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4-Hydroxy-3-nitro-5-ureido-benzenesulfonamides selectively target the tumor-associated carbonic anhydrase isoforms IX and XII showing hypoxia-enhanced anti-proliferative profiles.


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Abstract

Human carbonic anhydrases (CA, EC, 4.2.1.1) IX and XII are overexpressed in cancer cells as adaptive response to hypoxia and acidic conditions characteristic of many tumors. In addition, hypoxia facilitates the activity of specific oxido-reductases that may be exploited to selectively activate bio-reductive prodrugs. Here, new selective CA IX/XII inhibitors, as analogues of the anti-tumor phase II drug SLC-0111 are described, namely ureido-substituted benzenesulfonamides appended with a nitro-aromatic moiety to yield an anti-proliferative action increased by hypoxia. These compounds were screened for the inhibition of the ubiquitous hCA I/II and the target hCA IX/XII. Six X-ray crystallographies with CA II and IX/mimic allowed for the rationalization of the compounds inhibitory activity. The effects of some such compounds on the viability of HT-29, MDA-MB-231 and PC-3 human cancer cell lines in both normoxic and hypoxic conditions were examined, providing the initiation towards the development of hypoxia-activated anti-tumor CAIs.

Keywords: Hypoxia, tumor-associated carbonic anhydrase, inhibition, anti-proliferative.
Introduction

Hypoxia is a condition characterized by low levels of oxygen that commonly marks the microenvironment within solid tumors. Hypoxia results from dysfunctional microvasculature caused by the rapid growth of the tumor. As such, hypoxia promotes several effects in the tumor, including a switch to glycolytic metabolism, extracellular acidosis, resistance to apoptosis, increased mutation upon inhibition of DNA repair, up-regulation of angiogenesis, enhanced local invasiveness, metastatic spread, and promotion of cancer cells stemness. The hypoxia-inducible factor 1 (HIF-1) programs such an orchestra of events, which results in cancer cells survival and proliferation. Additionally, tumor hypoxia plays a role in resistance to radiotherapy and chemotherapy. As a result, the hypoxic microenvironment of solid tumors has become of interest to the scientific community for the development of novel anti-cancer agents.

Since the majority of normal tissues are devoid of hypoxic regions (although mild physiological hypoxia can be found in several tissues), bio-reductive prodrugs can be designed for selective activation under low oxygen conditions typical of many solid tumors. Hypoxia-activated prodrugs (HAP) are designed to target and kill hypoxic cells. The enzymes responsible for the activation of these prodrugs include a variety of specific one-electron and two-electron oxido-reductases (e.g. POR), whose catalysis differs depending on the bioreductive drug class. Inhibition of this process by molecular oxygen imparts specificity for the hypoxic tumor regions and ensures that the bio-reductive drugs exhibit a reduced toxicity to normal tissues. The majority of HAP metabolites lead to DNA damage by interfering with DNA replication.

Among the five identified classes of bioreductive compounds that can undergo enzymatic reduction to active species, several nitro-aromatic derivatives (which also constitute important therapeutic agents against a variety of protozoan and bacterial infections of humans and animals) have been evaluated in clinical trials, including the nitroimidazoles developed for hypoxic cell imaging using immuno-histochemistry or positron emission tomography (PET). Thereafter, reduction of a nitro group (NO₂) to a hydroxylamine (NHOH) or amine (NH₂) moiety has been utilized to design
the hypoxic cytotoxin **PR-104** (in phase II clinical trials), whose cytotoxic metabolites give rise to DNA interstrand cross-links, which can kill tumor cells.\(^{16-17}\)

![Chemical structures](https://example.com/

**Figure 1.** Structures of nitroaromatic HAPs, clinically used CAIs and **SLC-0111** the sulfonamide CA IX/XII inhibitor in Phase I/II clinical trials.

Two isoforms of carbonic anhydrase (CAs, EC 4.2.1.1), CA IX and CA XII, are overexpressed in hypoxic cancers and contribute to tumor physiology by supporting an acidic extracellular microenvironment suited for hypoxic tumor cell survival and proliferation, but detrimental to normal cells.\(^{18-20}\) Particularly, CA IX is normally expressed in few tissues, but is upregulated in many tumor types (e.g. colorectal, breast, brain, etc.) mainly due to its strong transcriptional activation by hypoxia mediated by the HIF-1 transcription factor.\(^{20}\) CA IX and XII have been validated as markers of disease progression in many hypoxic tumors and their targeted inhibition has been associated with a significant reduction of the growth of both primary tumors and metastases.\(^6\) Hence, these CA isoforms have become attractive targets for the design of anti-neoplastic therapies, as recently proposed by several groups. Not surprisingly, CA IX/XII have been the main focus of the last decade research over other CA isoforms.\(^{5,18-20}\)

CA IX and XII are transmembrane, multi-domain proteins whose extracellular catalytic domain is similar to that of the other cytosolic, mitochondrial, secreted or membrane-anchored hCA isoforms.\(^{21,22}\) Ubiquitous cytosolic isoforms CA I and II are the main off-target isoforms when CA
IX and XII are the targets. Primary sulfonamides are the most investigated class of CAIs and have been in clinical use for almost 50 years for the treatment of glaucoma, epilepsy, and as diuretics. Acetazolamide (AAZ) and Dorzolamide (DRZ, Figure 1) are the prototypical first, and second-generation drugs. Their lack of selectivity amongst the human CA isoforms is a major issue in the therapeutic anti-tumor applications of CA IX/XII sulfonamide-based inhibitors, due to the risk of unwanted side effects.

The lead methods that have been applied for the identification of isoform selective sulfonamide-based CAIs are the ring and the tail approach, which respectively consist of modulating a ring (mainly its chemical nature) directly linked to the sulfonamide group and appending different tails to the aromatic/heterocyclic ring present in the scaffold of the CAIs. In particular, this allows the modulation of interactions with the variable active site regions present towards the middle and the edge of the CAs binding sites.

Extensive application of the tail approach, over the last decades, has greatly enriched the database of CAIs, although only a small subset of CA isoform-selective derivatives was found. Linkers of the ureido-type stood out amongst a plethora of available functional groups for ease of preparation, increased water solubility of derivatives and high flexibility of the spacer. For these reasons, incorporation of linkers of the ureido-type have been widely pursued for sulfonamides and their bioisoster sulfamates, leading to several CAIs with selective inhibition profiles for the tumor-associated isoforms.

Among such series of derivatives, SLC-0111, a simple, ureido-substituted benzenesulfonamide successfully completed Phase I clinical trials for the treatment of advanced, metastatic hypoxic tumors over-expressing CA IX and has been scheduled for Phase II trials later last year.

Previous studies have reported hypoxia-activated sulfonamides incorporating disulfide functionalities, as well as a series of 3-nitro-2-substituted benzenesulfonamides, with the aim of designing bio-reductive inhibitors targeting the hypoxia regulated, tumor-associated isozymes, but *in vitro/vivo* studies of these compounds were not reported. Rami et al. reported a series of
nitroimidazoles incorporating sulfonamide-like moieties as radio/chemosensitizing agents targeting the tumor-associated CA IX and CA XII.\textsuperscript{36}

Here we explore the possibility of designing selective CA IX/XII inhibitors of the ureido-substituted benzenesulfonamide-type incorporating a nitro-aromatic moiety to yield a cytotoxic effect increased by hypoxia. The derivatives were obtained from 3-nitro-4-hydroxy-5-aminobenzenesulfonamide that was procured by a selective mono-reduction of the di-nitro precursor. The series of compounds was screened for the inhibition of the physiologically relevant CA isoforms I, II, and the tumor-associated CA IX and XII. X-ray crystallography was employed for rationalizing the CA inhibitory profiles and the adduct of three inhibitors with both CA II and IX/mimic analysed at the molecular level. A hypoxia-enhanced anti-proliferative activity of some such derivatives, albeit not striking, was shown by studying their effects on viability of human colorectal HT-29, breast adenocarcinoma MDA-MB-231, prostate PC-3 cancer cell lines in both normoxic and hypoxic conditions.

\textbf{Result and Discussion}

\textbf{Chemistry}

The derivatives were firstly designed to selectively inhibit CA IX and XII over the cytosolic I and II. Thus, the incorporation of the nitro group should be considered from the structure-activity relationship point of view. The nitro group represents a unique functional group with a variety of chemical and biological actions. Its strong electro-withdrawing nature creates localized or regional electron deficient areas within the molecules.\textsuperscript{37} Appending a nitro group at the benzenesulfonamide scaffold might be considered as an application of the ring approach in that the chemical nature of the main scaffold is influenced. Overall, the acidity of the sulfonamide group is enhanced. In addition, the nitro moiety can participate in both intermolecular and intramolecular hydrogen bonding, increasing the possible interaction points within the binding site pockets.\textsuperscript{37}
Of note, Mori et al. recently reported a series of nitro-benzoic acid as potent and selective CA IX and XII inhibitors, although the carboxy moiety generally has worse CA inhibition properties than the sulfonamide group.\textsuperscript{38}

A structural development of SLC-0111 recently reported by us led to the identification of new promising CA IX/XII selective ureido-CAIs. The compounds, of the A type (Figure 2), exhibit a tail incorporated in \textit{meta} with respect to the sulfonamide moiety and an additional \textit{para}-OH. The latter increases CAs enzymatic selectivity by rotational restriction of precise intramolecular bonds and enhances the derivatives water solubility.\textsuperscript{39} The fluoro-phenyl derivative was investigated \textit{in vivo} using an orthotopic syngeneic breast tumor model that robustly expresses hypoxia-inducible CA IX. It was found to inhibit tumor growth in a dose-dependent manner, reaching levels that matched those observed with SLC-0111 treatment.\textsuperscript{39}

Considering this evidence, the aim was to incorporate a nitro-aromatic moiety in an A type structure to yield additional anti-hypoxic tumor activities.

