Synthesis and inhibition potency of novel ureido benzenesulfonamides incorporating GABA as tumor-associated carbonic anhydrase IX and XII inhibitors

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INTRODUCTION

Sixteen human (h) α-carbonic anhydrase (CA, EC 4.2.1.1) isoforms have been discovered till now. They belong to a large family of metalloenzymes, which comprises six genetic families, i.e. α, β, γ, δ, ζ and η classes. Carbonic anhydrases are mainly involved in the reversible hydration reaction of carbon dioxide to release bicarbonate and proton. Among the various biological functions, CAs are involved in pH buffering of the intra- and extra-cellular spaces. Mainly two carbonic anhydrase isoforms hCA IX and XII, possessing extracellular active site, are over-expressed in most of hypoxic tumors, such as gliomas/ependymomas, mesotheliomas, carcinomas of the bladder, cervix, kidneys, lungs, breast, and other dysplasia resistant to classic ependymomas, mesotheliomas, carcinomas of the bladder, cervix, and other dysplasia resistant to classic antitumor therapy.

The most studied and clinically used pharmacophores as carbonic anhydrase inhibitor (CAI) are the sulfonamides, which is their mechanism of action within the enzymatic cavity is well reported with many representatives in clinical use for decades. However, one of the main side effects of sulfonamide CAIs, especially those ones belonging to the first generation of such drugs, i.e. acetazolamide, is the fact that they are not able to discriminate different CA isoforms. The most efficient sulfonamide CAIs indeed inhibit the majority of the mammalian CA isoforms, thus leading to a wide range of side effects.

Only recently it has been developed several novel generations of sulfonamide CAIs, which posses a remarkable selectivity profile for inhibiting prevalently the two transmembrane hCA IX and XII isoforms. Among such isoform-selective benzenesulfonamide CAIs, the ureido-containing inhibitors are very interesting, as some representatives of this class (among which compounds A–D) possessed nanomolar activity for the inhibition of the tumor-associated isoforms hCA IX and XII; whereas they were much weaker inhibitors of the widespread, cytosolic off-target isoforms hCA I and II. The hCA IX inhibition with these sulfonamides in vitro, in cell cultures, and in animals with the over-expression of hCA IX and XII isoforms, they are considered as interesting druggable target for imaging and treatment of hypoxic tumors. Therefore, the selective inhibition of two transmembrane isoforms CA IX and XII over the ubiquitous and cytosolic ones, CA I/II, represents a promising approach for the development of an efficient and low-side effect antitumor therapy.

The most efficient sulfonamide CAIs, the ureido-containing inhibitors are very interesting, as some representatives of this class (among which compounds A–D) possessed nanomolar activity for the inhibition of the tumor-associated isoforms hCA IX and XII; whereas they were much weaker inhibitors of the widespread, cytosolic off-target isoforms hCA I and II. The hCA IX inhibition with these sulfonamides in vitro, in cell cultures, and in animals with
transplanted tumors, leads to a return to more normal extracellular pH values, with consequential delay of tumor growth. One of these sulfonamide CA IX/XII inhibitors is currently in Phase I clinical trials for the treatment of primary tumors/metastases overexpressing these enzymes.

A large number of ureido-substituted benzenesulfonamide derivatives have been extensively studied from our group showing an interesting and excellent inhibition profile against the tumor-associated human carbonic anhydrase. Indeed, by only one-step synthesis occurring as well in high yield between sulfonamide and stoichiometric amount of aryl/alkyl isocyanates a large number of such derivatives were prepared. Generally, the ureido containing derivatives, among which compounds A–D are considered the most interesting representatives of the series (Chart 1), shown to possess an excellent low nanomolar inhibition potency demonstrated by a significant regression of the tumor volume in vivo experiments. It has also been shown that depending on the substitution pattern on the urea side it was possible to obtain a good inhibition selectivity of the transmembrane CA isoforms over the cytosolic ones.

