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3-Hydroxy-1H-quinazoline-2,4-dione as a New Scaffold to Develop Potent and Selective Inhibitors of the Tumor-associated Carbonic Anhydrases IX and XII

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ABSTRACT

The paper describes the discovery of the 3-hydroxyquinazolin-2,4-dione as a useful scaffold to obtain potent inhibitors of the tumor-associated human carbonic anhydrases (hCAs) IX and XII. A set of derivatives (**1-29**), bearing different substituents on the fused benzo ring (Cl, NO_2, NH_2, CF_3, ureido, amido, heterocycles), were synthesized and several of them showed nanomolar activity in inhibiting the hCA IX and XII isoforms while they were ineffective in inhibiting the cytosolic enzymes hCAs I and II. Some selected compounds were tested for their antiproliferative activity on HT-29 colon cancer cell lines. After 48 h treatment with the lower dose (30 μM), derivatives **12**, **14**, **15** and **19** were significantly active inducing a mortality by about 50% in both normoxia and hypoxia. This finding let us to hypothesize for these compounds more than one mechanism of action involving both CAs IX-XII and other not yet identified target(s).

INTRODUCTION

The human (h) carbonic anhydrases (CAs) are ubiquitous zinc enzymes distinguished in at least 15 isoforms and differing in molecular properties, subcellular localization and tissue distribution. hCA activities are involved in the regulation of physiological functions related to carbon dioxide/bicarbonate transport, pH, electrolyte secretion in tissues and organs and to many metabolic processes.[1] The hCA isoforms IX and XII are membrane-bound enzymes whose expression is significantly enhanced in a large variety of cancer cells, being strongly induced by hypoxia, a common feature of solid tumor, such as glioma, breast cancer and colon carcinoma. Hypoxia, due to the discrepancy between the oxygen demand of proliferating tumor cells and oxygen supply from the vasculature, shifts metabolism towards glycolysis and anaerobic fermentation affording a high production of lactic acid. Oncogenic metabolism also generates excessive formation of carbon dioxide and protons, thus inducing a progressive acidification of the extracellular environment. This condition can lead to apoptosis in the surrounding healthy cells thus benefiting the expansion of tumor cells which in turn develop different mechanisms to counteract acidosis and maintain a slightly alkaline intracellular pH, favorable for the cell proliferation. Transporters and ion pumps lead to the extrusion of lactate and protons from the cell and the entrance of bicarbonate ions,[1,2,4-5] these last being produced by carbon dioxide hydration, catalyzed by the transmembrane hCAs IX and XII. Hence, both hCA isoforms concur to maintain a) a more alkaline resting intracellular pH and b) the acidic extracellular pH which, in turn, contributes to tumor progression via up-regulation of proteases, angiogenic and cell-growth factors, and through impaired immune functions.[4-8 h]CA IX activity has been associated with chemoresistance which might be due to two mechanisms.[9] First, chemotherapy causes intracellular acidosis, and hCA IX prevent apoptosis by maintaining a normal intracellular pH in response to chemotherapy. Second, hCA IX-induced extracellular acidosis is connected to a chemoresistance by a reduction in uptake of weakly basic anticancer drugs.

Coherently, acetazolamide (AZA), a classical hCA inhibitor, was reported to reduce tumor growth and to delay the development of the tumor, when combined with chemotherapeutic drugs.

Nevertheless, AZA, as well as other classical aromatic sulfonamides, are non-selective inhibitors, thus leading to side effects by blocking the cytosolic isoenzymes of physiological relevance, i.e. hCA I and II. Hence, selective targeting of the tumor-associated hCA isoenzymes is a promising strategy to obtain effective and safer agents in cancer therapy.[1,2,8-10]

Up to now several classes of inhibitors directly/indirectly interacting with the Zn^{2+} ion from the active hCA site have been disclosed, including sulphonamides, metal-complexing anions, phenols and hydroxamic acids.[11,12] Within the last class, benzohydroxamic acid (Chart 1) inhibited all the hCA isoforms with potencies spanning the low to medium micromolar range.[11] In particular, it showed K_i values of 45.9 and 9.51 μM at the membrane-bound hCA IX and XII isoforms, respectively, while lower potencies were found for the cytosolic highly abundant hCA I and II (K_i= 83.1 and 179 μM). X-ray crystallographic studies on the adduct that benzohydroxamic acid forms with the best characterized and easily crystallizable hCA isoform, i.e. the hCA II, highlighted that the carbonyl and hydroxyl groups coordinate to the zinc ion to form a 5-member chelate complex.[11]

In the search for new selective hCA inhibitors we envisaged the 3-hydroxy-1H-quinazoline-2,4-dione **1** (Chart **1**) as a cyclic analogue of the benzohydroxamic acid. We hypothesized that the less flexible structure of the bicyclic scaffold **1** might have led to an increased selectivity toward the targeted CA IX and XII isoforms. Moreover, the 3-hydroxy group and either the 2- or 4-carbonyl function were thought to be able to coordinate the Zn^{2+} from the enzyme active site. Anticipating our results, compound **1**, notwithstanding its simple structure, turned out to be an inhibitor of hCAs IX and XII endowed with higher potencies (K_i= 480 nM and 104 nM, respectively) than those of benzohydroxamic acid and high selectivity versus hCA I and hCA II isoforms (both K_i> 10000 nM). These interesting findings suggested that 3-hydroxy-1H-quinazoline-2,4-dione **1** could be a versatile scaffold to develop new non-sulfonamide hCA inhibitors and prompted us to keep on studying this class of compounds. Thus, taken derivative **1** as lead, the set of 3-hydroxy-1H-quinazoline-2,4-dione derivatives **2-27** (Chart 1), bearing different substituents on the fused

benzo ring, were synthesized and evaluated as hCA inhibitors. Moreover, the 3-O-modified quinazoline-2,4-dione derivatives **28** and **29** were synthesized to evaluate the importance of the free 3 hydroxy group for hCA inhibition. Some selected compounds (**3**, **5**, **6**, **12**, **14**, **15** and **19**) were also pharmacological evaluated to assess their antiproliferative effect on human colorectal cancer HT-29 cells.

RESULTS AND DISCUSSION

Chemistry. The 3-hydroxyquinazoline-2,4-dione derivatives **1-29** were prepared as outlined in Schemes 1-5. The synthesis of the lead compound **1**[13] and derivatives **2-8**, featuring simple substituents (Cl, NO_2, NH_2, CF_3) on different positions of the fused benzo ring, is depicted in Scheme 1. The synthetic pathway to prepare the 3-hydroxy-1H-quinazoline-2,4-dione derivatives **4-7** was already described by us,[14,15] but neither the experimental procedures nor the characterization data of **4-6** were previously reported. Hence, they are described herein in detail. Derivatives **1-8** were prepared starting from the suitably substituted 1,2-dihydro-3,1-benzoxazine-2,4-diones **30-36**, commercially available (**30-33**) or synthesized following reported procedures (**34-36**).[14-16] The 7-trifluoromethyl-3-hydroxyquinazoline-dione **7** was obtained as previously described[14] from **36**. To prepare **1-6** and **8**, derivatives **30-35** were reacted with O-benzylhydroxylamine in refluxing ethanol to give the 2-amino-N-benzyloxybenzamides **37-42**[17] which were cyclized with triphosgene to afford the 3-benzyloxyquinazoline-2,4-dione derivatives **43-48**. Reduction of the 6-nitroquinazoline derivative **45** with stannous chloride led to the corresponding amino-substituted compound **49**. Debenzylation of **43-49** with 48% hydrobromic acid in glacial acetic acid gave the desired compounds **1-8**.[13,14,15]

The 3-hydroxyquinazoline-2,4-dione derivatives **9-13**, characterized by the presence of different groups (1,2,4-triazol-4-yl, amido and ureido) at position 6, were synthesized starting from the 6-aminoquinazoline derivative **49** (Scheme 2). Treatment of **49** with diformylhydrazine and

trimethylchlorosilane in anhydrous pyridine afforded the 6-(1,2,4-triazol-4-yl)-derivative **50**. Reaction of **49** with succinic anhydride in glacial acetic acid at 60 °C and in the presence of sodium acetate gave, after acidification, the 6-succinamido- compound **51**. The same reaction with phthalic anhydride, afforded the 6-phthalamido- derivative **52** which was obtained through a different work-up (see Experimental procedure for details). When derivative **49** was reacted with benzyl- and phenyl-isocyanate in anhydrous boiling tetrahydrofuran, the related 6-ureido derivatives **53** and **54** were obtained. Derivatives **50-54** were 3-O-debenzylated through catalytic hydrogenation giving rise to the desired 3-hydroxy derivatives **10-13**.