![Figure 2. Design of 4-hydroxy-3-nitro-5-ureido-benzenesulfonamides.](image)

To nitrate a benzenesulfonamide ring, it is important to consider the reactivity of the aromatic core as well as instability of other groups to the nitration conditions. Sulfonamide rapidly decomposes to sulfonic acid passing through N-nitrosulfonamide, requiring a proper protection that was afforded by using N,N-dimethylformamide diethyl acetal.\textsuperscript{40}
Conversely, protection of aromatic amines with the latter reactant \textsuperscript{41} or acetyl/Boc did not prevent decomposition in the nitrating mixture. As a result, the 3-amino-4-hydroxybenzenesulfonamide could not be used as a starting point in the synthetic strategies.

On the other hand, phenolic moieties are known to exhibit stability towards nitration\textsuperscript{40} making it possible to the synthetic pathway with 4-hydroxybenzenesulfonamide. It should be considered that heating was necessary to achieve a dinitration of the aromatic scaffold since that is impaired by the benzene ring deactivation elicited by strong electron-withdrawing groups, such as the sulfonamide and first forming nitro moiety. For instance, attempts to di-nitrate the unsubstituted benzenesulfonamide crashed with the strong deactivation of the intermediate 3-nitrobenzenesulfonamide.

The synthetic routes planned to afford the main series of ureido-derivatives led to possible deviations from the main synthetic pathway, which were also explored to generate further SAR.

**Scheme 1.** Employed methods to achieve the selective mono-reduction of di-nitro derivative 3 to aminophenol 7.

The key intermediate 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 was obtained by the dinitration of the protected 4-hydroxybenzenesulfonamide 2, followed by the reduction of a unique nitro group with formation of the amino phenol 7 (Scheme 1) and successive sulfonamide deprotection in acidic media. The attempt to directly nitrate the 4-hydroxybenzenesulfonamide 1 in
the sulfonitric mixture led to sulfonamide decomposition to the corresponding di-nitro sulfonic acid. Indeed, we recently reported a similar behavior in the synthesis of N-nitrosulfonamides.40

The mono-nitro derivative 3a was also isolated in low yields and thus deprotected, which was taken into account for the in vitro assays. Diverse reducing agents and reactions conditions were investigated in order to afford the selective mono reduction of derivative 3.42-45 The better, although still low yields, were achieved when Na2S2O4 was used according to the literature method.42 Derivative 3 was also deprotected in acidic media to give the primary sulfonamide 5. The latter was used as the starting material for one of the previously mentioned deviations from the main synthetic pathway. Unfortunately, our aim to obtain a set of di-nitro esters crashed with the rather low stability of most such compounds.46 Uniquely the benzoyl derivative 6 was obtained.

The key intermediate 8 was subjected to diverse functionalizations in addition to the originally planned ureas production.

First, acylation reactions of the amino group did not produce high yields of the planned amide derivatives. As a likely result of the strong acidity of the phenol group and the poor nucleophilicity of the amine, the di-benzoyl compounds 9 and 10 were obtained. The pyridinium salt 11 was prepared by the reaction of 8 with the proper pyrylium compound.47 The light-sensitive derivative 12 was achieved by diazonium salt formation and N2 release in aqueous NaNO2.
Scheme 2. General synthetic procedure of nitrobenzenesulfonamides 4-24.

The main set of compounds, namely ureas 13-24, were prepared by the reaction of 8 with commercially available isocyanates, in addition to the freshly prepared one obtained from 1,3,4,6-tetra-O-acetyl-glucosamine. Thereafter, compound 24 was de-acetylated with a sodium methoxide to give the glycoside 25 (Scheme 3). All derivatives were characterized by 1H-NMR, 19F-NMR 13C-NMR and mass spectrometry.
Of note, the set of ureido-derivatives feature peculiar spectroscopic properties. Indeed, it was not possible to observe any phenolic signal in the $^1$H NMR of ureas 13-24 and their precursors 3, 7 and 8. In addition, ureas 13-24 showed chromatic changes depending on the conditions to which they were subjected. Derivative 8, which appears as a red solid, turned yellow after reaction with different isocyanates. The resulting yellow powder urea turned red again after purification by silica gel chromatography. MS was performed to confirm the derivatives identity was maintained. $^1$H NMR signals of aromatic and exchangeable protons shifted to lower frequencies passing from the yellow to the red solid. Such a behavior could be related to intramolecular movement of electrons and intramolecular H-bond repositioning.

![Scheme 3. Synthesis of derivative 25.](image)

**Carbonic anhydrase inhibition**

The CA inhibitory profiles of compounds 4-6 and 8-25 were evaluated by applying a stopped flow carbon dioxide hydrase assay, in comparison to AAZ as standard CAI, against four physiologically significant isoforms CA I, II, IX and XII. The following SAR can be gathered from the inhibition data shown in Table 1:

<table>
<thead>
<tr>
<th>Compound</th>
<th>CA I</th>
<th>CA II</th>
<th>CA IX</th>
<th>CA XII</th>
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<tr>
<td>4-25</td>
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<td></td>
<td></td>
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<tr>
<td>AAZ</td>
<td></td>
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</table>

**Table 1.** Inhibition data of CA isoforms CA I, II, IX and XII with sulfonamides 4-25 reported here and the standard sulfonamide inhibitor AAZ by a stopped flow CO$_2$ hydrase assay.
(i) The cytosolic and off-target hCA I is the least inhibited isoform by sulfonamides 4-6, 8-25 among those herein screened. Most derivatives act as low micromolar inhibitors, with KIs spanning between 0.69 and 9.29 µM. The pyridinium derivative 11 and urea derivative 19 and 23 did not inhibit CA I up to 50 µM. On the contrary, the pentafluorinated urea derivative 17 is the most efficient hCA I inhibitor with a Ki value of 0.69 µM. The simplest nitro compound 4 was also found to exhibit a sub-micromolar inhibitory efficiency. The remaining derivatives inhibit CA I in a rather narrow range that do not allow to compile further SAR.

(ii) Most nitro compounds inhibit the ubiquitous, off-target isoform CA II in a medium nanomolar to low micromolar range that spans from 0.18 to 5.15 µM. Among the non-ureido derivatives, the pyridinium 11 acts as micromolar CA II inhibitor with a Ki of 1.81µM. The substitution pattern of

<table>
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<tr>
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<td>0.10</td>
<td>0.21</td>
</tr>
<tr>
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<td>1.81</td>
<td>0.15</td>
<td>0.22</td>
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<td>0.64</td>
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<tr>
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<td>0.86</td>
<td>0.17</td>
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<td>0.012</td>
<td>0.025</td>
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* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5% of the reported values).
the ureido moiety actively affects the CA II inhibition profiles within the urea subset. Incorporation of a methylene or ethylene spacer between the ureido-linker and the aromatic portion lowers the inhibitory efficiency of 22 and 23 (K\textsubscript{Is} of 3.08 and 2.53 µM) with respect to compounds that bear a directly linked aromatic tail. Acetylation of the alcoholic groups on the glycosidic tail of 25 decreases the inhibition from 0.86 to 1.90 µM (24). Most evaluated substitution patterns at the ureidophenyl ring enhanced the enzymatic inhibition. Exceptions are compounds 18 and 19 that incorporate a 3-MeO or 3,4-(methylenedioxy) moiety, whose K\textsubscript{Is} decreased from 2.78 (13) to 5.15 and 4.30 µM, respectively. The incorporation of fluorine atoms (14, 16, 17) or trifluoromethyl groups (15, 21) on the benzene ring favoured CA II inhibition (K\textsubscript{Is} of 0.20-0.53 µM) when compared to the unsubstituted 13.

(iii) The tumor-associated isozymes CA IX and XII are the most efficiently inhibited by the reported nitro compounds, since all measured K\textsubscript{Is} ranged between low to medium nanomolar range (0.10-0.94 µM). CA IX was found to be potently inhibited by all non-ureido derivatives, among which the di-nitrobenzoyl 6 exhibits a 95 nM K\textsubscript{i}, representing the most active CA IX inhibitor herein reported. Likewise, its dinitro precursor 5 as well as the di-4-F-benzoylated nitro derivative 10 were measured to act as comparably potent inhibitors with K\textsubscript{Is} of 0.10 µM. Again, the substitution pattern of the ureido moiety actively elicits the inhibitory trend spanning in the range 0.11-0.94 µM. The latter value belongs to derivative 13 that incorporates an unsubstituted, directly linked phenyl portion. The most favorable modifications in terms of CA IX inhibition include the separation of the phenyl ring from the urea by an ethylene spacer (22, K\textsubscript{i} of 0.11 µM), the incorporation of a 3,4-(methylenedioxy) moiety at the benzene ring (19, K\textsubscript{i} of 0.15 µM), or its replacement with a glycosidic portion (25, K\textsubscript{i} of 0.17 µM). Acetylation of the alcoholic groups in the glycosidic tail lowers CA IX inhibition by 1.7-fold. Inclusion of a trifluoromethyl group at the \textit{para} position of the phenyl moiety (15) was the least efficient substitution among those evaluated (K\textsubscript{i} of 0.63 µM).
(iv) The target CA XII was potently inhibited by nitro derivatives 4-6 and 8-25, with KIs ranging between 0.08 and 0.95 µM. As for the previous isoform, non-ureido derivatives 4-6, 8-12 generally afforded the most efficient inhibition. The presence of two nitro groups in 5 and its benzyolated derivatives 6 appeared to be favourable towards CA XII inhibition eliciting the lowest KIs herein reported, 76 and 72 nM respectively. Of note, the catechol derivative 12 was also shown to act as a strong CA XII inhibitor, with a KI of 93 nM. CA XII features a region rich in Thr and Ser residues at the edge of the active site. 22 H-bonded interactions taking place between such residues and nitro and/or hydroxy moieties of 5, 6 and 12 could ease the formation of the ligand-target adduct and likely justify the observed inhibition profiles. Ureido-derivatives 13-25 showed KI inhibition values slightly higher and ranging between 0.08 and 0.95 µM when compared to compounds 4-12. It is worth highlighting that the most efficient derivatives within this subset incorporate a pentafluorobenzene (17, KI of 82 nM) and a glycosidic portion (25, KI of 160 nM) on the ureido moiety. Accordingly, both portions were able to establish most of H-bonds contacts with the enzymatic counterpart. Conversely, other substitutions did not significantly affect the CA XII inhibition profile with respect to the unsubstituted, directly linked phenyl-bearing derivative 13 (KI of 0.83). For instance, ureidophenyl compounds 15, 16, 20 and the O-acetylglycoside 24 inhibit CA XII in a comparable manner to 13 (KIs in the range 0.79-0.95 µM). Remarkable inhibition is also shown by compound 16, featuring the 3,4-(methylenedioxy) moiety at the benzene ring (KI of 0.25 µM) and 22, which incorporates an ethylene spacer between the urea and the aromatic tail (KI of 0.31 µM).