X-ray crystallographic studies of adduct formed between hCA II enzyme with ureido benzenesulfonamides, such as A–D derivatives, pointed out some important features of their inhibition mechanism. It is clear that the benzenesulfonamide moiety of various inhibitors belonging to this series was entirely superposable in the enzyme active site with the deprotonated sulfonamide portion (SO\_2NH\_2 anion) coordinated to the Zn (II) ion, whereas the tails (R) of the ureido moiety adopted different orientations based on their specific chemical nature.

Therefore, for sulfanilamide derivatives incorporating the ureido linker, it has been shown that the tails attached to the urea portion are located in various hydrophobic pockets of CA II catalytic site and interacted with different amino acid residues according to the chemical properties of the R moiety.

Considering the inhibition profiles, the selectivity and the mechanism of inhibition of these compounds, it appeared of great interest to further explore these scaffolds for obtaining more potent CAIs. We extended consequently our earlier investigations on ureido benzenesulfonamide CAIs synthesizing and testing the inhibition potency of a small series of derivatives containing the same scaffold but incorporating instead also an elongation between the ureido and the sulfonamide moieties, such as a GABA portion.

Also, in this new series of GABA containing ureido benzenesulfonamide derivatives many low nanomolar hCA IX or CA XII inhibitors have been identified.

Since, in our previous studies some ureido benzenesulfonamide derivatives containing GABA were found highly efficient and selective CA IX/XII inhibitors and distinguished as excellent potential anticancer agents, we decided to continue our interest to the same pathway.

Therefore, in this paper we expanded the synthesis of the derivatives incorporating GABA as a linker between the ureido and the benzenesulfonamide moieties. The inhibition potency of these novel compounds against that over-expressed in both hypoxic tumors hCA IX and XII isoforms over the physiologically dominant, cytosolic hCA I and II ones have been determined and thus compared with the efficiency obtained from similar derivatives previously studied and their smaller congeners containing sulfanilamide instead.

### Materials and methods

#### Chemistry

Anhydrous solvents and all reagents were purchased from Sigma-Aldrich (Milan, Italy), Alfa Aesar (Milan, Italy) and Merck (Darmstadt, Germany). All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. Nuclear magnetic resonance (\(^1\)H-NMR and \(^13\)C-NMR) spectra were recorded using a Bruker Advance III 400 MHz spectrometer (Flawil, Switzerland) in DMSO-d_6. Chemical shifts are reported in parts per million (ppm) and the coupling constants (J) are expressed in Hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; sept, septet; t, triplet; q, quadruplet; m, multiplet; brs, broad singlet; brm, broad multiplet; dd, double of double; td, triplet of double; tt, triplet of triplet; appd, apparent double; appt, apparent triplet.

The assignment of exchangeable protons (NH) was confirmed by the addition of D\(_2\)O. Electron ionization mass spectra (70 eV) were recorded on a Hewlett-Packard 5989 Mass Engine Spectrometer (Milan, Italy). Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates.

The synthesis and the full characterization of all compounds investigated here as hCA inhibitors are reported elsewhere, derivatives 6–11 in reference 24 and 12–20 in Supplemental information.
CA inhibition studies

An Applied Photophysics stopped-flow instrument (Oxford, UK) has been used for assaying the CA catalyzed CO₂ hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as an indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.4), 10 mM Tris.HCl and 0.1 M NaSO₄ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO₂ hydration reaction for a period of 10–100 s. The CO₂ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 mM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at RT prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier, were determined the inhibition potency of some ureido sulfonamide derivatives, such as compounds A–D, which incorporate a sulfanilamide moiety in one side and an ureido tail in the other (Chart 1). It has been demonstrated that this series of compounds act as highly effective hCA II inhibitors with potencies that correlate well with the orientation of the R moiety present in the ureido tail of the compound.

Similar to the leads A–D just reported, the new compounds obtained by the above-mentioned reactions showed as well remarkable properties as CAIs, against the cytosolic ubiquitous hCA I and CA II isoforms and the tumor-associated hCA IX and CA XII ones. Indeed, many such compounds were low nanomolar hCA IX/XII inhibitors whereas their affinity for the cytosolic off-target isozyme I was not as good, making them moderate tumor CA-selective inhibitors.