The 3-hydroxyquinazoline-2,4-diones **14-26** were prepared as outlined in Scheme 3. These compounds are featured by the presence of a chlorine atom or a trifluoromethyl group at position 7 combined with different group at position 6, i.e. nitro (**14, 15**), amino (**16**), heterocyclic moieties (**17-21**), acylamino- (**22-25**) and benzylureido- (**26**) substituents. The 7-trifluoromethyl-derivatives **15** and **21** were prepared as previously described.[14] Within the 7-chloro-substituted compounds, derivative **20** was a new chemical entity while the others (**14, 16-19**, **22-26**) were previously reported by us.[15,19] Nevertheless, for derivatives **14** and **16**, as well as for their precursor **55-57**, no experimental data were provided.[15] Thus, the synthesis of **14** and **16** is herein reported in detail, together with that of **20**. Briefly, the 7-chloro-3-hydroxyquinazoline-2,4-dione **4** was treated with acetic anhydride to give the corresponding 3-acetoxy derivative **55** whose nitration afforded the 6-nitro compound **56**. This last was either transformed into the 3-hydroxy-6-nitro derivative **14** or reduced to the related 6-amino derivative **57**. Treatment of **57** with NaOH solution afforded the corresponding 3-hydroxy derivative **16**. Reaction of **57** with formaldehyde and glyoxal, in the presence of ammonium acetate, gave the (6-imidazol-1-yl)-substituted quinazoline **58** which was desacetylated to yield the desired compound **20**. The quinazoline-2,4-dione derivatives **27** and **28** were obtained as depicted in Scheme 4. To prepare the 6-phenylureido-substituted compound **27**, the starting 7-chloro-1,2-dihydro-3,1-benzoxazine-2,4-dione **33** was nitrated to yield the 7-chloro-6-nitro derivative **59**[18] which was reacted with O-

benzylhydroxylamine in refluxing ethanol to give the 2-amino-4-chloro-6-nitro-N-benzyloxybenzamide **60**. This compound was cyclized with triphosgene to provide the related quinazoline-2,4-dione **61** which was transformed into the corresponding amino derivative **62** by using stannous chloride in refluxing ethanol. Reaction of **62** with phenylisocyanate in refluxing anhydrous tetrahydrofuran led to the 6-phenylureido derivative **63** which was debenzylated by catalytic hydrogenation to afford compound **27**. The 7-chloro-3-methoxyquinazoline-2,4-dione **28** was synthesized starting from the 7-chloro-benzoxazine-2,4-dione derivative **33** which was reacted with O-methylhydroxylamine in refluxing ethanol to give the 2-amino-4-chloro-N-methoxybenzamide **64** whose cyclization with triphosgene afforded the desired compound **28**. Finally, derivative **29** was synthesized as outlined in Scheme 5. The 7-chloroquinazoline derivative **65**[19] was transformed into the corresponding 7-chloro-6-nitro derivative **66** which was then hydrolyzed at the ester function to give the related carboxylic acid **29**.

CA Inhibitory Activity. The quinazoline-2,4-dione derivatives **1-29** were tested for their efficacy toward the targeted hCAs IX and XII and also against the physiologically relevant hCA I and II, which are cytosolic isoforms abundantly expressed through the body, thus responsible of the side effects of non-selective hCA inhibitors. The data shown in Table 1 indicate that the 3-hydroxy-1H-quinazoline-2,4-dione **1** is a useful scaffold to develop non-classical inhibitors of the tumor-associated hCAs. In fact, most of the tested derivatives inhibited both hCA IX and XII at low nanomolar concentrations, while being inactive at the off-target hCAs I and II. As anticipated above, the 3-hydroxyquinazoline-2,4-dione **1** was prepared as cyclic analogue of the non-selective hCA inhibitor benzohydroxamic acid with the aim that the reduced flexibility of **1** might increase the compound selectivity and that the NHCO function, inserted to cyclize the lead, might reinforce interaction of **1** with the enzyme. Very interestingly, compound **1** showed enhanced potency and selectivity toward the targeted hCAs IX and XII, thus prompting us to continue the investigation of this class of compounds. Hence, small substituents with different lipophilic and electronic

properties were singularly introduced at position 6 (Cl, NO_2, NH_2) and 7 (Cl, CF_3) or combined at the 6,7 (Cl) or 5,7 (Cl) positions. The obtained compounds **2-8** displayed the same selectivity of the lead **1** versus the hCA I and II isoforms and showed, on the whole, a significant increase of activity at both the targeted enzymes, most of compounds displaying K_i values in the low nanomolar range (K_i= 3.4-8.2 nM). There are only two exceptions, i.e. the 6-chloro- and 6-amino- derivatives **2** and **8**, which are less active than **1** as hCA XII inhibitors.

It has to be noted that the presence of the 7-chloro substituent (compound **4**) gave rise to a potent hCA XII inhibitor (K_i= 3.4 nM), 20-fold selective versus the hCA IX isoform, while introduction of a second chlorine atom, either at the 6- or 5-position (compounds **5** and **6**, respectively) made the compound equally potent at the two targeted hCAs. Also the 6-nitro group was particularly advantageous, being derivative **3** one of the most potent dual hCAs IX and XII inhibitor, among those herein reported.

Subsequently, the 6-amino group of **8** was exploited to be transformed into the 1,2,4-triazol-1-yl moiety (compound **9**) or used as linker for acyl groups (compounds **10**, **11**) and carbamoyl residues (derivatives **12-13**). All these transformations were carried out to probe groups with different properties and, consequently, ability to interact with the catalytic site of hCAs. The above mentioned substituents made compounds **9-13** more active than the unsubstituted compound **1** against the hCA IX while worsened ability to inhibit the isoform XII. It is worth noting that the 6-benzylureido derivative **12** display the highest inhibitor activity against the hCA IX isoform (K_i= 26.7 nM). Instead, its inferior homologue, the 6-phenylureido derivative **13**, was about 10-fold less potent at this isoform.

When the 6-nitro substituent was combined with either a chlorine atom or a trifluoromethyl residue at position 7 (compounds **14** and **15**, respectively) two new dual potent hCA IX-XII endowed with similar activity were obtained. The trifluoromethyl and chloro substituents elicited a similar effect also when they were combined with the 6-(1,2,4-triazol-4-yl) moiety (compare compound **17** to **21**).

The 6-amino-7-chloro substitution turned out profitable for the hCA IX inhibition, being

derivative **16** more active (K_i= 43.1 nM) than 7-chloro derivative **4** (K_i= 69.7 nM) but especially than the 6-amino derivative **8** (K_i= 272 nM). This finding evidenced the profitable role of the 7-chlorine atom that was confirmed when this substituent was introduced on derivatives **9-13** to give compounds **17**, **23**, **25-27**. These latter inhibitors, in fact, were significantly more potent than the former (**9-13**) and showed K_i values in the low nanomolar range (6.9-42.8 nM) on both the targeted hCA isoforms, the only exception being the 6-phenylureido derivative **27**, scarcely active at the hCA XII.

Compounds **18-20** were synthetized as analogues of **17** whose 6-(1,2,4-triazol-4-yl) group was replaced with other five-membered heterocyclic rings. The best group proved to be the pyrrol-1-yl, in fact compound **18** showed an increased activity on the hCA IX, with respect to **17**. Instead, the imidazol-1-yl substituent afforded the lowest activity against the hCA IX and especially the hCA XII (compound **20**).

Comparing the data of derivatives **22-24**, it can be observed the beneficial role of the acidic carboxylic function on the 6-substituent. In fact, the 6-(carboxybenzoylamino)-substituted compounds **23** and **24** are significantly more potent as hCA IX-XII inhibitors than the 6-benzoylamino-derivative **22**. Derivative **25**, whose 6-(3-carboxypropanoylamino) chain is more flexible and less lipophilic than the 6-substituent of compounds **23** and **24**, maintained a similar activity profile, being a potent inhibitor of both the hCA isoforms (K_i= 7 and 8.9 nM). Concerning the effects of the 6-benzylureido (**26**) and 6-phenylureido (**27**) moieties, they resemble those discussed above for the corresponding compounds **12-13**, lacking the 7-chloro substituent. In fact, the 6-benzylureido derivative **26** is a potent dual inhibitor of the targeted hCAs and it is more active than the 6-phenylureido **25**. The latter, in any case, showed a good hCA IX potency (K_i= 42.8 nM) and 17-fold selectivity versus the hCA XII isoform.

The 3-methoxy-quinazoline derivatives **28** was synthesized to evaluate the importance of the 3-hydroxy group in this series of hCA inhibitors as we hypothesized that this group might be involved in the coordination of the zinc ion in the catalytic site. The complete inactivity of **28** at the hCA XII

and the significantly reduced potency against the hCA IX, with respect to the 3-hydroxy derivative **4**, point out the important role of the hydroxy substituent and seems to confirm the hypothesis that this group, partially deprotonated at physiological pH, together with the 2- or 4-carbonyl function of the inhibitor, might coordinate the zinc ion. Actually, also the 3-methoxy group might interact with the metal ion but with lower strength than that of the 3-hydroxyl, due to both electronic and steric factors. When the 3-hydroxy group of derivative **14** was alkylated with an acetic chain (derivative **29**) the ability to inhibit the targeted enzymes remained almost the same, compound **29** and the respective 3-hydroxy derivative **14** being equally active towards both the isoforms.