(v) The present inhibitory profiles differ by those previously reported for ureido-tailed benzenesulfonamides. 28-32 In fact, the data in Table 1 clearly report weaker inhibitions of all the considered isoforms. Though the nitro group at the meta-position of the benzene sulfonamide scaffold appears to be detrimental for CA inhibition, several derivatives maintain nanomolar efficacy and show interesting selective inhibition profiles depending on the substitution pattern.
The unsubstituted, directly linked phenyl-bearing derivative 13 exhibits a 3-fold selective action for CA IX/XII (Kis of 0.94 and 0.83 µM) over CA II (Ki of 2.78 µM) and much greater over CA I (Ki > 50 µM). Fluorination of the ureido-aromatic portion reduces the selectivity of 14-17 for the tumor-associated isoforms by enhancing their CA I/II inhibitory potency (CA II: Kis in the range 0.20-0.53 µM). As an exception, compound 21, that bears a 3,5-di-CF3-phenyl substituent showed an increased 5-fold CA IX/XII selectivity over II. Incorporation of methyl groups at the same positions of the aromatic ring increases the target promiscuity of derivative 20. The selectivity for the tumor-associated isozymes also increased by appending 3-MeO (18) or 3,4-methylenedioxy (19) moieties at the aromatic portion, with the CA IX/II selectivity index (SI) of 19 reaching the value of 30. The detachment of the aromatic ring from the ureido linker by a methylene or ethylene spacer enhances again the preferential efficacy of 22 and 23 for CA IX and XII by 10-fold when the SI are compared to those of derivative 13. The acetylation of the glycoside alcoholic groups of 25 markedly enhanced the inhibition of the tumor-associated isoforms in comparison to hCA II.

**X-ray crystallography**

![Figure 3](image)

**Figure 3.** Crystal structures of CA II (gray) in complex with inhibitors **A)** 15 (pink), **B)** 16 (green) and **C)** 17 (cyan). Hydrogen bonds (2.5 - 3.5 Å) are depicted as black dotted lines. The zinc is shown as a magenta sphere.
The X-ray crystal structures of CA II and CA IX/mimic were determined in complex with inhibitors 15, 16 and 17 (Figures 3 and 4). All six datasets had overall redundancies in the range of 2.4-3.9 with completeness above 90% (Table 1). Of note, the $R_{pim}$ values were consistently higher in the CA II structures in comparison to the CA IX/mimic structures. Also, the resolution was consistently higher in the CA IX/mimic structures. The quality of resulting electron density for the compounds were similar in CA II and CA IX/mimic (Supplementary Figure S1). Interestingly, a single inhibitor molecule was bound in the active site of each CA II structure (Site I) whereas additional inhibitors (Sites II and III) were observed to bind around the CA IX/mimic active site (Supplementary Figure S2).

All three inhibitors (Site I) were observed to bind directly to the active site zinc through the N1 atom of the sulfonamide group (~2.0 Å), displacing the catalytic zinc-bound solvent (Figures 3 and 4). As previously observed in similar CA sulfonamide complexes, additional hydrogen bonds from T199 N and OG to the sulfonamide oxygen atoms were present. An unusual orientation of the benzene ring was observed with respect to most of the previously observed CA-benzenesulfonamide adducts. (Supplementary Figure S3). Indeed, the presence of the nitro group in position 3 of the scaffold elicits a torsion to the ligand that enables conservation of the coordination, but impairs hydrophobic and π-π interactions with the aromatic residues nearby. The nitro group protrudes toward H64, forming H-bonds with residues in the hydrophilic side of the cavity. Such a deviation from the usual aromatic ring position can justify the generally reduced CA inhibition profiles reported in Table 1 when compared to previously reported ureido-substituted benzenesulfonamides, such as SLC-0111.

The orientation of the tails extending out of the active site differed between the three compounds (Figures 3 and 4). In CA II, inhibitors 15 and 17 were observed to bind in the hydrophobic region, whereas 16 oriented toward the interface of the hydrophobic and hydrophilic regions of the active site. Conversely, inhibitors 17 and 16 bound in the hydrophobic region and 15
was bound at the hydrophobic/hydrophilic interface of CA IX/mimic (Figure 4A-C). Multiple inhibitors of 15 and 17 were bound in the CA IX/mimic active site (Supplementary Figure S2).

Figure 4. Crystal structures of CA IX/mimic (cyan) in complex with inhibitors A) 15 (pink), B) 16 (green) and C) 17 (cyan). Hydrogen bonds (2.5 - 3.5 Å) are depicted as black dotted lines. The zinc and solvent are shown as magenta and red spheres, respectively.

For inhibitor 15 bound in CA II (site I), additional hydrogen bonds were observed between Q92 and the amine group of the linker (3.4 Å), and between N67 and N62 and the nitro oxygen of the inhibitor (3.0 and 3.1 Å, respectively). In CA IX/mimic, hydrogen bonds existed between Q67 and Q92 and the carboxyl group of the linker (3.3 and 2.6 Å, respectively), Q67 and the hydroxyl group of inhibitor (2.8 Å), and N62 and the hydroxyl and oxygen of NO2 (3.5 and 2.9 Å, respectively). Additional van der Waal interactions with residues F131, V135, and L198 in CA II and S65, V131 and L198 in CA IX/mimic further stabilize the tail of the compound (Fig 4A-B). The surface area covered by 15 was 326 Å in CA II and 296 Å in CA IX/mimic. Fewer van der Waals interactions are expected in CA IX/mimic due to the smaller inhibitor-bound surface area, likely caused by the F131V variation, contributing to the 3-fold selectivity of 15 for CA II over CA IX (Table 1).

For inhibitor 17 bound in CA II (site I), hydrogen bond interactions were observed between Q92 and the hydroxyl group of the inhibitor (3.4 Å), N67 and N62 and the nitro oxygen of the
inhibitor (3.5 and 3.2 Å, respectively). In CA IX/mimic, interactions existed between Q67 and the inhibitor hydroxyl group (2.7 Å) and N62 and nitro oxygen of the inhibitor (2.9 Å). Van der Waal interactions were observed between the inhibitor and L198 in CA II and V131, V135, L198 and P202 in CA IX/mimic (Fig 4C-D). The tail was observed to flip approximately 90° in CA II to accommodate the steric hindrance by F131. The surface area covered by the ligand in the active site of CA II was 353 Å² and 330 Å² in CA IX/mimic. The increased number of van der Waals interactions observed in CA IX/mimic were balanced by the greater number of hydrogen bonds in CA II, suggesting the similar affinities of 17 for both CA II and CA IX/mimic (Table 1).

For inhibitor 16 bound in CA II (site I), hydrogen bond exists between Q92 and the amine group of the linker (2.8 Å) and T200 and nitro oxygen of the inhibitor (3.3 Å). In CA IX/mimic, these interactions were observed between N62 and nitro oxygen of 16 (2.8 Å). Van der Waal interactions existed between 16 and Q69, F131 and L198 in CA II and L198 in CA IX/mimic. The surface area covered by 16 was 373 Å² and 358 Å² in CA II and CA IX/mimic, respectively. The greater number of van der Waals interactions and hydrogen bonds observed in CA II support the 2-fold selectivity of 16 for CA II over CA IX/mimic (Table 1).

Table 2: Crystallographic statistics

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$R_{\text{rim}}$ - the precision of the averaged intensity measurements which gives the standard error of the mean.

$R_{\text{sym}}$ - measure of agreement among the independent measurements of symmetry-related reflections in a crystallographic data set.

$R_{\text{cryst}}$ - measures the agreement between the model and the observed data.

**Cytotoxicity Assay**

Several compounds that exhibited the best inhibitory profiles within the ureas series (14, 17, 19, 21, 22, 24) and the non-ureido subset (5, 8, 10, 11) were chosen (30-200 µM) to evaluate their effects on the viability of human colon cancer HT-29 cells, breast adenocarcinoma MDA-MB-231 cells and prostatic cancer PC-3 cells via MTT assay. The efficacy of the compounds over 48h is summarized in Figure 6 (and Table S1-3 in ESI). The untreated control showed 100% viability. The effect of SLC-0111 on viability of the three cell lines is also reported (Table S4 in ESI).
Figure 6. (A) HT-29, (B) MDA-MB-231, (C) PC-3 cells (1x10⁴/well) were treated with compounds 5, 8, 10, 11, 14, 17, 19, 21, 22, 24 (30 - 200 μM). Incubation was allowed for 48 h in normoxic (20% O₂) and hypoxic conditions (1% O₂). Control condition was arbitrarily set as 100% and values are expressed as the mean ± S.E.M. of three experiments. *P<0.05, **P<0.01 and ***P<0.001 in comparison to control (0 μM, not shown).