As mentioned above all hypoxic tumors overexpressed CA IX and CA XII isoforms after activation of the hypoxia inducible factor-1 (HIF-1) with a pH imbalance between the tumor tissue and the normal tissue as main consequence. The hCA IX/XII isoforms inhibition with sulfonamides was recently shown to reverse the effect of tumor acidification, leading to inhibition of the primary tumor and metastases growth, and CA IX/XII have been proposed as novel therapeutic antitumor targets.

Reported here, there are novel ureido-benzene sulfonamide CAIs obtained considering compounds A–D as leads, some of which incorporate the same R-ureido linker, where R was phenyl, benzyl, cyclopentyl and 4-iodophenyl together with a GABA portion connecting the 4-aminomethyl or 4-aminomethylbenzene sulfonamide and the ureido moieties. Therefore, the present findings extend our previous studies and report here the synthesis of a series of ureido benzene sulfonamide derivatives-containing the same ureido portion of such compounds or new ureido linker.

The reaction of γ-Boc-GABA with 4-(aminomethyl)-benzene sulfonamide and 4-(2-aminomethyl)-benzene sulfonamide and consequently hydrolysis of the coupling products afforded the key intermediates as hydrochlorides and 5, which were reacted with one equivalent of various arylisocyanates, such as 4-methylbenzyl, 4-iodophenyl and 4-ethylphenyl isocyanates, as well as 4-

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Isocyanates used for the preparation of compounds 6–20.

| Isocyanates | R |
|-------------|---|
| A1          | \( \text{N=C=O} \)
| A2          | \( \text{N=C=O} \)
| A3          | \( \text{N=C=O} \)
| A4          | \( \text{N=C=O} \)
| A5          | \( \text{N=C=O} \)
| A6          | \( \text{N=C=O} \)
| A7          | \( \text{N=C=O} \)
| A8          | \( \text{N=C=O} \)

Table 1. Isocyanates used for the preparation of compounds 6–20.

nitrophenyl one (Table 1) in the presence of N-ethylisopropylamine (Scheme 1). These isocyanates were firstly chosen in such a way as to contain moieties which were shown earlier to lead to interesting CA inhibitory compounds in the ureido benzenesulfonamide derivatives\(^{21}\) for comparison reason.

Reaction of the key intermediate sulfonamide hydrochlorides 4 and 5, possessing an amino group at the GABA moiety with arylisocyanates A1–8 reported in Table 1 in the molar ratio of 1:1 in the presence of a weak base afforded the formation of a large series of ureido benzenesulfonamide derivatives (6–20) (Scheme 1). The equivalent amount of base used in this synthesis was required to form the nucleophilic free base of the amine group. Due to its low reactivity, a tertiary amine such as diisopropylethylamine (DIPEA) is always used as a base in this case (Scheme 1).

The characterization of new compounds by \(^1\text{H}-\) and \(^{13}\text{C}-\)NMR as well as by mass spectroscopy, confirmed their chemical structures (For further details, see Experimental Protocols reported in reference 24 and Supplemental information).

CA inhibition

Inhibition data with the new group of ureido benzenesulfonamides 6–20 reported here, against the human (h) CA isozymes hCA I, II (cytosolic) and hCA IX, XII (transmembrane and tumor associated), as well as the derivatives A–D reported earlier (for comparison reasons)\(^{21}\) are shown in Table 2. Therefore, the following structure activity relationship (SAR) can be observed from data reported in Table 2:

(i) The cytosolic and widespread isoform hCA I was moderately inhibited by almost all the compounds reported here, with inhibition constants in the range 192.7–373.4 nM. The best hCA I inhibitors in the series were the phenyl and the 4-nitrophenyl-ureido derivatives of 4-aminophenylbenzenesulfonamide (7 and 11), with \(K_I\)s of 32.2 and 41.8 nM, respectively. The ethylbenzene-ureido derivative of 4-aminomethylbenzenesulfonamide (10) (\(K_I\) of 52.6 nM) was also a very effective hCA I inhibitor, and revealed an inhibition potency 5.4-fold higher compared to its longer congener 12 (\(K_I\) of 286.1 nM). On the other hand, the remaining substitution patterns R led to compounds with inhibition constants of the same order of magnitude as the clinically used drug acetazolamide, AAZ, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide (Table 2). The low inhibition of this isoform may be considered as a positive characteristic of this new series of compounds (shared also with the corresponding 4-substituted-ureido-benzenesulfonamide derivatives A, C and D reported earlier)\(^{21}\) since the ubiquitous isoform hCA I is abundant in red blood cells, it can be certainly considered an important off-target when research of antitumor CAIs is involved\(^{24}\).