Anti-proliferative assays. Some selected compounds (**3**, **5**, **6**, **12**, **14**, **15** and **19**), chosen among those possessing the best inhibitory profile, were tested (30 – 300 μM) to evaluated their effects on viability of human colon cancer HT-29 cells, over the time (16 – 72 h) in both normoxic and hypoxic conditions. The efficacy of the tested compounds at the lower concentration (30 μM) was reassumed in Figure 1. Cell viability was compared after 48 h treatment of each compound. Derivatives **12**, **14**, **15** and **19** were significantly active inducing a mortality by about 50%. Interestingly, no differences were highlighted between normoxic and hypoxic environments. Table 2 shows the detailed results obtained over time. The 72 h long incubation of cells with the tested derivatives highlighted the activity of **12**, **14**, **15** and **19** at 30 μM, while **5** and **6** were effective starting from 100 and 300 μM, respectively. Hypoxia did not modify activities. To note, a decrease of efficacy was measured at high concentrations after long incubation. This phenomena could be related to a reduced stability of the tested solutions rather than an intrinsic variation of efficacy. After 16 h incubation, a significant decrease of cell viability was induced by **12** only (Supplementary Table S1). It should be mentioned that it is difficult to explain why some of these compounds showed such significant cytotoxic effects not only in hypoxia (when hCA IX is overexpressed) but also in normoxia, when the enzyme is not present in these cells. Thus, it is not improbable that, besides interference with the hCA IX/XII activity in hypoxia, they may exert their

cytotoxic effects through other mechanisms of action, not yet identified, involving pathways in normoxia. This behavior was in fact observed also for some sulfocoumarins, hCA inhibitors possessing a diverse inhibition mechanism compared to the zinc binders, as they anchor to the zinc-coordinated water molecule.[10a,20]

CONCLUSION

This study has led to the discovery of the 3-hydroxyquinazolin-2,4-dione as a new scaffold for the development of potent inhibitors of the tumor-associated hCAs IX and XII. Several herein reported derivatives showed high inhibitory activity against these two hCA isoforms with K_i values in the low nanomolar range while they were ineffective in inhibiting the cytosolic enzymes hCAs I and II. Some compounds proved to be effective as antiproliferative agents on HT-29 colon cancer cell lines, both in normoxic and hypoxic conditions. This finding let us to hypothesize for these derivatives more than one mechanism of action, involving hCAs IX and XII in hypoxia and other not identified target(s) in normoxia.

EXPERIMENTAL PROCEDURES

Chemistry. All the commercially available reagents and solvents were used as purchased from Sigma-Aldrich and Alfa Aesar (Italy) without further purification. Analytical silica gel plates (Merck F254) and silica gel 60 (Merck, 70-230 mesh) were used for analytical TLC and for column chromatography, respectively. All melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Elemental analyses were performed with a Flash E1112 Thermofinnigan elemental analyzer for C, H, N and the results were within ± 0.4% of the theoretical values. All final compounds revealed purity not less than 95%. The IR spectra were recorded with a Perkin-Elmer Spectrum RX I spectrometer in Nujol mulls and are expressed in cm^{-1}. NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz for ^1H NMR). The chemical shifts are reported in δ (ppm) and are relative to the central peak of the solvent which was

$CDCl_3$ or $DMSOd_6$. The following abbreviations are used: s= singlet, d= doublet, t= triplet, q= quartet, m= multiplet, br= broad and ar= aromatic protons.

General Procedure for the Synthesis of 3-Hydroxy-1H-quinazoline-2,4(1H,3H)-dione derivatives 1-6. The title compounds **1-6** were prepared as previously described for **7**,[14] i.e. by heating at reflux for about 3-5 h a suspension of the 3-benzyloxy-5,7-dione derivatives **43-48** (3.3 mmol) in 48% hydrobromic acid (6 mL) and glacial acetic acid (6 mL). The resulting solution was cooled at room temperature and diluted with water (50 mL), the obtained solid was collected by filtration, washed with water and recrystallized.

*3-Hydroxy-1H-quinazoline-2,4(1H,3H)-dione (**1**).*[13] Yield 75%; mp > 300 °C (2-Methoxyethanol). ^{1}H NMR ($DMSOd_6$) 7.20-7.24 (m, 2H, ar), 7.66 (t, 1H, J = 8.0 Hz), 7.94 (d, 1H, ar, J = 8.0 Hz).

*6-Chloro-3-hydroxy-1H-quinazoline-2,4(1H,3H)-dione (**2**).* Yield 95%; mp > 300°C (2-Methoxyethanol). ^{1}H NMR ($DMSOd_6$) 7.22 (d, 1H, ar, J = 8.8 Hz), 7.71 (dd, 1H, ar, J = 2.5 and 8.8 Hz), 7.88 (d, 1H, H-5, J = 2.5 Hz), 10.68 (br s, 1H, OH), 11.68 (br s, 1H, NH). Anal. Calcd for $C_8H_5ClN_2O_3$.

*3-Hydroxy-6-nitro-1H-quinazoline-2,4(1H,3H)-dione (**3**).* Yield 85%; mp 273-275 °C (Acetonitrile). ^{1}H NMR ($DMSOd_6$) 7.36 (d, 1H, ar, J = 8.4 Hz), 8.45 (dd, 1H, ar, J = 2.5 and 8.8 Hz), 8.65 (d, 1H, H-5, J = 2.5 Hz), 10.87 (br s, 1H, OH), 12.17 (br s, 1H, NH). IR 1691, 1714, 3200-3500. Anal. Calcd for $C_8H_5N_3O_5$.

*7-Chloro-3-hydroxy-1H-quinazoline-2,4(1H,3H)-dione (**4**).* Yield 80%; mp >300 °C (EtOH). ^{1}H NMR ($DMSOd_6$) 7.18 (d, 1H, ar, J = 1.8 Hz), 7.25 (dd, 1H, ar, J = 8.4 and 1.8 Hz), 7.92 (d, 1H, ar, J= 8.4 Hz), 10.70 (br s, 1H, OH), 11.70 (br s, 1H, NH). Anal. Calcd for $C_8H_5ClN_2O_3$.

*6,7-Dichloro-3-hydroxy-1H-quinazoline-2,4(1H,3H)-dione (**5**).* Yield 72%; mp >300 °C (EtOH). ^{1}H NMR ($DMSOd_6$) 7.34 (s, 1H, ar), 8.03 (s, 1H, ar), 10.75 (br s, 1H, OH), 11.75 (br s, 1H, NH). Anal. Calcd for $C_8H_4Cl_2N_2O_3$.

*5,7-Dichloro-3-hydroxy-1H-quinazoline-2,4(1H,3H)-dione (**6**).* Yield 70%; mp °C (EtOH). ^{1}H NMR ($DMSOd_6$) 7.15 (d, 1H, ar, J = 1.0 Hz), 7.38 (d, 1H, ar, J = 1.0 Hz), 10.63 (br s, 1H, OH), 11.75 (br s, 1H; NH). IR 1680, 1750, 3415. Anal. Calcd for $C_8H_4Cl_2N_2O_3$.

6-Amino-3-hydroxy-1H-quinazoline-2,4(1H,3H)-dione (8). A suspension of the 3-benzyloxy-derivative **43** (3.3 mmol) in 48% hydrobromic acid (6 mL) and glacial acetic acid (6 mL) was heated at reflux for 6 h. The solvent was distilled off at reduced pressure and the resulting residue was treated with water (30 mL) and neutralized with a $NaHCO_3$ saturated solution. The obtained solid was collected by filtration, washed with water and recrystallized. Yield 70%; mp > 300 °C (DMF). ^{1}H NMR ($DMSOd_6$) 5.20 (br s, 2H, NH_2), 6.93 (br s, 2H, ar), 7.10 (s, 1H, ar), 10.40 (br s, 1H, OH), 11. 08 (br s, 1H, OH). Anal. Calcd for $C_8H_7N_3O_3$.

General Procedure for the Synthesis of 3-Hydroxy-1H-quinazoline-2,4(1H,3H)-dione derivatives 9-13. 10% Pd/C was added (10% w/w with respect to the 3-benzyloxy derivative) to a solution of **50-52** (0.87 mmol) in hot EtOH (about 250 mL) or **53**, **54** (0.87 mmol) in DMF (5 mL). The mixture was hydrogenated in a Parr apparatus at 40-45 psi for 4-6 h (for **9**, **10**, **12**, **13**) and 24 h (for **11**), then the catalyst was filtered off. Evaporation of ethanol at reduced pressure or dilution of the DMF solution with water (about 20 mL) gave a solid which was collected and recrystallized.