The greatest anti-proliferative action was displayed after an incubation of 48h. Short incubation time (16h) with the compounds were tested for HT-29 cell lines and are reported in ESI (Figure S4). Although partially, hypoxia enhanced the efficacy of many of the screened compounds in reducing cancer cell viability depending on the concentration and type of cell line. It is worth stressing that other CAIs, such as sulfocoumarins, 3-hydroxyquinazoline-2,4-diones, selenoureido-benzensulfonamides, chromeno[4,3-c]pyrazol-4-ones and nitrogenous base-bearing benzenesulfonamides did not show hypoxia-enhanced anti-proliferative efficacy in vitro,
although they exhibited potent inhibitory action against the hypoxia-induced CA IX and CA XII. It could be speculated that the brief time that cells spend in low oxygen conditions does not produce sufficient CA IX/XII overexpression with respect to cells treated in normoxic conditions. However, this evidence validates the herein reported data. As a result, the increase of cytotoxicity of nitrobenzenesulfonamides in hypoxic conditions likely shows the introduction of alternative mechanism of action, such as prodrugs activation.

In detail, of the ten assayed nitro-aromatics, six compounds exhibited hypoxia-increased cytotoxic effects against HT-29 cells, eight against MDA-MB-231 cells, and four against PC-3 cells in a concentration-dependent manner (Figure 6, Table S1-3 in ESI). Among the non-ureido derivatives, the di-(4-F-benzoyl) derivative 10 was the best anti-proliferative agent, reducing the number of living cells up to 6% in HT-29, 13% in MDA-MB-231 and 8% in PC-3 cell lines. Whereas this strong effect resulted not to be related to hypoxia in HT-29 and PC-3 cells, a strong hypoxic activation arose with breast cancer cells. Interestingly, the pyridinium salt 11 showed a better in vitro efficacy in normoxic conditions with PC-3 cells and mostly with MDA-MB-231 ones, reducing the viability of the culture up to 40% at 30 µM and 3% at 100 µM. These results are in agreement with the behavior of pyridinium-bearing benzenesulfonamides, whose permanent positive charge does not allow sufficient crossing of the membrane. Precursors 5 and 8 do not shown remarkable effects.

The di-3,5-CF₃-phenyl derivative 21 exhibits the best anti-proliferative profile among the tested ureas. Its efficacy against MDA-MB-231 cell is 4-fold enhanced by hypoxia at 100 µM, whereas it is equal in normoxic and hypoxic conditions for HT-29 and PC-3 cells. The 3,4-(methylenedioxy)phenylurea 19 proved to be more active against HT-29 in hypoxic conditions in comparison to normoxic. Most remaining ureido compounds demonstrated weak to medium cytotoxic effects increased by hypoxia. The hypothesis of bio-reductive pathways undergone by the nitrobenzenesulfonamides is partially supported by the reported data. Literature data would suggest that hydroxilamine and nitroso derivatives are the most likely produced cytotoxic species.
Nevertheless, further studies are currently ongoing to properly typify the nature of the cytotoxins as well as the activation mechanisms taking place in hypoxic conditions.

Conclusions

Hypoxia promotes a multitude of effects on tumor biology. Overexpression of CA IX and CA XII aids the tumor cells to maintain a suitable intra/extracellular pH for cancer cell survival and growth. Hypoxia also furthers the activity of specific one-electron and two-electron oxido-reductases, that may be exploited to achieve a selective bio-reductive prodrugs activation to cytotoxin. Herein, we designed new selective CA IX/XII inhibitors, as analogues of the anti-tumor phase II entering drug SLC-0111. Derivatives of the ureido-benzenesulfonamide-type were appended with a nitro-aromatic moiety to yield an anti-proliferative action increased by hypoxia. Several such derivatives display nanomolar efficacy and show interesting CA IX/XII selective inhibition profiles over the off-target CA I/II. X-ray crystallography of the adduct of three inhibitors with both CA II and IX/mimic rationalized the compounds generally worsened inhibitory profiles with respect to previously reported ureido-benzenesulfonamides. The effects of some selected compounds on the viability of human colorectal HT-29, breast adenocarcinoma MDA-MB-231, prostate PC-3 cancer cell lines have been evaluated in both normoxic and hypoxic conditions. It can be noted that hypoxia has a low to medium effect on the efficacy of many screened compounds in reducing cancer cells viability depending on the concentration and type of cell line. Since other CAIs, such as such as AAZ, SLC-0111, sulfocoumarins, 3-hydroxyquinazoline-2,4-diones, selenoureido-benzensulfonamides, chromeno[4,3-c]pyrazol-4-ones and nitrogenous base-bearing benzenesulfonamides, did not show hypoxia-enhanced anti-proliferative efficacy in vitro in spite of a potent inhibitory action against the hypoxia-induced CA IX and CA XII, the herein reported data support evidence of a hypoxia-strengthened cytotoxic action by nitrobenzenesulfonamides. The present study provided the initiation towards the development of hypoxia-activated anti-tumor CAIs. Further experiments are currently ongoing to prove that a bioreduction process is taking
place. New nitro-derivatives have been designed which incorporate the tail in para position of the benzenesulfonamide scaffold to overcome the steric hindrance of the nitro moiety and yield a greater CA IX/XII inhibition and as a result, more marked in vitro cytotoxicity.

Experimental protocols

Chemistry

Anhydrous solvents and all reagents were purchased from Sigma-Aldrich, Fluorochem and TCI. 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, \( ^1\)F-NMR, \( ^13\)C-NMR) spectra were recorded using a Bruker Advance III 400 MHz spectrometer 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, quartet; m, multiplet; brs, broad singlet; dd, doublet of doublets. The assignment of exchangeable protons (OH and NH) was confirmed by the addition of D\(_2\)O. Analytical thin-layer chromatography (TLC) was carried out on Sigma silica gel F-254 plates. Flash chromatography purifications were performed on Sigma Silica gel 60 (230-400 mesh ASTM) as the stationary phase and MeOH/DCM were used as eluents. Melting points (mp) were measured in open capillary tubes with a Gallenkamp MPD350.BM3.5 apparatus and are uncorrected. The HPLC analysis was performed by using an Agilent 1200 Series equipped by autosampler, binary pump system and diode array detector (DAD). The column used was a Luna PFP 30 mm length, 2 mm internal diameter and 3 \( \mu \)m particle size (Phenomenex, Bologna, Italy) at constant flow of 0.25 mL min\(^{-1} \), employing a binary mobile phase elution gradient. The solvents used were 10 mM formic acid and 5 mM ammonium formate in mQ water solution (solvent A) and 10 mM formic acid and 5 mM ammonium formate in methanol (solvent B) according to the elution gradient as follow: initial at 90% solvent A, which was then decreased to 10% in 8 min, kept for 3 min,
returned to initial conditions in 0.1 min and maintained for 3 min for reconditioning, to a total run time of 14 min. The stock solution of each analyte was prepared in methanol at 1.0 mg mL\(^{-1}\) and stored at 4 °C. The sample solution of the analyte was freshly prepared by diluting its stock solution up to a concentration of 10 μg mL\(^{-1}\) in mixture of mQ water:methanol 50:50 (v/v) and 5 μL were injected in the HPLC system. The chromatographic profiles of each analyte (ESI p. S24) were monitored at 230 nm, that represents the maximum UV absorption for these compounds. All compounds reported were >95% HPLC pure. The solvents used in HPLC measures were methanol (Chromasolv grade), purchased from Sigma-Aldrich (Milan - Italy), and mQ water 18 MΩ, obtained from Millipore's Simplicity system (Milan-Italy). High resolution mass spectrometry (HR-MS) analysis were performed with a Thermo Finnigan LTQ Orbitrap mass spectrometer equipped with an electrospray ionization source (ESI). Analysis were carried out in negative ion mode monitoring the [M-H]\(^{-}\) species, and it was used a proper dwell time acquisition to achieve 60,000 units of resolution at Full Width at Half Maximum (FWHM). Elemental composition of compounds were calculated on the basis of their measured accurate masses, accepting only results with an attribution error less than 5 ppm and a not integer RDB (double bond/ring equivalents) value, in order to consider only the protonated species.\(^{55}\) None of the screened derivatives reported PAINS alerts determined by SwissADME server (www.swissadme.ch).

**N,N-dimethylaminomethylene-4-hydroxy-benzenesulfonamide (2).**\(^{56}\)

N,N-Dimethylformamide dimethyl acetal (1.2 eq) was added to a solution of compound 4-hydroxybenzenesulfonamide 1 (2.0 g, 1.0 eq) in DMF (1.5 ml) at 0°C and that was stirred at r.t. for 0.25h. The reaction mixture was quenched with EtOAc (40 ml) and the formed precipitate was filtered and purified by silica gel column chromatography eluting with MeOH/DCM 5% to afford compound 2 as a yellow solid. 76% yield; silica gel TLC \(R_f\) 0.22 (MeOH/DCM 5 % v/v); \(\delta_H\) (400 MHz, DMSO-\(d_6\)): 2.92 (s, 3H, CH3), 3.16 (s, 3H, CH3), 6.88 (d, \(J = 8.4. 2H\)), 7.61 (d, \(J = 8.4. 2H\)),


8.18 (s, 1H), 10.29 (bs, 1H, exchange with D₂O, OH); δC (100 MHz, DMSO-d₆): 35.9, 41.7, 116.2, 129.1, 134.2, 160.3, 161.3.