(ii) Against the physiologically dominant isoform hCA II; derivatives 6–8, 10, 11, 19 and 20 behaved as very strong inhibitors with \(K_I\)s in the range of 4.87–6.9 nM.

The slightly less effective compound was the benzyl ureido derivative of the ethylbenzenesulfonamide 9 with a \(K_I\) value of 41 nM, whereas the remaining ones such as 9 and 12–18, were all quite similar and medium potency hCA II inhibitors, with inhibition constants in a very narrow range 41.0–93.4 nM. Although these compounds possess a rather wide range of substitution patterns, SAR is almost impossible to define as all substituted moieties lead to effective inhibitors of this isoform (Table 2). Most probably, introduction of various substituents, such as halogens, methyl, ethyl and nitro group in the aromatic ring or cyclopropyl ring, leads to a not significant increase in the inhibition potency. Interestingly, the new series of ureido benzenesulfonamides derivatives were very effective hCA II inhibitors compared to those reported earlier A–D, showing how an elongation of the linker chain between the ureido and the sulfonamide moieties can largely modify the inhibition potency of such compounds.

(iii) Several nanomolar hCA IX inhibitors are reported here, such as 6, 7, 13, 14, 16, 17 and 20, derivatives with \(K_I\)s in the range of 42.2–53.4 nM and thus about two times higher compared to that of the clinically used AAZ. Although many of the highly effective hCA IX inhibitors reported here have not shown excellent selectivity ratios for inhibiting this transmembrane isoform over the cytosolic ones, the most interesting CA IX inhibitor listed here was derivative 12 which has shown selectivity ratios as high as 52.01 against hCA I and 16.98 against hCA II (Table 1). Indeed, this derivative resulted to be the best and the most isoform selective CA IX inhibitor. The least effective hCA IX inhibitors were derivatives 8, 10, 15, 18 and 19, which showed \(K_I\)s in the range of 289–370.4 nM, being thus low potency inhibitors of this isoform compared to the clinically
used inhibitor AAZ. The remaining derivatives 9 and 11
have showed moderate inhibitory potency against this
isoform (Kᵢ values of 106.4 and 138.7 nM, respectively).

Although some of the compounds reported here contain same
substitution pattern at the R moiety as part of ureido
sulfonamides previously studied possessed, it is interesting to notice that their
shorter congeners containing sulfanilamide B–D are generally
about 10-fold more effective as hCA IX inhibitors compared to the
new derivatives incorporating a GABA moiety in their
structures (such as in the case of derivatives 8, 9 and 10 with D).
However, only derivatives 6 and 7 showed higher
inhibition potency against hCA IX isoform with Kᵢ 10 times lower
compared to their shorter congener A (with Kᵢ of 41.6–46 nM for
compounds 6 and 7 versus 575 nM of derivatives A), revealing to
be more effective hCA IX inhibitors.

However, it is also interesting to observe that among the
ureido-derivatives incorporating a GABA moiety the ones con-
taining 4-aminophenylbenzenesulfonamide are generally stronger
hCA IX inhibitors than the shorter 4-aminomethylbenzenesulfo-
namide congeners of the same series (Table 2).