*3-Hydroxy-6-(1,2,4-triazol-4-y1)-1H-quinazoline-2,4(1H,3H)-dione (**9**).* Yield 55%; mp> 300 °C (AcOH/DMF); ^{1}H NMR ($DMSOd_6$) 7.33 (d, 1H, ar, J = 8.8 Hz), 7.98 (dd, 1H, ar, J = 2.6 and 8.8 Hz), 8.28 (d, 1H, H-5, J = 2.6 Hz), 9.17 (s, 2H, s, triazole proton), 10.72 (br s, 1H, OH), 11.76 (br s, 1H, NH). Anal. Calcd for $C_{10}H_7N_5O_3$.

*6-(3-Carboxypropanoylamino)-3-hydroxy-1H-quinazoline-2,4(1H,3H)-dione (**10**).* Yield 87%; mp 247-249 °C (2-Methoxyethanol/DMF). ^{1}H NMR ($DMSOd_6$) 2.55-2.58 (m, 4H, $2CH_2$), 7.14 (d, 1H, ar, J = 8.8 Hz), 7.77 (dd, 1H, ar, J = 2.3 and 8.8 Hz), 8.28 (d, 1H, H-5, J = 2.3 Hz), 10.16 (s, 1H,

NH), 10.54 (br s, 1H, OH), 11.47 (br s, 1H, NH), 12.14 (br s, 1H, OH). Anal. Calcd for $C_{12}H_{11}N_3O_6$.

*6-(2-Carboxybenzoylamino)-3-hydroxy-1H-quinazoline-2,4(1H,3H)-dione (**11**)*. Yield 72%; mp > 300 °C (EtOAc/MeOH). ^{1}H NMR (DMSOd_6) 7.18 (d, 1H, ar, J = 8.8 Hz), 7.56-7.68 (m, 3H, ar), 7.83-7.89 (m, 2H, ar), 8.42 (s, 1H, H-5), 10.57 (br s, 2H, NH + OH), 11.51 (s, 1H, NH), 13.07 (br s, 1H, COOH). Anal. Calcd for $C_{16}H_{11}N_3O_6$.

*6-(3-Benzylureido)-3-hydroxy-1H-quinazoline-2,4(1H,3H)-dione (**12**)*. Yield 80%; mp> 300 °C (2-Methoxyethanol). ^{1}H NMR (DMSOd_6) 4.30 (d, 2H, CH_2, J = 5.8 Hz), 6.66 (t, 1H, NH, J = 5.9 Hz), 7.09 (d, 1H, ar, J = 8.8 Hz), 7.22-7.35 (m, 5H, ar), 7.60 (dd, 1H, ar, J = 2.4 and 8.8 Hz), 8.11 (d, 1H, ar, J = 2.4 Hz), 8.80 (s, 1H, NH), 10.51 (br s, 1H, OH), 11.39 (br s, 1H, NH). Anal. Calcd for $C_{16}H_{14}N_4O_4$.

*6-(3-Phenylureido)-3-hydroxy-1H-quinazoline-2,4(1H,3H)-dione (**13**)*. Yield 78%; mp > 300 °C (DMF/H_2O). ^{1}H NMR (DMSOd_6) 7.01 (t, 1H, ar, J = 7.4 Hz), 7.19 (d, 1H, ar, J = 8.8 Hz), 7.32 (t, 2H, ar, J = 7.5 Hz), 7.49 (d, 2H, ar, J = 7.5 Hz), 7.67 (d, 1H, ar, J = 8.8 Hz), 8.20 (s, 1H, ar), 8.70 (br s, 1H, NH), 8.87 (br s, 1H, NH), 10.55 (br s, 1H, OH), 11.42 (br s, 1H, NH). Anal. Calcd for $C_{15}H_{12}N_4O_4$.

General Procedure for the Synthesis of 3-Hydroxy-1H-quinazoline-2,4(1H,3H)-dione derivatives 14, 16 and **20**. A suspension of the 3-acetoxy derivatives **56-58** (0.42 mmol) in aqueous 2.5% NaOH (3 mL) was stirred at room temperature for 1 h and then neutralized with 6N HCl. The resulting solid was collected, washed with water and recrystallized.

*7-Chloro-3-hydroxy-6-nitro-1H-quinazoline-2,4(1H,3H)-dione (**14**)*. Yield 50%; mp 287-289 °C (EtOH); ^{1}H NMR (DMSOd_6) 7.34 (s, 1H, ar), 8.55 (s, 1H, ar), 10.9 (s, 1H, OH), 12.22 (br s, 1H, NH). Anal. Calcd for $C_8H_4ClN_3O_5$.

*6-Amino-7-chloro-3-hydroxy-1H-quinazoline-2,4(1H,3H)-dione (**16**)*. Yield 55%; mp > 300 °C (EtOAc); ^{1}H NMR ($DMSOd_6$) 5.44 (br s, 2H, NH_2), 7.06 (s, 1H, ar), 7.34 (s, 1H, ar), 10.52 (br s, 1H, OH), 11.23 (br s, 1H, NH). Anal. Calcd for $C_8H_6ClN_3O_3$.

*7-Chloro-3-hydroxy-6-(1-imidazolyl)-1H-quinazoline-2,4(1H,3H)-dione (**20**).* Yield 49%; mp > 300 °C (2-Methoxyethanol); ^{1}H NMR ($DMSOd_6$) 7.11 (s, 1H, imidazole proton), 7.41 (s, 1H, ar), 7.45 (s, 1H, imidazole proton), 7.91 (s, 1H, imidazole proton), 8.17 (s, 1H, ar), 10.78 (br s, 1H, OH), 11.89 (br s, 1H, NH). Anal. Calcd for $C_{11}H_7ClN_4O_3$.

7-Chloro-3-hydroxy-6-(3-phenylureido)-1H-quinazoline-2,4(1H,3H)-dione (27). The title compound was prepared by catalytic hydrogenation of the 3-benzyloxy derivative **63** (0.87 mmol) in DMF solution (2.5 ml), in the same condition described above to prepare compounds **9-13**. The crude compound was purified by crystallization. Yield 62%; mp > 300 °C (DMF/ H_2O) ^{1}H NMR ($DMSOd_6$) 7.01 (t, 1H, ar, J = 7.4 Hz), 7.27 (s, 1H, ar), 7.31 (t, 2H, ar, J = 7.4 Hz), 7.48 (d, 2H, ar, J = 8.1 Hz), 8.4 (s, 1H, ar), 8.69 (br s, 1H, NH), 9.37 (br s, 1H, NH), 10.63 (br s, 1H, OH), 11.52 (br s, 1H, NH). Anal. Calcd for $C_{15}H_{11}ClN_4O_4$.

7-Chloro-3-methoxy-1H-quinazoline-2,4(1H,3H)-dione (28). Triethylamine (8.75 mmol) was dropwise added to a solution of derivative **64** (3.5 mmol) and triphosgene (2.45 mmol) in anhydrous tetrahydrofuran (20 mL). The mixture was stirred at room temperature for about 15 h, then most of the solvent evaporated at reduced pressure. The residue was treated with water (about 30-40 mL) to give a solid which was collected, washed with water and recrystallized. Yield 70%; mp 280-282 °C (EtOH) ^{1}H NMR ($DMSOd_6$) 3.88 (s, 3H, OCH_3), 7.19 (s, 1H, H-8), 7.27 (d, 1H, ar, J = 8.5 Hz), 7.94 (d, 1H, ar, J = 8.5 Hz), 11.72 (br s, 1H, NH) Anal. Calcd for $C_9H_7ClN_2O_3$.

(7-Chloro-2,4-dioxo-2,4-dihydro-6-nitro-1H-quinazolin-3-yl)oxyacetic acid (29). A suspension of the ethyl ester **66** (1.0 mmol) in 2.5% aqueous NaOH (20 mL) was stirred at room temperature for 1 h. The cooled (0 °C) solution was acidified to pH 1 with 6N HCl and the resulting solid was

collected, abundantly washed with water and recrystallized. Yield 95%; mp 263-265 °C (EtOH); ^{1}H NMR (DMSOd_6) 4.64 (s, 2H, CH_2), 7.35 (s, 1H, ar), 8.56 (s, 1H, ar), 12.25 (s, 1H, NH), 13.20 (br s, 1H, OH). IR 1680, 1730, 1770, 3300-3100. Anal. Calcd for $C_{10}H_6ClN_3O_7$.

General Procedure for the Synthesis of 2-Amino-N-(benzyloxy)-arylamide derivatives 37-42.