N,N-dimethylaminomethylene-4-hydroxy-3,5-dinitro-benzenesulfonamide (3) and N,N-dimethylaminomethylene-4-hydroxy-3-nitro-benzenesulfonamide (3a).

N,N-Dimethylaminomethylene-4-hydroxy-benzenesulfonamide (1.0 g, 1.0 eq) was added portion wise to a mixture of concentrated H₂SO₄ (2.2 mL) and fuming HNO₃ (1.6 mL) at 0°C and the obtained solution was stirred at 40°C for 4h. The reaction mixture was cooled and quenched with slush (40 ml) and the formed precipitate was filtered-off, dried under vacuo and purified by silica gel column chromatography eluting with MeOH/EtOAc from 5 to 10% to afford the titled compounds 3 and 3a as yellow solids. (3) 72% yield; m.p. >300°C; silica gel TLC Rf 0.46 (MeOH/DCM 20 % v/v); δH (400 MHz, DMSO-d₆): 2.95 (s, 3H, CH₃), 3.17 (s, 3H, CH₃), 8.22 (s, 1H), 8.29 (s, 2H); δC (100 MHz, DMSO-d₆): 36.1, 42.0, 128.1, 128.5, 141.8, 152.9, 161.1.

(3a) 4% yield; m.p. 195-198°C; silica gel TLC Rf 0.60 (MeOH/DCM 5 % v/v); δH (400 MHz, DMSO-d₆): 2.95 (s, 3H, CH₃), 3.18 (s, 3H, CH₃), 7.27 (d, J = 8.6, 1H), 7.92 (dd, J = 2.4, 8.6, 1H), 8.24 (d, J = 2.4, 1H), 8.24 (s, 1H), 11.94 (bs, 1H, exchange with D₂O, OH); δC (100 MHz, DMSO-d₆): 36.1, 41.9, 120.7, 124.4, 133.2, 134.6, 137.3, 155.4, 160.9.

4-Hydroxy-3-nitro-benzenesulfonamide (4).

A suspension of N,N-dimethylaminomethylene-4-hydroxy-3-nitro-benzenesulfonamide 3a (0.04 g, 1.0 eq) in MeOH (3 ml) was treated with HCl 12M (0.5 ml) and stirred at 90°C for 6h. The reaction mixture was concentrated in vacuo, treated with H₂O (5 ml) and extracted with EtOAc (3x10 ml). The combined organic layers were dried over Na₂SO₄, filtered-off and concentrated in vacuo to give the titled compound 4. 98% yield; m.p. 201-203°C; silica gel TLC Rf 0.26 (MeOH/DCM 5 % v/v); δH (400 MHz, DMSO-d₆): 7.31 (d, J = 8.6, 1H), 7.47 (s, 2H, exchange with D₂O, SO₂NH₂), 7.96 (dd, J = 2.4, 8.6, 1H), 8.32 (d, J = 2.4, 1H), 12.01 (bs, 1H, exchange with D₂O, OH); δC (100 MHz,
4-Hydroxy-3,5-dinitro-benzenesulfonamide (5).

A suspension of N,N-dimethylaminomethylene-4-hydroxy-3,5-dinitro-benzenesulfonamide 3 (0.8 g, 1.0 eq) in MeOH (20 ml) was treated with HCl 12M (4 ml) and stirred at 90°C for 5h. The reaction mixture was concentrated in vacuo, treated with H2O (20 ml) and extracted with EtOAc (3x20 ml). The combined organic layers were dried over Na2SO4, filtered-off and concentrated under vacuo to give a residue that was purified by silica gel column chromatography eluting with MeOH/DCM 10% to afford the titled compound 5 as a yellow solid. 95% yield; m.p. >300°C; silica gel TLC Rf 0.23 (MeOH/DCM 20 % v/v); δH (400 MHz, DMSO-d6): 7.14 (s, 2H, exchange with D2O, SO2NH2), 8.19 (s, 2H); δC (100 MHz, DMSO-d6): 118.6, 128.8, 143.2, 160.7; ESI-HRMS (m/z) [M-H]-: calculated for C6H4N3O7S 261.9765; found 261.9760.

Benzoic acid 2,6-dinitro-4-sulfamoyl-phenyl ester (6).

Benzoyl chloride (1.5 eq) was added dropwise to a solution of 4-hydroxy-3,5-dinitro-benzenesulfonamide 5 (0.1 g, 1.0 eq) in CH3CN (4 ml) under a nitrogen atmosphere and that was stirred at 90°C for 5h. The reaction mixture was concentrated in vacuo and the obtained residue was purified by silica gel column chromatography eluting with EtOAc/Hexane 60% to afford the titled compound 6 as a white solid. 43% yield; m.p. 202-203°C; silica gel TLC Rf 0.28 (EtOAc/n-hexane 40 % v/v); δH (400 MHz, MeOD-d4): 7.63 (t, J = 7.8, 2H), 7.79 (t, J = 7.8, 1H), 8.18 (d, J = 7.8, 2H), 8.89 (s, 2H); δC (100 MHz, MeOD-d4): 127.5, 127.8, 129.3, 130.9, 135.3, 140.9, 143.9, 144.3, 163.3; ESI-HRMS (m/z) [M-H]-: calculated for C13H8N3O8S 366.0027; found 366.0029.

N’-((3-Amino-4-hydroxy-5-nitrophenyl)sulfonyl)-N,N-dimethylformimidamide (7).
Sodium hydrosulfite (3.0 eq) was added portion wise to a suspension of N,N-dimethylaminomethylene-4-hydroxy-3,5-dinitro-benzenesulfonamide 3 (0.5g, 1.0 eq) in H$_2$O/MeOH ¼ (12 ml). The reaction mixture was stirred at r.t. for 4h, quenched with HCl 0.5M (25 ml) and extracted with EtOAc (3x30 ml). The organic layers were dried over Na$_2$SO$_4$, filtered-off and concentrated under vacuo to give a residue that was purified by silica gel column chromatography eluting with MeOH/DCM 3% to afford the titled compound 7 as a red solid. 47% yield; m.p. 222-225°C; silica gel TLC $R_f$ 0.32 (MeOH/DCM 10 % v/v); $\delta$H (400 MHz, DMSO-$d_6$): 2.95 (s, 3H, CH$_3$), 3.18 (s, 3H, CH$_3$), 7.29 (d, $J$ = 8.4, 1H), 7.40 (bs, 2H, exchange with D$_2$O, NH$_2$, overlap with signal at 7.29 and 7.50), 7.50 (dd, $J$ = 2.4, 8.8, 1H), 8.19 (s, 1H); $\delta$C (100 MHz, DMSO-$d_6$): 36.0, 41.8, 109.6, 114.9, 135.1, 135.6, 141.94, 143.4, 160.5; ESI-HRMS (m/z) [M-H]: calculated for C$_9$H$_{11}$N$_4$O$_5$S 287.0445; found 287.0448.

3-Amino-4-hydroxy-5-nitro-benzenesulfonamide (8).

A suspension of 3-amino-N,N-dimethylaminomethylene-4-hydroxy-5-nitro-benzenesulfonamide 7 (0.4 g, 1.0 eq) in MeOH (10 ml) was treated with HCl 12M (2 ml) and stirred at 90°C for 8h. The reaction mixture was concentrated in vacuo, treated with H$_2$O (20 ml) and extracted with EtOAc (3x20 ml). The combined organic layers were dried over Na$_2$SO$_4$, filtered-off and concentrated under vacuo to give a residue that was purified by silica gel column chromatography eluting with MeOH/DCM 10% to afford compound 8 as a red solid. 95% yield; m.p. 232-234°C; silica gel TLC $R_f$ 0.11 (MeOH/DCM 5 % v/v); $\delta$H (400 MHz, DMSO-$d_6$): 7.30 (d, $J$ = 1.8, 1H), 7.34 (s, 2H, exchange with D$_2$O, SO$_2$NH$_2$) 7.60 (dd, $J$ = 1.8, 1H); $\delta$C (100 MHz, DMSO-$d_6$): 109.7, 114.0, 135.4, 135.6 142.2, 144.3; ESI-HRMS (m/z) [M-H]: calculated for C$_6$H$_6$N$_3$O$_5$S 232.0023; found 232.0026.

**General synthetic procedure of benzoic acid 2-benzoylamino-6-nitro-4-sulfamoyl-phenyl esters 9-10.**
The proper benzoyl chloride (1.5 eq) was added dropwise to a solution of 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) in dry pyridine (1.5 ml) under a nitrogen atmosphere and that was stirred at r.t. for 0.5h. The reaction mixture was quenched with 1M HCl (10 ml) and extracted with EtOAc (2x15 ml) The organic layers were dried over Na2SO4, filtered-off and concentrated under vacuo to give a residue that was purified by silica gel column chromatography eluting with EtOAc/n-hexane 50% to afford the titled compounds 9-10.

**Benzoic acid 2-benzyloamino-6-nitro-4-sulfamoyl-phenyl ester (9).**

Benzoic acid 2-benzyloamino-6-nitro-4-sulfamoyl-phenyl ester 9 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and benzoyl chloride (1.5 eq) in dry pyridine. 53% yield; m.p. 216-217°C; silica gel TLC \( R_f \): 0.72 (MeOH/DCM 20 % v/v); \( \delta_H \) (400 MHz, DMSO-\( d_6 \)): 7.53 (t, \( J = 7.2 \), 2H), 7.63 (m, 3H), 7.81 (t, \( J = 7.2 \), 1H), 7.86 (s, 2H, exchange with D\(_2\)O, SO\(_2\)NH\(_2\)), 7.90 (d, \( J = 7.2 \), 2H), 8.17 (d, \( J = 7.2 \), 2H), 8.46 (d, \( J = 2.4 \), 1H), 8.66 (d, \( J = 2.4 \), 1H), 10.71 (s, 1H, exchange with D\(_2\)O, N\( H \)CO); \( \delta_C \) (100 MHz, DMSO-\( d_6 \)): 120.2, 128.7, 128.8, 128.9, 129.5, 130.1, 131.1, 133.2, 134.4, 134.9, 135.8, 139.8, 142.9, 143.5, 163.7, 167.12; ESI-HRMS (m/z) [M-H]: calculated for C\(_{20}\)H\(_{14}\)N\(_3\)O\(_7\)S 440.0547; found 440.0540.