(iv) Most of the compounds reported here are shown to be very
efficient hCA XII inhibitors possessing inhibition activity at
low nanomolar range in the same order of magnitude as
AAZ (Table 2). Indeed, it is interesting to notice that this
second transmembrane tumor-associated isoform, hCA
XII, was much better inhibited by the new ureido
benzenesulfonamides investigated here, compared to the
other transmembrane tumor-associated isoform, hCA IX.
The best inhibitors were derivatives 7, 12, 13, 15–17, 19
and 20 with Kᵢ values in a narrow range between 4.9 and
10.4 nM. The remaining derivatives were slightly less
effective hCA XII inhibitors, with inhibition constants in
the range of 28.5–58.3 nM, which is about 10-fold higher
compared to that of the standard drug AAZ. However, the
elongation of the main skeleton between the ureido linker
and the benzenesulfonamide portion with a GABA moiety
did not significantly improve the inhibition potency of
these compounds compared to their smaller congeners
(such as in the case of derivatives 8 and 9 with B, 13 and
14 with C, 19 and 20 with D). Indeed, benzyl ureido
derivatives 8 and 9 have shown extremely similar inhibi-
tion potency against hCA XII isoform compared to their
sulfanilamide congener B, revealing Kᵢ values of 58.3 and
50.7 nM, respectively, like that one of the compound B
equal to 49.5 nM. The same occurred both for the
cyclopentyl derivatives 13 and 14, which have shown Kᵢ
values of 10 and 28.5 nM, respectively, revealing to be
slightly less effective hCA XII inhibitors than their
conger C with Kᵢ of 7 nM and for the 4-iodophenyl
derivatives 19 and 20, with inhibition constants of 6.1 and
5.1 nM of the same order of magnitude as compound D
possessing a Kᵢ value equal to 4.3 nM.

Table 2. Inhibition data of human CA isoforms hCA I, II, IX and XII with ureido-sulfonamides 6–20
reported here and the standard sulfonamide inhibitor acetazolamide (AAZ) by a stopped flow CO₂ hydrase
assay.²⁶

| Compound | n  | R                  | hCA I (nM) | hCA II (nM) | hCA IX (nM) | hCA XII (nM) |
|----------|----|--------------------|------------|-------------|-------------|--------------|
| 6       | 1  | C₆H₅                | 373.4      | 4.9         | 46          | 37.4         |
| 7       | 2  | C₆H₅                | 32.2       | 1.1         | 41.6        | 6.2          |
| 8       | 1  | C₆H₅CH₂             | 359        | 6.1         | 456.6       | 58.3         |
| 9       | 2  | C₆H₅CH₂             | 214.4      | 41          | 106.4       | 50.7         |
| 10      | 1  | C₆H₅(CH₂)₂          | 52.6       | 6.9         | 300.9       | 43.5         |
| 11      | 2  | 4-NO₂C₆H₄           | 41.8       | 6.8         | 138.7       | 43           |
| 12      | 2  | C₆H₅(CH₂)₂          | 286.1      | 93.4        | 5.5         | 10.4         |
| 13      | 1  | C₆H₅                | 254.1      | 70.5        | 42.2        | 10           |
| 14      | 2  | C₆H₅                | 240.4      | 60          | 45.3        | 28.5         |
| 15      | 1  | 4-C₆H₅Ph            | 247.9      | 71.4        | 289         | 5.4          |
| 16      | 2  | 4-C₆H₅Ph            | 232.4      | 57.5        | 53.4        | 6            |
| 17      | 1  | 4-CH₃PhCH₂          | 203.2      | 73.5        | 50.2        | 4.9          |
| 18      | 2  | 4-CH₃PhCH₂          | 192.7      | 64.3        | 328.2       | 6.2          |
| 19      | 1  | 4-IC₆H₄             | 217.8      | 6.8         | 370.4       | 6.1          |
| 20      | 2  | 4-IC₆H₄             | 213.4      | 6.3         | 46.7        | 5.1          |
| AAZ     | –  | –                  | 250        | 12          | 25          | 5.7          |
| A³      | –  | C₆H₅                | 760        | 3730        | 575         | 67.3         |
| B⁴      | –  | C₆H₅CH₂             | 92         | 2200        | 41.4        | 49.5         |
| C⁴      | –  | C₆H₅                 | 470        | 226         | 7.3         | 7.0          |
| D⁴      | –  | 4-C₆H₅H₄            | 5500       | 2634        | 24.5        | 4.3          |