The title compounds were prepared as described for 2-amino-N-(benzyloxy)arylamides **37** and **39**.[17] Briefly, triethylamine (4.3 mmol) was added to a suspension of O-benzylhydroxylamine hydrochloride (4.3 mmol) in ethanol (15 mL). The proper 2,4-dihydro-1H-3,1-benzoxazine-2,4-dione derivative **30-35** (4.3 mmol), commercially available with the exceptions of the 6,7-dichloro- **34**[15] and 5,7-dichloro- **35**,[16] was added to the solution. The mixture was heated at reflux for about 3 h, then cooled at room temperature and diluted with water (about 50 mL) to give a solid that was collected, washed with water and recrystallized.

*2-Amino-N-(benzyloxy)-5-chlorobenzamide **(38)**.* Yield 35%; mp 159-160 °C (Cyclohexane/EtOAc). ^{1}H NMR (DMSOd_6) 4.90 (s, 2H, CH_2), 6.43 (br s, 2H, NH_2), 6.73 (d, 1H, ar, J = 8.8 Hz), 7.18 (d, 1H, ar, J = 8.5 Hz), 7.33-7.46 (m, 6H, ar). 11.55 (br s, 1H, NH). Anal. Calcd for $C_{14}H_{13}ClN_2O_2$.

*2-Amino-N-(benzyloxy)-4-chlorobenzamide **(40)**.* Yield 95%; mp 140-142 °C (EtOH). ^{1}H NMR (DMSOd_6) 4.88 (s, 2H, CH_2), 6.47 (d, 1H, ar, J = 2.2 Hz), 6.51 (br s, 2H, NH_2), 6.75 (d, 1H, ar, J = 2.2 Hz), 7.27-7.42 (m, 6H, ar), 11.46 (br s, 1H, NH). Anal. Calcd for $C_{14}H_{13}ClN_2O_2$.

*2-Amino-N-(benzyloxy)-4,5-dichlorobenzamide **(41)**.* Yield 75%; mp 158-160 °C (Cyclohexane/EtOAc). ^{1}H NMR (DMSOd_6) 4.88 (s, 2H, CH_2), 6.60 (br s, 2H, NH_2), 6.93 (d, 1H, ar, J= 2.9 Hz), 7.35-7.48 (m, 6H, ar), 11.60 (br s, 1H, NH). Anal. Calcd for $C_{14}H_{12}Cl_2N_2O_2$.

*2-Amino-N-(benzyloxy)-4,6-dichlorobenzamide **(42)**.* Yield 83%; mp 150-152 °C (Cyclohexane/EtOAc). ^{1}H NMR (DMSOd_6) 4.95 (s, 2H, CH_2), 5.55 (br s, 2H, NH_2), 6.66-6.68 (m, 2H, ar), 7.33-7.42 (m, 5H, ar), 11.65 (s, 1H, NH). Anal. Calcd for $C_{14}H_{12}Cl_2N_2O_2$.

General Procedure for the Synthesis of 3-Benzyloxy-1H-quinazoline-2,4(1H,3H)-dione derivatives 43-48. Triethylamine (3.29 mol) was dropwise added to a solution of 2-amino-N-(benzyloxy)arylamides **37-42** (1.37 mmol) and triphosgene (0.53 mmol) in anhydrous tetrahydrofuran (20 mL). The mixture was stirred at room temperature for about 1-4 h, then it was diluted with water (about 50 mL) to give a solid which was collected, washed with water and recrystallized.

*3-Benzyloxy-1H-quinazoline-2,4(1H,3H)-dione (**43**)*[13] (noto ma fatto per altra via). Yield 75%; mp 207-209 °C (MeOH). ^{1}H NMR ($DMSOd_6$) 5.10 (s, 2H, CH_2), 7.21-7.26 (m, 2H, ar), 7.40-7.44 (m, 3H, ar), 7.57-7.59 (m, 2H, ar), 7.69 (t, 1H, ar, J = 8.4 Hz), 7.97 (d, 1H, ar, J = 7.7 Hz), 11.65 (br s, 1H, NH). Anal. Calcd for $C_{15}H_{12}N_2O_3$.

*3-Benzyloxy-6-chloro-1H-quinazoline-2,4(1H,3H)-dione (**44**).* Yield 71%; mp 254-255 °C (EtOAc). ^{1}H NMR ($DMSOd_6$) 5.10 (s, 2H, CH_2), 7.23 (d, 1H, ar, J = 8.7 Hz), 7.56-7.58 (m, 5H, ar), 7.57 (d, 1H, ar, J = 6.7 Hz), 7.74 (dd, 1H, H-5, J = 2.4 and 8.7 Hz), 11.80 (br s, 1H, NH). Anal. Calcd for $C_{15}H_{11}ClN_2O_3$.

*3-Benzyloxy-6-nitro-1H-quinazoline-2,4(1H,3H)-dione (**45**).* Yield 65%; mp 298-299 °C (CH_3NO_2). ^{1}H NMR ($DMSOd_6$) 5.13 (s, 2H, CH_2), 7.36 (d, 1H, ar, J = 9.0 Hz), 7.42-7.46 (m, 5H, ar), 7.59 (d, 1H, ar, J = 7.3 Hz), 8.49 (dd, 1H, ar, J = 2.7 and 9.0 Hz), 8.65 (d, 1H, H-5, J = 2.7 Hz). Anal. Calcd for $C_{15}H_{11}N_3O_5$.

*3-Benzyloxy-7-chloro-1H-quinazoline-2,4(1H,3H)-dione (**46**).* Yield 92%; mp 294-295 °C (EtOH). ^{1}H NMR ($DMSOd_6$) 5.10 (s, 2H, CH_2), 7.22 (s, 1H, ar), 7.30 (d, 1H, ar, J = 8.5 Hz), 7.40-7.43 (m, 3H, ar), 7.56-7.58 (m, 2H, ar), 7.97 (d, 1H, ar, J = 8.5 Hz), 11.77 (br s, 1H, NH). Anal. Calcd for $C_{15}H_{11}ClN_2O_3$.

*3-Benzyloxy-6,7-dichloro-1H-quinazoline-2,4(1H,3H)-dione (**47**).* Yield 85%; mp 245-246 °C (EtOH) ^{1}H NMR ($DMSOd_6$) 5.09 (s, 2H, CH_2), 7.39-7.42 (m, 4H, ar), 7.54-7.56 (m, 2H, ar), 7.95 (d, 1H, ar, J = 8.4 Hz), 11.75 (br s, 1H, NH). Anal. Calcd for $C_{15}H_{10}Cl_2N_2O_3$.

*3-Benzyloxy-5,7-dichloro-1H-quinazoline-2,4(1H,3H)-dione (**48**)*. Yield 87%; mp 264-265 °C (EtOH) ^{1}H NMR (DMSOd_6) 5.05 (s, 2H, CH_2), 7.16 (d, 1H, ar, J= 1.1 Hz), 7.40-7.42 (m, 4H, ar), 7.55-7.57 (m, 2H, ar), 11.90 (br s, 1H, NH). Anal. Calcd for $C_{15}H_{10}Cl_2N_2O_3$.

6-Amino-3-benzyloxy-1H-quinazoline-2,4(1H,3H)-dione (49). A suspension of 3-benzyloxy-6-nitro-1H-quinazoline-2,4-dione **45** (2.72 mmol) and $SnCl_2$ (16.3 mmol) in anhydrous EtOH (10 mL) was heated at reflux for 3 h, then most of the solvent was removed under vacuum. The resulting mixture was treated with water (15 mL) and neutralized with a $NaHCO_3$ saturated solution. The obtained solid was collected by filtration, then, to separate the desired compound **49** from inorganic salts, it was treated with three-four portions of boiling EtOAc (30 mL). From the collected organic solutions the solvent was distilled off at reduced pressure to yield a solid which was recrystallized. Yield 65%; mp 231-233 °C (EtOH). ^{1}H NMR (DMSOd_6) 5.07 (s, 2H, CH_2), 5.45 (br s, 2H, NH_2), 6.94-7.00 (m, 2H, ar), 7.15 (s, 1H, H-5), 7.41-7.42 (m, 3H, ar), 7.54-7.56 (m, 2H, ar), 11.26 (br s, 1H, NH) Anal. Calcd for $C_{15}H_{13}N_3O_3$.