**4-F-Benzoic acid 2-(4-F-benzoyl)amino-6-nitro-4-sulfamoyl-phenyl ester (10).**

4-F-Benzoic acid 2-(4-F-benzoyl)amino-6-nitro-4-sulfamoyl-phenyl ester 10 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and 4-F-benzoyl chloride (1.5 eq) in dry pyridine. 41% yield; m.p. 217-220°C; silica gel TLC \( R_f \) (EtOAc/n-hexane 50 % v/v); \( \delta_H \) (400 MHz, DMSO-\( d_6 \)): 7.39 (t, \( J = 8.8 \), 2H), 7.48 (t, \( J = 8.8 \), 2H), 7.86 (s, 2H, exchange with D\(_2\)O, SO\(_2\)NH\(_2\)), 7.98 (m, 2H), 8.25 (m, 2H), 8.46 (d, \( J = 2.4 \), 1H), 8.66 (d, \( J = 2.4 \), 1H), 10.72 (s, 1H, exchange with D\(_2\)O, N\( H \)CO); \( \delta_F \) (376 MHz, DMSO-\( d_6 \)): -107.47 (s, 1F), -103.07 (s, 1F);\( \delta_C \) (100 MHz, DMSO-\( d_6 \)): 116.6 (d, \( J^{\text{CF}} = 22.0 \),
117.4 (d, J^2 CF = 22.2), 120.3, 125.3, 129.0, 130.8, 131.6 (d, J^3 CF = 9.2), 134.2 (d, J^4 CF = 9.9),
134.8, 139.7, 142.9, 143.4, 162.8, 165.4 (d, J^1 CF = 248.8), 166.1, 166.9 (d, J^1 CF = 252.3); ESI-
HRMS (m/z) [M-H]: calculated for C_{20}H_{12}F_{2}N_{3}O_{7}S 476.0359; found 476.0362.

1-(2-Hydroxy-3-nitro-5-sulfamoyl-phenyl)-2,4,6-trimethyl-pyridinium, perchlorate salt (11).
2,4,6-Trimethyl-pyridinium, tetrafluoroborate salt (1.2 eq) was added to a solution of 3-amino-4-
hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) in dry MeOH (4 ml) under a nitrogen
atmosphere and that was stirred at 70°C overnight. The reaction mixture was quenched with H_2O
(10 ml) and treated with 1M NaClO_4 aqueous solution (3 eq). The formed precipitate was filtered
and purified by silica gel column chromatography eluting with MeOH/DCM 15% to afford the
titled compounds 11. 53% yield; m.p. 230°C dec.; silica gel TLC R_f 0.05 (MeOH/DCM 10 % v/v);
δ_H (400 MHz, DMSO-d_6): 2.39 (s, 6H, 2 x CH_3), 2.61 (s, 3H, CH_3), 7.10 (s, 2H, exchange with
D_2O, SO_2NH_2), 7.83 (d, J = 2.4, 1H), 7.89 (s, 2H), 8.41 (d, J = 2.4, 1H); δ_C (100 MHz, DMSO-d_6):
21.7, 22.2, 120.7, 127.9, 128.7, 129.4, 134.5, 138.7, 156.2, 159.5, 161.6; ESI-HRMS (m/z) [M-H]:
calculated for C_{14}H_{15}ClN_{3}O_{9}S 436.0212; found 436.0208.

3,4-Dihydroxy-5-nitro-benzenesulfonamide (12).
NaNO_2 (1.2 eq) was added portion wise to a solution of 3-amino-4-hydroxy-5-nitro-
benzenesulfonamide 8 ( 0.06 g, 1.0 eq) in HCl 2M (4 ml) at 0°C and that was stirred at r.t. for 15
min. The reaction mixture was extracted with EtOAc (3x10), the organic layers were dried over
Na_2SO_4, filtered-off and concentrated under vacuo to give a residue that was purified by silica gel
column chromatography eluting with MeOH/DCM 15% to afford the titled compound 12 as a
brown solid. 60% yield; m.p. 200°C dec.; silica gel TLC R_f 0.21 (MeOH/DCM 10 % v/v); δ_H (400
MHz, DMSO-d_6): 7.56 (s, 2H, exchange with D_2O, SO_2NH_2) 8.59 (d, J = 2.6, 1H), 8.71 (d, J = 2.6,
1H); δ_C (100 MHz, DMSO-d_6): 98.3, 125.6, 137.4, 135.7, 142.6, 165.7; ESI-HRMS (m/z) [M-H]:
calculated for C_{6}H_{5}N_{2}O_{6}S 232.9863; found 232.9861.
General synthetic procedure of 4-hydroxy-3-nitro-5-ureido-benzenesulfonamides 13-25.\textsuperscript{29}

The proper isocyanate (1.1 eq) was added to a suspension of 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) in dry CH\textsubscript{3}CN (1.5 ml) under a nitrogen atmosphere and that was stirred at r.t. until starting material was consumed (TLC monitoring). The solvent was removed under \textit{vacuo} and the obtained residue was purified by silica gel column chromatography eluting with MeOH/DCM from 5 to 15%.

4-Hydroxy-3-nitro-5-(3-phenyl-ureido)-benzenesulfonamide (13).

Compound 13 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and phenyl isocyanate (1.1eq) in dry CH\textsubscript{3}CN (1.5 ml). The reaction mixture was stirred at r.t. overnight to afford the titled compound 13 as a red solid. 80% yield; m.p. 225-227°C; silica gel TLC \textit{Rf} 0.40 (MeOH/DCM 20 % \textit{v/v}); δ\textit{H} (400 MHz, DMSO-\textit{d}6): 7.05 (t, \textit{J} = 7.6, 1H), 7.35 (t, \textit{J} = 7.6, 2H), 7.51 (d, \textit{J} = 7.6, 2H), 7.52 (s, 2H, exchange with D\textsubscript{2}O, SO\textsubscript{2}NH\textsubscript{2}), 8.04 (d, \textit{J} = 2.4, 1H), 8.84 (s, 1H, exchange with D\textsubscript{2}O, NH\textsubscript{CO}), 8.99 (d, \textit{J} = 2.4, 1H), 9.66 (s, 1H, exchange with D\textsubscript{2}O, NH\textsubscript{CO}); δ\textit{C} (100 MHz, DMSO-\textit{d}6): 115.8, 119.1, 119.6, 123.2, 129.7, 129.9, 133.6, 134.9, 135.6, 140.3, 153.1; ESI-HRMS (m/z) [M-H]: calculated for C\textsubscript{13}H\textsubscript{11}N\textsubscript{4}O\textsubscript{6}S 351.0394; found 351.0387.

3-[3-(4-Fluoro-phenyl)-ureido]-4-hydroxy-5-nitro-benzenesulfonamide (14).

Compound 14 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and 4-F-phenyl isocyanate (1.1 eq) in dry CH\textsubscript{3}CN (1.5 ml). The reaction mixture was stirred at r.t. overnight to afford the titled compound 14 as a red solid. 84% yield; m.p. 250°C dec.; silica gel TLC \textit{Rf} 0.26 (MeOH/DCM 20 % \textit{v/v}); δ\textit{H} (400 MHz, DMSO-\textit{d}6): 6.92 (s, 2H, exchange with D\textsubscript{2}O, SO\textsubscript{2}NH\textsubscript{2}), 7.13 (t, \textit{J} = 8.8, 2H), 7.51 (m, 2H), 7.93 (d, \textit{J} = 2.4, 1H), 8.33 (d, \textit{J} = 2.4, 1H), 8.91 (s, 1H, exchange with D\textsubscript{2}O, NH\textsubscript{CO}), 9.73 (s, 1H,
exchange with D$_2$O, NHCO); $\delta_F$ (376 MHz, DMSO-$d_6$): -121.99 (s, 1F); $\delta_C$ (100 MHz, DMSO-$d_6$): 113.2, 116.2 (d, $J^{CF} = 22.0$), 118.9, 120.6 (d, $J^{CF} = 7.0$), 122.6, 123.5, 136.9, 137.4, 153.4, 158.1 (d, $J^{CF} = 236.0$), 160.9; ESI-HRMS (m/z) [M-H]$^{-}$: calculated for C$_{13}$H$_{10}$FN$_4$O$_6$S 369.0300; found 369.0295.

4-Hydroxy-3-nitro-5-[3-(4-trifluoromethyl-phenyl)-ureido]-benzenesulfonamide (15).

Compound 15 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and 4-CF$_3$-phenyl isocyanate (1.1eq) in dry CH$_3$CN (1.5 ml). The reaction mixture was stirred at r.t. overnight to afford 15 as a red solid. 75% yield; m.p. 225-226°C; silica gel TLC $R_f$ 0.13 (MeOH/DCM 10 % v/v); $\delta_H$ (400 MHz, DMSO-$d_6$): 6.94 (s, 2H, exchange with D$_2$O, SO$_2$NH$_2$), 7.65 (d, $J = 8.8$, 2H), 7.72 (d, $J = 8.8$, 2H), 7.96 (d, $J = 2.4$, 1H), 8.36 (d, $J = 2.4$, 1H), 9.04 (s, 1H, exchange with D$_2$O, NHCO), 10.11 (s, 1H, exchange with D$_2$O, NHCO); $\delta_F$ (376 MHz, DMSO-$d_6$): -59.99 (s, 3F); $\delta_C$ (100 MHz, DMSO-$d_6$): 113.7, 118.6, 119.2, 122.3 (q, $J^{CF} = 31.8$), 122.7, 122.9 (q, $J^{CF} = 270.0$), 126.9 (q, $J^{CF} = 3.7$), 132.6, 136.5, 144.8, 153.1, 160.8; ESI-HRMS (m/z) [M-H]$^{-}$: calculated for C$_{14}$H$_{10}$F$_3$N$_4$O$_6$S 419.0268; found 419.0276.

