calculations

The compounds in deprotonated form were built using the *SKETCH* module, as implemented in *SYBYL* (version 8.0),[29] and their geometry was optimized using the *MINIMIZE* module. The minimization process uses the *POWELL* method with the *TRIPOS* force field (dielectric constant 1*r*) to reach a final convergence of 0.01 kcal mol^{-1}. Docking simulation was then performed into hCA IX (RCSB Protein Data Bank 3IAI) with the automated *GOLD* program.[30] The active site was defined including all residues in a volume of 10 Å around acetazolamide taken as reference.

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