3-Benzyloxy-6-(1,2,4,triazol-4-y1)-1H-quinazoline-2,4(1H,3H)-dione (50). Diformylhydrazine (3.72 mmol) and then, drop by drop, trimethylsilylchloride (18.68 mmol) and triethylamine (8.68 mmol) was added to suspension of the 6-amino derivative **49** (1.24 mmol) in anhydrous pyridine (10 mL). The solution was heated at reflux for 22 h, then most of the solvent was distilled off at reduced pressure. The residue was added with water (about 20 mL) to yield a solid which was collected and recrystallized. Yield 84%; mp > 300 °C (EtOAc/EtOH); ^{1}H NMR (DMSOd_6) 5.13 (s, 2H, CH_2), 7.34 (d, 1H, ar, J = 8.8 Hz), 7.42-7.45 (m, 3H, ar), 7.58-7.59 (m, 2H, ar), 8.01 (dd, 1H, ar, J = 2.5 and 8.8 Hz), 8.23 (d, 1H, H-5, J = 2.5 Hz), 9.19 (s, 2H, s, triazole proton), 11.87 (br s, 1H, NH). Anal. Calcd for $C_{17}H_{13}N_5O_3$.

General Procedure for the Synthesis of 6-Acylamino--3-benzyloxy-1H-quinazoline-2,4-diones 51-52. A mixture of the 6-amino derivative **49** (1.06 mmol), succinic anhydride (1.06 mmol) or phthalic anhydride (3.18 mmol) and sodium acetate (1.06 mmol) in glacial acetic acid (5 mL) was heated at 60° C for about 6 h. After cooling at room temperature, the suspension was diluted with water (30 mL) and acidified to pH 4 with 2N HCl to give a solid which was collected, washed with water, dried and recrystallized (compound **51**). To obtain compound **52**, the reaction mixture was filtered and the isolated solid was stirred in 2.5 % aqueous NaOH (10 mL) at room temperature for about 30 min. The suspension was acidified to pH 1 with 6N HCl and the resulting solid was collected, washed with water and recrystallized.

*3-Benzyloxy-6-(3-carboxypropanoylamino)-1H-quinazoline-2,4(1H,3H)-dione (**51**).* Yield 86%; mp 288-290 °C (EtOH). ^{1}H NMR (DMSOd_6) 2.55-2.57 (m, 4H, 2CH_2), 5.10 (s, 2H, CH_2), 7.15 (d, 1H, ar, J = 8.8 Hz), 7.39-7.44 (m, 3H, ar), 7.57-7.59 (m, 2H, ar), 7.81 (dd, 1H, ar, J = 2.2 and 8.8 Hz), 8.28 (d, 1H, H-5, J = 2.1 Hz), 10.27 (s, 1H, NH), 11.59 (br s, 1H, NH), 12.14 (br s, 1H, OH). Anal. Calcd for $C_{19}H_{17}N_3O_6$.

*3-Benzyloxy-6-(2-carboxybenzoylamino)-1H-quinazoline-2,4(1H,3H)-dione (**52**).* Yield 83%; mp > 300 °C (EtOH) ^{1}H NMR (DMSOd_6) 5.12 (s, 2H, CH_2), 7.19 (d, 1H, ar, J = 8.8 Hz), 7.35-7.45 (m, 3H, ar), 7.57-7.69 (m, 4H, ar), 7.89 (d, 1H, ar, J = 6.9 Hz), 8.40 (d, 2H, ar, J = 2.2 Hz), 8.42 (d, 1H, H-5, J= 2.2 Hz), 10.57 (br s, 1H, NH), 11.63 (br s, 1H, NH), 13.09 (br s, 1H, COOH). Anal. Calcd for $C_{23}H_{17}N_3O_6$.

General Procedure for the Synthesis of 3-Benzyloxy-6-ureido-1H-quinazoline-2,4(1H,3H)-dione derivatives 53-54. A mixture of the 6-amino derivative **49** (1.59 mmol) and benzyl- or phenyl-isocyanate (2.54 mmol) in anhydrous tetrahydrofuran (10 mL) was refluxed for 3 h. The suspension was cooled at room temperature and the solvent evaporated at reduced pressure. The residue was treated with Et_2O (20 mL) and the solid obtained was collected by filtration and recrystallized.

*3-Benzyloxy-6-(3-benzylureido)-1H-quinazoline-2,4(1H,3H)-dione derivative (**53**)*. Yield 68%; mp > 300 °C (2-Methoxyethanol). ^{1}H NMR (DMSOd_6) 4.30 (d, 2H, CH_2, J = 5.8 Hz), 5.09 (s, 2H, CH_2O), 6.66 (t, 1H, NH, J = 5.8 Hz), 7.11 (d, 1H, J = 8.8 Hz), 7.24-7.44 (m, 8H, 7ar + NH), 7.56-7.58 (m, 2H, ar), 7.65 (dd, 1H, ar, J = 2.4 and 8.8 Hz), 8.11 (d, 1H, J = 2.4 Hz), 8.80 (s, 1H, NH), 11.50 (br s, 1H, NH). Anal. Calcd for $C_{23}H_{20}N_4O_4$.

*3-Benzyloxy-6-(3-phenylureido)-1H-quinazoline-2,4(1H,3H)-dione derivative (**54**)*. Yield 77%; mp > 300 °C (EtOH/DMF). ^{1}H NMR (DMSOd_6) 5.11 (s, 2H, CH_2), 6.97 (t, 1H, ar, J = 7. 2 Hz), 7.17 (d, 1H, ar, J = 8.7 Hz), 7.29 (t, 2H, ar, J = 7.6 Hz), 7.42-7.48 (m, 5H, ar), 7.57-7.59 (m, 2H, ar, J = 7.4 Hz), 7.67 (d, 1H, ar, J = 8.7 Hz), 8.16 (s, 1H, ar), 8.70 (br s, 1H, NH), 8.90 (br s, 1H, NH), 11.55 (br s, 1H, NH). Anal. Calcd for $C_{22}H_{18}N_4O_4$.

3-Acetoxy-7-chloro-1H-quinazoline-2,4(1H,3H)-dione (55). A suspension of derivative **4** (1.4 mmol) in acetic anhydride (15 mL) was refluxed for 2 h. Evaporation of the excess of acetic anhydride at reduced pressure gave a residue that was stirred with water (40 mL). The solid obtained was collected, washed with water and recrystallized. Yield 85%; mp 266-268 °C (EtOH); ^{1}H NMR (DMSOd_6) 2.39 (s, 3H, CH_3), 7.27 (s, 1H, H-8), 7.35 (d, 1H, ar, J = 8.5 Hz), 7.95 (d, 1H, ar, J = 8.5 Hz), 12.07 (br s, 1H, NH). Anal. Calcd for $C_{10}H_7ClN_2O_4$.

3-Acetoxy-7-chloro-6-nitro-1H-quinazoline-2,4(1H,3H)-dione (56). Compound **55** (7.9 mmol) was portion wise added to a cold (-10 °C) solution of 90% HNO_3 (25 mL). After the addition was completed, the solution was stirred at -10 °C for about 30 min, then it was poured onto iced water (about 30-50 mL) and the solid obtained was collected by filtration, abundantly washed with water, dried and recrystallized. Yield 85 %; mp 264-266 °C (EtOH); ^{1}H NMR (DMSOd_6) 2.41 (s, 3H, CH_3), 7.41 (s, 1H, ar), 8.59 (s, 1H, ar), 12.6 (br s, 1H, NH). Anal. Calcd for $C_{10}H_6ClN_3O_6$.

3-Acetoxy-6-amino-7-chloro-1H-quinazoline-2,4(1H,3H)-dione (57). A suspension of the 6-nitro derivative **56** (3.34 mmol) and $SnCl_2$ (20 mmol) in anhydrous EtOH (20-30 mL) was heated at reflux for 3 h, then most of the solvent was removed under vacuum. The resulting mixture was treated with water (15 mL) and neutralized with a $NaHCO_3$ saturated solution. The obtained solid was collected by filtration and then, to separate the desired compound **57** from inorganic salts, it was treated with three-four portions of boiling EtOAc (about 30 mL). From the collected organic solutions the solvent was distilled off at reduced pressure to yield a solid which was recrystallized. Yield 54%; mp 246-248 °C (EtOH); ^{1}H NMR ($DMSOd_6$) 2.37 (s, 3H, CH_3), 5.58 (br s, 2H, NH_2), 7.12 (s, 1H, ar), 7.35 (s, 1H, ar), 11.60 (s, 1H, NH). Anal. Calcd for $C_{10}H_8ClN_3O_4$.

3-Acetoxy-7-chloro-6-(1-imidazolyl)-1H-quinazoline-2,4(1H,3H)-dione (58). Equimolar amounts (2.96 mmol) of formaldehyde, glyoxal and ammoniun acetate were added to a suspension of the 6-amino-derivative **57** (1.18 mmol). The mixture was stirred at 50°C for 4h, then cooled at room temperature, diluted with water (about 20 mL) and neutralized with a $NaHCO_3$ saturated solution. The mixture was extracted with EtOAc (30 mL x 4) and the organic phase was washed with water (50 mL) and anhydrified (Na_2SO_4). Evaporation of the solvent at reduced pressure gave a solid which was used as such for the next step. Yield 60%; ^{1}H NMR ($DMSOd_6$) 2.09 (s, 3H, CH_3), 7.11 (s, 1H, imidazole proton), 7.41-7.46 (m, 2H, 1 ar + 1 imidazole proton), 7.91-7.95 (m, 2H, 1 ar + 1 imidazole proton), 10.81 (br s, 1H, NH). Anal. Calcd for $C_{13}H_9ClN_4O_4$.