3-[3-(4-Fluoro-3-methyl-phenyl)-ureido]-4-hydroxy-5-nitro-benzenesulfonamide (16).

Compound 16 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and 4-F-3-methyl phenyl isocyanate (1.1 eq) in dry CH$_3$CN (1.5 ml). The reaction mixture was stirred at r.t. overnight to afford 16 as a red solid. 81% yield; m.p. 240°C dec.; silica gel TLC $R_f$ 0.27 (MeOH/DCM 20 % v/v); $\delta_H$ (400 MHz, DMSO-$d_6$): 2.42 (d, $J = 1.2$, 3H, CH$_3$), 6.87 (s, 2H, exchange with D$_2$O, SO$_2$NH$_2$), 7.05 (t, $J = 9.2$, 1H), 7.28 (m, 1H), 7.45 (m, 1H), 7.93 (d, $J = 2.4$, 1H), 8.30 (d, $J = 2.4$, 1H), 8.87 (s, 1H, exchange with D$_2$O, NHCO), 9.63 (s, 1H, exchange with D$_2$O, NHCO); $\delta_F$ (376 MHz, DMSO-$d_6$): -126.41 (s, 1F); $\delta_C$ (100 MHz, DMSO-$d_6$): 15.4 (d, $J^{CF} = 3.0$), 112.6, 115.7 (d, $J^{CF} = 22.8$), 117.9 (d, $J^{CF} = 7.6$),
118.9, 121.9 (d, $\delta^1\text{CF} = 4.2$), 122.0, 124.9 (d, $\delta^2\text{CF} = 17.8$), 132.7, 137.0, 131.1, 153.4, 156.7 (d, $\delta^1\text{CF} = 236.0$), 161.7; ESI-HRMS (m/z) [M-H]: calculated for C$_{14}$H$_2$FN$_4$O$_6$S 383.0456; found 383.0461.

4-Hydroxy-3-nitro-5-(3-pentafluorophenyl-ureido)-benzenesulfonamide (17).

Compound 17 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and pentafluorophenyl isocyanate (1.1 eq) in dry CH$_3$CN (1.5 ml). The reaction mixture was stirred at r.t. overnight to afford 17 as a red solid. 79% yield; m.p. 250°C dec.; silica gel TLC $R_f$ 0.31 (MeOH/DCM 20 % v/v); $\delta_H$ (400 MHz, DMSO-$d_6$): 7.01 (s, 2H, exchange with D$_2$O, SO$_2$NH$_2$), 7.98 (d, $J = 2.2$, 1H), 8.52 (d, $J = 2.2$, 1H), 9.88 (bs, 1H, exchange with D$_2$O, NHCO), 10.73 (bs, 1H, exchange with D$_2$O, NHCO); $\delta_F$ (376 MHz, DMSO-$d_6$): -164.84 (t, $J = 22.6$, 2F), -160.02 (t, $J = 22.6$, 1F), -145.82 (d, $J = 22.6$, 2F); ESI-HRMS (m/z) [M-H]: calculated for C$_{13}$H$_6$F$_5$N$_4$O$_6$S 440.9923; found 440.9930.

4-Hydroxy-3-[3-(3-methoxy-phenyl)-ureido]-5-nitro-benzenesulfonamide (18).

Compound 18 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and 3-methoxy phenyl isocyanate (1.1 eq) in dry CH$_3$CN (1.5 ml). The reaction mixture was stirred at r.t. overnight to afford 18 as a red solid. 71% yield; m.p. 255°C dec.; silica gel TLC $R_f$ 0.15 (MeOH/DCM 10 % v/v); $\delta_H$ (400 MHz, DMSO-$d_6$): 3.78 (s, 3H, OCH$_3$), 6.64 (dd, $J = 2.4$,7.8, 1H), 6.97 (dd, $J = 2.4$, 7.8, 1H), 7.24 (t, $J = 7.8$, 1H), 7.26 (s, 1H), 7.51 (s, 2H, exchange with D$_2$O, SO$_2$NH$_2$), 8.03 (dd, $J = 2.2$, 1H), 8.40 (s, 1H, exchange with D$_2$O, NHCO), 8.97 (d, $J = 2.2$, 1H), 9.68 (s, 1H, exchange with D$_2$O, NHCO); $\delta_C$ (100 MHz, DMSO-$d_6$): 55.8, 104.5, 108.1, 111.2, 113.1, 118.9, 122.4, 130.4, 132.4, 136.9, 142.4, 153.3, 160.6, 161.2; ESI-HRMS (m/z) [M-H]: calculated for C$_{14}$H$_{13}$N$_4$O$_7$S 440.9923; found 440.9930.

3-(3-Benz[1,3]dioxol-5-yl-ureido)-4-hydroxy-5-nitro-benzenesulfonamide (19).
Compound 19 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and 5-isocyanato-benzo[1,3]dioxole (1.1 eq) in dry CH₃CN (1.5 ml). The reaction mixture was stirred at r.t. overnight to afford 19 as a red solid. 78% yield; m.p. 225-228°C; silica gel TLC Rf 0.21 (MeOH/DCM 10 % v/v); δH (400 MHz, DMSO- d₆): 6.02 (s, 2H, C₆H₂), 6.79 (dd, J = 2.0, 8.4, 1H), 6.89 (d, J = 8.4, 1H), 7.27 (d, J = 2.0, 1H), 7.51 (s, 2H, exchange with D₂O, SO₂NH₂), 8.01 (d, J = 2.2, 1H), 8.76 (s, 1H, exchange with D₂O, NHCO), 8.96 (d, J = 2.2, 1H), 9.55 (s, 1H, exchange with D₂O, NHCO); δC (100 MHz, DMSO-d₆): 101.7, 101.9, 109.2, 112.0, 115.5, 120.1, 133.3, 134.5, 135.8, 135.6, 143.4, 145.1, 148.3, 153.1; ESI-HRMS (m/z) [M-H]-: calculated for C₁₄H₁₁N₄O₈S 395.0292; found 395.0289.

3-[3-(3,5-Dimethyl-phenyl)-ureido]-4-hydroxy-5-nitro-benzenesulfonamide (20).

Compound 20 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and 3,5-dimethylphenyl isocyanate (1.1 eq) in dry CH₃CN (1.5 ml). The reaction mixture was stirred at r.t. overnight to afford 20 as a red solid. 82% yield; m.p. 215°C dec.; silica gel TLC Rf 0.54 (MeOH/DCM 20 % v/v); δH (400 MHz, DMSO-d₆): 2.26 (s, 6H, 2 x C₆H₃), 6.62 (s, 1H), 6.91 (s, 2H, exchange with D₂O, SO₂NH₂), 7.15 (s, 2H), 7.93 (d, J = 2.8, 1H), 8.36 (d, J = 2.8, 1H), 8.88 (s, 1H, exchange with D₂O, NHCO), 9.52 (s, 1H, exchange with D₂O, NHCO); δC (100 MHz, DMSO-d₆): 22.1, 113.4, 116.8, 118.6, 123.0, 124.1, 132.5, 36.9, 138.5, 140.9, 153.4, 160.6; ESI-HRMS (m/z) [M-H]-: calculated for C₁₅H₁₅N₄O₆S 379.0707; found 379.0706.

3-[3-(3,5-Bis-trifluoromethyl-phenyl)-ureido]-4-hydroxy-5-nitro-benzenesulfonamide (21).

Compound 21 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and 3,5-bis-trifluoromethylphenyl isocyanate (1.1 eq) in dry CH₃CN (1.5 ml). The reaction mixture was stirred at r.t. overnight to afford 21 as a red solid. 80% yield; m.p. 245°C dec.; silica gel TLC Rf 0.14 (MeOH/DCM 10 %
4-Hydroxy-3-nitro-5-(3-phenethyl-ureido)-benzenesulfonamide (22).

Compound 2 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and phenethyl isocyanate (1.1eq) in dry CH$_3$CN (1.5 ml). The reaction mixture was stirred at r.t. overnight to afford 22 as a red solid. 62% yield; m.p. 245-248°C; silica gel TLC $R_f$ 0.10 (MeOH/DCM 5% v/v); $\delta$H (400 MHz, DMSO-$d_6$): 2.77 (t, $J = 7.6$, 2H, $CH_2$), 3.30 (t, $J = 7.6$, 2H, overlap with water signal, $CH_2$), 6.87 (s, 2H, exchange with D$_2$O, SO$_2$NH$_2$), 7.28 (m, 5H), 7.90 (d, $J = 2.2$, 1H), 7.96 (s, 1H, exchange with D$_2$O, NHCO), 8.38 (d, $J = 2.2$, 1H), 8.91 (s, 1H, exchange with D$_2$O, NHCO); $\delta$C (100 MHz, DMSO-$d_6$): 37.4, 41.8, 112.3, 118.0, 122.6, 126.9, 129.2, 129.6, 132.2, 138.1, 140.8, 156.3, 161.8; ESI-HRMS (m/z) [M-H]: calculated for C$_{15}$H$_9$F$_6$N$_4$O$_6$S 487.0142; found 487.0143.