²From reference 24; ³From reference 23; *Mean from three different assay, by a stopped flow technique
(errors were in the range of ±5–10% of the reported values).
respectively. Approximately 2 and 10 times lower than that shown by AAZ ratios shown by A–D compared to those of the corresponding sulfanilamide derivatives transmembrane isoforms over the cytosolic ones is lower to tumor-associated isoforms over the cytosolic isoforms compared to those of the same series of derivatives. The selectivity ratio for inhibiting the target isoforms over the off-target isoforms for most of this series of derivatives is rather low compared to that shown by their smaller congeners A–D (Figures 1 and 2). However, only one compound is shown to be very selective inhibitor against both the tumor-associated CA isoforms. Indeed, the phenethyl derivative 12 possessed a selectivity ratio of 52.01 for inhibiting hCA IX over hCA I and of 16.98 for inhibiting hCA IX over hCA II, thus being the best selective inhibitor of the series against the tumor-associated isoforms over the cytosolic ones, as shown in Figure 1. It should also be noted that acetazolamide has quite low selectivity ratios for inhibiting the tumor-associated isoforms against hCA I and II isozymes, as shown in Figure 1. It should also be noted that acetazolamide has quite low selectivity ratios for inhibiting the tumor-associated isoforms against hCA I and II isozymes, as shown in Figure 1.

(v) Regarding the selectivity of the new derivatives reported here against the two transmembrane tumor-associated hCA IX and XII isoforms over the off-target cytosolic hCA I and II ones, some interesting results emerged. Firstly, the selectivity ratio for inhibiting the target isoforms over the off-target isoforms for most of this series of derivatives is rather low compared to that shown by their smaller congeners A–D (Figures 1 and 2). However, only one compound is shown to be very selective inhibitor against both the tumor-associated CA isoforms. Indeed, the phenethyl derivative 12 possessed a selectivity ratio of 52.01 for inhibiting hCA IX over hCA I and of 16.98 for inhibiting hCA IX over hCA II, thus being the best selective inhibitor of the series against the tumor-associated isoforms over the cytosolic ones, as shown in Figure 1. It should also be noted that acetazolamide has quite low selectivity ratios for inhibiting the tumor-associated isoforms against hCA I (and as well hCA II), which are 10 and 0.48, respectively. Although the selectivity ratios of derivative 12 against the transmembrane isoforms over the cytosolic ones is lower compared to those of the corresponding sulfanilamide derivatives A–D, those value are still significantly higher than the selectivity ratios shown by AAZ. Therefore, this result revealed that compound 12 has better selectivity ratios for inhibiting the tumor-associated isoforms over the cytosolic isoforms compared to AAZ.

We can conclude that in this new series of compounds an elongation of the linker between the ureido and the sulfonamide moieties with a GABA scaffold, mostly brought an increase of the inhibition potency against the tumor-associated target isoforms, decreasing on the other hand their selectivity ratio against the off-target cytosolic isoforms.

Conclusions

A novel series of ureido benzenesulfonamides incorporating GABA scaffold and some of them containing same ureido linker as their sulfanilamide smaller congeners, were explored here as a new generation of selective tumor-associated hCA IX and XII inhibitors. The compounds investigated contain a GABA portion as linker between the ureido and the benzenesulfonamide moieties. Most of the new derivatives were medium potency inhibitors of the ubiquitous cytosolic hCA I isoform and efficient inhibitors against hCA II, but they showed significant inhibition potency against both the transmembrane isoforms hCA IX and XII, with inhibition constants in nanomolar range. The inhibition profile of these new GABA containing ureidosulfonamides is very different from the corresponding analogous not incorporating GABA, which were previously investigated as inhibitors of these enzymes.

Considering the selectivity ratios of the derivatives reported here for inhibiting the tumor-associated hCA IX over the physiologically dominant hCA I and II isoforms, the phenethyl derivative is the most selective inhibitor, thus representing an interesting tool for the development of new anticancer agents.

Declaration of interest

Most of the authors declare no financial interest. CTS reports conflict of interest as author of many patents on CA inhibitors. This project was funded by a 7th FP EU grant (METOXIA). MC and CTS also thank the Erasmus project for a mobility grant.

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Supplementary material available online

Supplemental Information.