2-Amino-N-(benzyloxy)-4-chloro-5-nitrobenzamide (60). The title compound was prepared by reacting 7-chloro-1,4-dihydro-6-nitro-2H-3,1-benzoxazine-2,4-dione **59** (4.3 mmol),[18] prepared by nitration of **33** (4.3 mmol), and the O-benzylhydroxylamine (4.3 mmol) in the experimental condition described above to prepare **37-42**. Yield 85%; mp 168-170 °C (cyclohexane/EtOAc) ^{1}H NMR ($DMSOd_6$) 4.92 (s, 2H, CH_2), 6.94 (s, 1H, ar), 7.39-7.45 (m, 5H, ar), 7.63 (br s, 2H, NH_2), 8.28 (s, 1H, ar), 11.93 (br s, 1H, NH). Anal. Calcd for $C_{14}H_{12}ClN_3O_4$.

3-Benzyloxy-7-chloro-6-nitro-1H-quinazoline-2,4(1H,3H)-dione (61). The title compound was obtained by cyclizing the 2-aminobenzamide derivative **60** (1.37 mmol) with triphosgene (0.53 mmol) in anhydrous tetrahydrofuran, in the same experimental conditions described above to prepare compounds **44-50**. Yield 90%; mp > 300 °C (2-methoxyethanol/DMF) ^{1}H NMR (DMSOd_6) 5.11 (s, 2H, CH_2), 7.39-7.44 (m, 4H, ar) 7.57-7.58 (m, 2H, ar), 8.60 (s, 1H, ar), 12.26 (br s, 1H, NH). Anal. Calcd for $C_{15}H_{10}ClN_3O_5$.

6-Amino-3-benzyloxy-7-chloro-1H-quinazoline-2,4(1H,3H)-dione (62). The title compound was obtained from the corresponding 6-nitro derivative **61** (2.72 mmol) and $SnCl_2$ (16,3 mmol) in anhydrous EtOH (10 mL) in the same experimental conditions described above to prepare derivative **49**. The crude compound was purified by crystallization. Yield 63%; mp > 300 °C (2-methoxyethanol) ^{1}H NMR (DMSOd_6) 5.07 (s, 2H, CH_2), 5.50 (br s, 2H, NH_2), 7.09 (s, 1H, ar), 7.38-7.42 (m, 4H, ar), 7.55-7.57 (m, 2H, ar), 11.31 (br s, 1H, NH). Anal. Calcd for $C_{15}H_{12}ClN_3O_3$.

3-Benzyloxy-7-chloro-6-(3-phenylureido)-1H-quinazoline-2,4(1H,3H)-dione 63. The title compound was prepared by reacting the 6-amino derivative **62** (1.59 mmol) with phenylisocyanate (2.54 mmol) in anhydrous tetrahydrofuran (10 mL) in the same experimental conditions described above for the preparation of compounds **53** and **54**. The crude compound was purified by crystallization. Yield 80%; mp > 300 °C (2-Methoxyethanol) ^{1}H NMR (DMSOd_6) 5.11 (s, 2H, CH_2), 7.02 (t, 1H, ar, J = 7.1 Hz), 7.16-7.32 (m, 3H, ar), 7.42-7.59 (m, 7H, ar), 8.44 (s, 1H, H-5), 8.73 (br s, 1H, NH), 9.40 (br s, 1H, NH) 11.63 (br s, 1H, NH). Anal. Calcd for $C_{22}H_{17}ClN_4O_4$.

2-Amino-4-chloro-N-methoxybenzamide (64). Triethylamine (5.08 mmol) was added to a suspension of O-methylhydroxylamine hydrochloride (5.08 mmol) in ethanol (40 mL). The commercially available 7-chloro-2,4-dihydro-1H-3,1-benzoxazine-2,4-dione derivative **33** (4.3

mmol) was added to the solution and the mixture was heated at reflux for 3 h. After cooling at room temperature, the solvent was evaporated at reduced pressure and the residue was treated with water (about 50 mL) to give a mixture that was extracted with EtOAc (30 mL x 3). The organic phase was anhydrified (Na_2SO_4) and the solvent evaporated under vacuum to afford an oily residue which was purified by column chromatography (eluent cyclohexane/EtOAc/MeOH, 6:4:1). Compound **64** was obtained as an oil that was used as such for the next step. Yield 69%; ^{1}H NMR ($DMSOd_6$) 3.71 (s, 3H, OCH_3), 6.57 (d, 1H, ar, J = 7.3 Hz), 6.59 (br s, 2H, NH_2), 6.88 (s, 1H, H-3), 7.68 (d, 1H, ar, J = 7.3 Hz), 11.50 (br s, 1H, NH).

Ethyl (7-chloro-2,4-dioxo-2,4-dihydro-6-nitro-1H-quinazolin-3-yl)oxyacetate (66). Compound **65**[19] (1.0 mmol) was portion wise added to cool (T= -10 °C) HNO_3 (90%, 4 mL). After the addition was completed, the mixture was stirred for about 30 min, then the solution was poured onto ice (about 50 g). The obtained solid was collected, abundantly washed with water and recrystallized. Yield 87%; mp 193-194 °C (EtOH). ^{1}H NMR ($DMSOd_6$) 1.21 (s, 3H, CH_3, J = 7.0 Hz), 4.17 (q, 2H, CH_2, J = 7.0 Hz), 4.72 (s, 2H, CH_2), 7.35 (s, 1H, ar), 8.56 (s, 1H, ar), 12.25 (br s, 1H, NH). Anal. Calcd for $C_{12}H_{10}ClN_3O_7$.

CA inhibition. An applied photophysics stopped-flow instrument has been used for assaying the hCA catalyzed CO_2 hydration activity.[21] 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 7.4) as buffer, and 20 mM Na_2SO_4 for maintaining constant the ionic strength (this anion is not inhibitory and has a K_i>200mM against these enzymes), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each measurement three traces of the initial 5–10% of the reaction have been used for determining the initial velocity (which was the

mean of the three traces), working with 10-fold decreasing inhibitor concentrations ranging between 0.1nM and 10–100 mM (depending on the inhibitor potency, but at least five points at different inhibitor concentrations were employed for determining the inhibition constants). 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 0.1 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 the Cheng–Prusoff equation, and represent the mean from three independent experiments. All human isoforms were recombinant enzymes produced as described earlier in our laboratory.[22]

Cytotoxicity Assay.

Cell culture and treatments. Human colon cancer cell lines HT-29 were obtained from American Type Culture Collection (Rockville, MD). HT-29 were cultured in DMEM high glucose with 20% FBS in 5% CO_2 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). HT-29 cells were plated in 96-wells cell culture ($1 \cdot 10^4$/well) and, 24 h after, treated with the tested compounds (0-300 μM) for 16, 48 and 72 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 0.1% O2 (hypoxia), 5% CO_2, and residual N_2. In parallel, normoxic (20% O_2) dishes were incubated in air with 5% CO_2.

Cell viability assay. HT-29 cell viability was evaluated by the reduction of 3-(4,5-dimethylthiozol-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.

Supporting information

-Combustion analysis data of the newly synthesized compounds.

-Effect of some selected hCA inhibitors on HT-29 cell viability after 16 h incubation.

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ACKNOWLEDGMENTS. The work was financially supported by the University of Florence.