3-(3-Furan-2-ylmethyl-ureido)-4-hydroxy-5-nitro-benzenesulfonamide (23).

Compound 23 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and furfuryl isocyanate (1.1eq) in dry CH$_3$CN (1.5 ml). The reaction mixture was stirred at r.t. overnight to afford 23 as a red solid. 44% yield; m.p. 223-226°C; silica gel TLC $R_f$ 0.12 (MeOH/DCM 5% v/v); $\delta$H (400 MHz, DMSO-$d_6$): 4.33 (d, $J = 5.6$, 2H, $CH_2$), 6.22 (s, 1H, Ar-$H$), 6.39 (s, 1H, Ar-$H$), 6.86 (s, 2H, exchange with D$_2$O, SO$_2$NH$_2$), 7.56 (s, 1H, Ar-$H$), 7.89 (d, $J = 2.4$, 1H, Ar-$H$), 8.19 (bs, 1H, exchange with D$_2$O, NHCO), 8.34 (d, $J = 2.4$, 1H, Ar-$H$), 8.94 (s, 1H, exchange with D$_2$O, NHCO); $\delta$C (100 MHz,
DMSO-$d_6$): 36.9, 106.8, 111.3, 112.8, 132.3, 138.0, 142.4, 155.2, 156.3, 161.5; ESI-HRMS (m/z) [M-H]$: calculated for C$_{12}$H$_{11}$N$_4$O$_7$S 355.0343; found 355.0339.


Compound 24 was obtained according the general procedure earlier reported using 3-amino-4-hydroxy-5-nitro-benzenesulfonamide 8 (0.06 g, 1.0 eq) and acetic acid 2,5-diacetoxy-6-acetoxymethyl-3-isocyanato-tetrahydro-pyran-4-yl ester 26 (3.0 eq) in dry CH$_3$CN (1.5 ml). The reaction mixture was stirred at r.t. for 48h to afford 24 as a red solid. 63% yield; m.p. 191-194°C; silica gel TLC $R_f$ 0.20 (MeOH/DCM 10 % v/v); δ$_H$ (400 MHz, DMSO-$d_6$): 1.92 (s, 3H, COCH$_3$), 2.02 (s, 3H, COCH$_3$), 2.06 (s, 3H, COCH$_3$), 2.06 (s, 3H, COCH$_3$), 3.90 (m, 1H, CH), 4.05 (m, 2H, 2 x CH), 4.24 (dd, $J = 4.4$, 12.4, 1H, CH), 4.94 (t, $J = 9.6$, 1H, CH), 5.36 (m, 1H, CH), 5.92 (m, 1H, CH), 6.97 (s, 2H, exchange with D$_2$O, SO$_2$NH$_2$), 7.74 (bs, 1H, exchange with D$_2$O, NH$_2$); δ$_C$ (100 MHz, DMSO-$d_6$): 21.3, 21.4, 21.5, 21.6, 55.8, 62.6, 69.4, 72.4, 73.1, 93.0, 113.7, 118.2, 124.0, 132.6, 137.1, 155.8, 160.1, 169.9, 170.3, 170.5, 171.0; ESI-HRMS (m/z) [M-H]$: calculated for C$_{21}$H$_{25}$N$_4$O$_{15}$S 605.1032; found 605.1033.

4-Hydroxy-3-nitro-5-(3-((3S,4S,5R,6S)-2,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)ureido)benzenesulfonamide (25).

Compound 24 (0.04g, 1.0 eq) was added to a freshly prepared sodium methoxide (7.0 eq) methanolic solution (5 ml) under a nitrogen atmosphere at 0°C and that was stirred at 0°C for 0.5h. Neutralization of the solution with Amberlite IR-120-H$^+$ ion exchange resin, followed by filtration (the resin washed several times with methanol) and evaporation of the filtrate to dryness, afforded compound 25.74% yield; ESI-HRMS (m/z) [M-H]$: calculated for C$_{13}$H$_{17}$NaO$_{15}$S 437.0609; found 437.0611.
4.2. CA inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO₂ hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na₂SO₄ (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 (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature 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 and the Cheng-Prusoff equation, as reported earlier, and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier.

X-ray Crystallography

CA II and CA IX/mimic were transformed in E. coli BL21 (DE3) competent cells in Luria broth medium containing 100mg/ml of Ampicillin at 37°C. CA IX/mimic is a CA II variant with seven amino acid substitutions (A65S, N67Q, E69T, I91L, F131V, K170E and L204A) in the active site to mimic that of CA IX. Protein expression was induced by 0.1mg/ml isopropyl β-D thiogalactoside (IPTG). After incubating for 3 h at 37°C, the cells were harvested by centrifugation. Purification
was performed using a \( p \)-aminomethyl-benzenesulfonamide agarose affinity column and the protein eluted with 400mM sodium azide, 50 mM Tris, pH 7.8. Both CA II and CA IX/mimic were buffer exchanged (into 50mM Tris-HCl, pH 7.8) and concentrated using Amicon ultra-filtration centrifugal tubes with a 10 kDa molecular weight cut-off. Purity was verified by SDS-PAGE stained with coomassie brilliant blue and concentration determined by UV spectroscopy at 280 nm with a molar extinction coefficient of 54800 cm\(^{-1}\) mol\(^{-1}\).

The CA II and CA IX/mimic crystals were grown via the hanging drop vapor diffusion method with a precipitant solution of 1.6 M sodium citrate, 50 mM Tris and pH 7.8 at room temperature (RT). Crystal drops were set up in a 1:1 ratio with 2.5\( \mu \)l of protein and 2.5 \( \mu \)l of precipitant solution. The stock concentrations of CA II and CA IX/mimic were 10 mg/ml, resulting in a final concentration of 5 mg/ml protein in the drop.

The inhibitors were dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted 10-fold in the precipitant solution to a final concentration of 1.2, 1.3 and 1.1 mM for 15, 16 and 17, respectively. The CA II and CA IX/mimic crystals were soaked overnight with the inhibitors.

X-ray diffraction data were collected at RT in-house using a Rigaku RU-H3R rotating Cu anode (wavelength of 1.5418 Å) operating at 50 kV and 22 mA with osmic mirrors and an R-Axis IV\(^{++}\) image detector. A total of 180 images were collected for each data set with a crystal to detector distance of 100 mm, exposure time of 10 min, and oscillation angle of 1°. Data collection was performed at RT. The data were indexed and integrated using the HKL2000 software package. Molecular replacement was used for initial phasing in PHENIX (Phaser-MR One-Component Interface) using CAII (PDB ID: 3KS3) as a search model. The model refinements and generation of ligand restraint files were also performed using the PHENIX programs. COOT (Crystallographic object oriented toolkit) was utilized to observe refinements and evaluate the structure. The percentage of inhibitor-bound surface area was calculated using PDB e-PISA. All the figures were generated using PyMol.
Cell culture and treatments

Human prostate cancer cell line PC3, human breast cancer cell line MDA-MB-231, and human colon cancer cell line HT-29 were obtained from American Type Culture Collection (Rockville, MD). PC3, MDA-MB-231 and HT-29 were cultured in DMEM high glucose with 20% FBS in 5% CO₂ atmosphere at 37° C. Media contained 2 mM L-glutamine, 1% essential amino acid mix, 100 IU ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Sigma, Milan, Italy). Cells were plated in 96-wells cell culture (1.10⁴/well) and, 24 h after, treated with the tested compounds (0-200 μM) for 48 h. Low oxygen conditions were acquired in a hypoxic workstation (Concept 400 anaerobic incubator, Ruskinn Technology Ltd., Bridgend, UK). The atmosphere in the chamber consisted of 1% O₂ (hypoxia), 5% CO₂, and residual N₂. In parallel, normoxic (20% O₂) dishes were incubated in air with 5% CO₂.

Cell viability assay

PC3, MDA-MB-231 and HT-29 cell viability was evaluated by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an index of mitochondrial compartment functionality. Cells were plated and treated as described. Post-treatments, after extensive washing, 1 mg/ml MTT was added into each well and incubated for 30 minutes at 37 °C. After washing, the formazan crystals were dissolved in 150 μl DMSO. The absorbance was measured at 550 nm. Experiments were performed in quadruplicate on at least three different cell batches.

Statistical analysis

Results were expressed as mean ± S.E.M. and the analysis of variance was performed by one way ANOVA. A Bonferroni’s significant difference procedure was used as post-hoc comparison. P values of less than 0.05 were considered as significant. Data were analyzed using the “Origin® 9.1” software.
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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: additional synthetic procedures and characterization of compounds, NMR spectra, HPLC-purity chromatograms, supplemental X-ray crystallographic figures, anti-proliferative assays data. SMILES representation for compounds (CSV).

ASSOCIATED CONTENT

Coordinates and structure factors for hCA II and hCA IX-mimic complexes with 15, 16 and 17 have been deposited in the Protein Data Bank (PDB) with accession codes: hCAII_15: 6EBE, hCAII_16: 6EDA, hCAII_17: 6ECZ, CA IX-mimic_15: 6EEA, CA IX-mimic_16: 6EEO, A CA IX-mimic_17: 6EEH. Authors will release the atomic coordinates and experimental data upon article publication.

ABBREVIATIONS USED

HIF-1, hypoxia-inducible factor 1; HAP, hypoxia-activated prodrug; CA, carbonic anhydrase; CAI, CA inhibitor; Ki, inhibition constant; Boc, tert-butyloxycarbonyl; EtOAc, ethyl acetate; DMF, dimethyl formamide; DCM, dichloromethane; MeOH, methanol.
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Caption: Bioreductive 4-hydroxy-3-nitro-5-ureido-benzenesulfonamides, designed on the anti-tumor phase II drug SLC-0111, selectively inhibit CA IX and XII and show hypoxia-enhanced anti-proliferative profiles.