ABBREVIATIONS USED

CA, carbonic anhydrase; AZA, acetazolamide; MTT, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide

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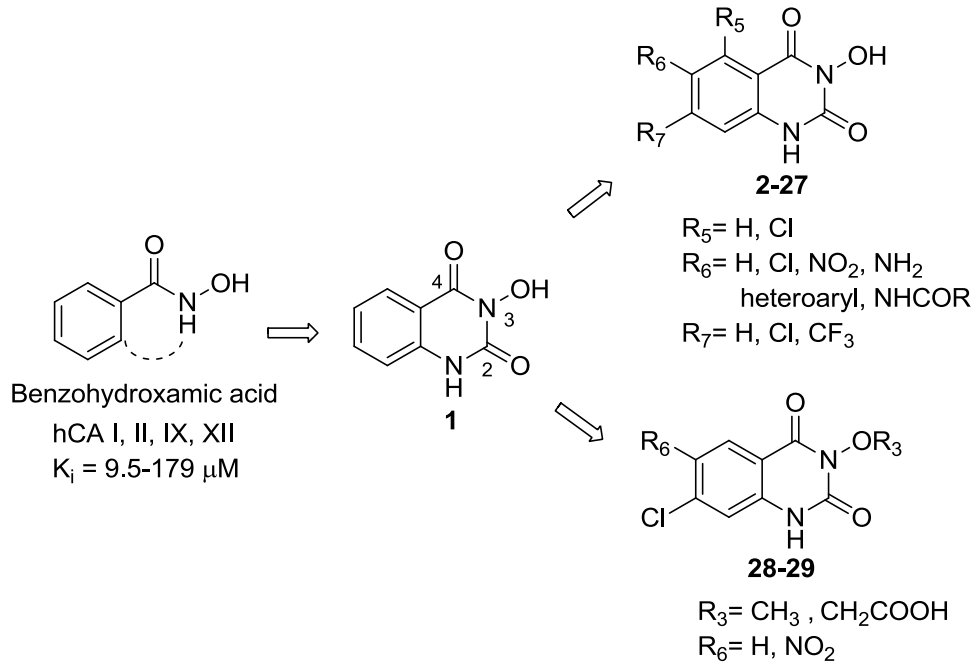
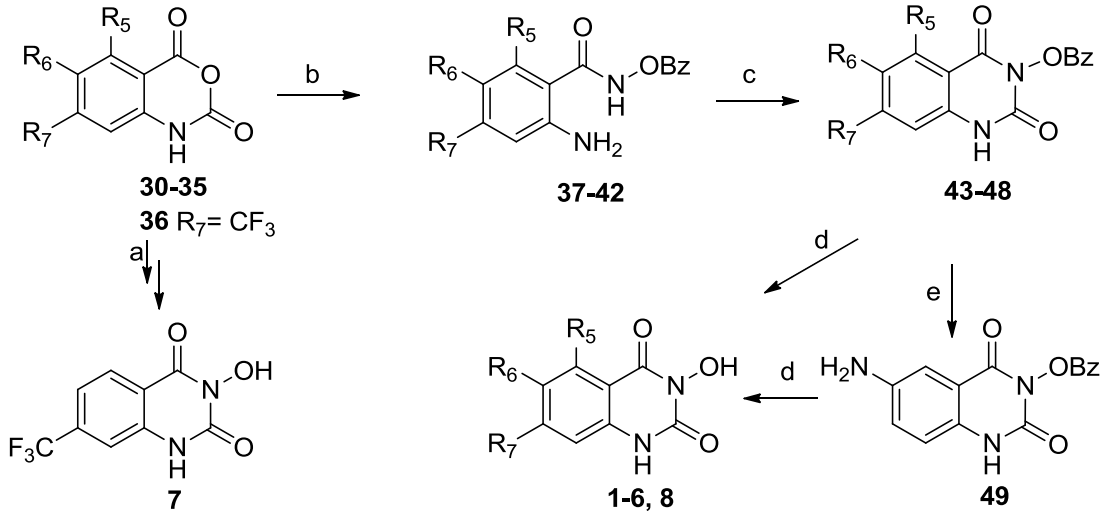
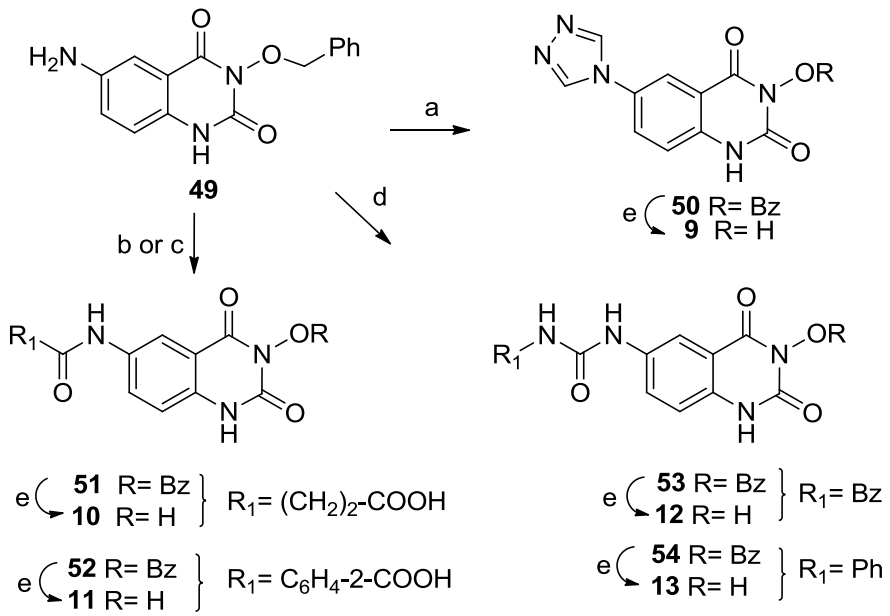


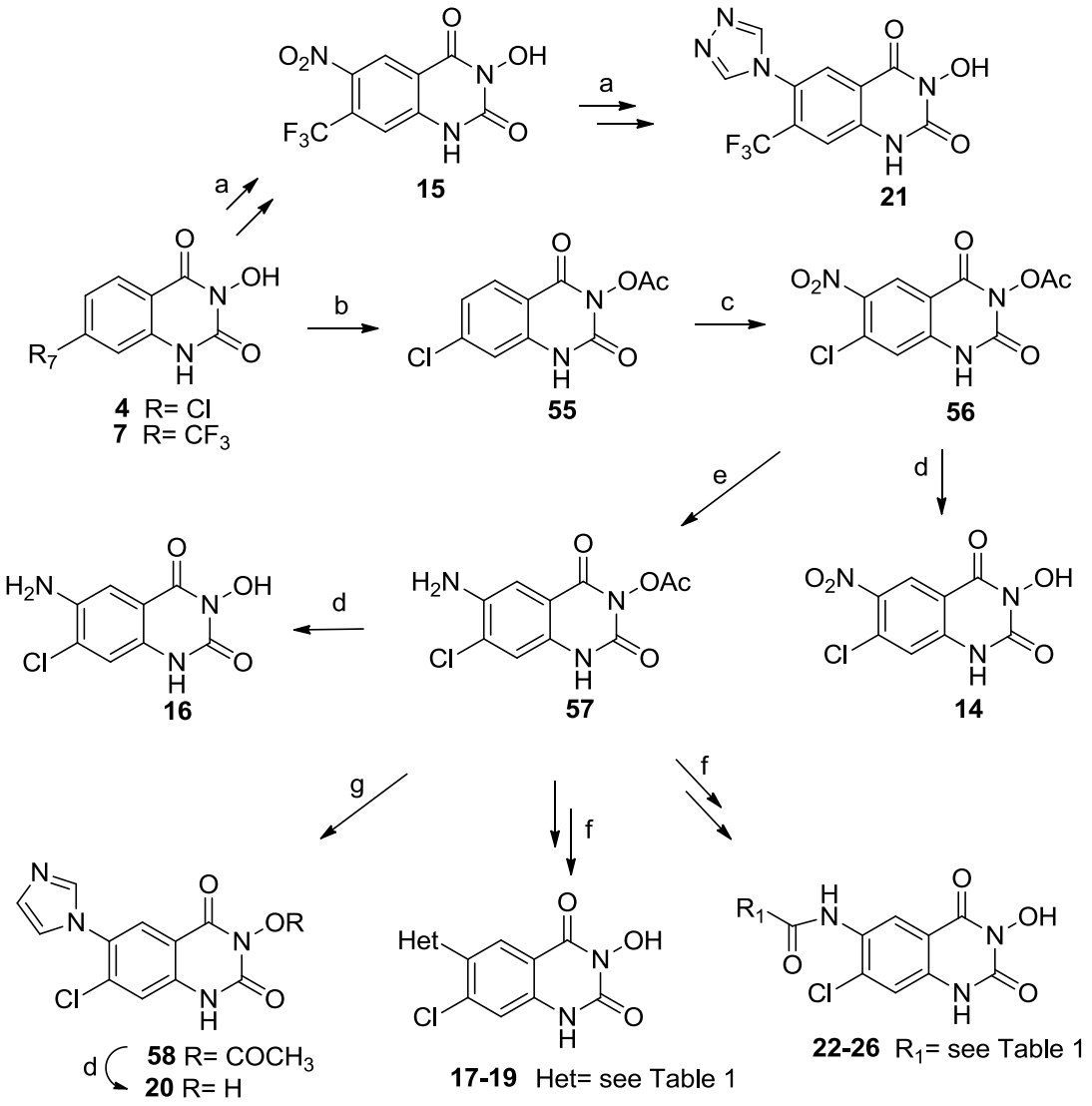
Chart 1. 3-Hydroxyquinazolin-2,4-dione **1** as cyclic analog of benzohydroxamic acid and derivatives **2-29**, new inhibitors of the human carbonic anhydrases IX and XII.



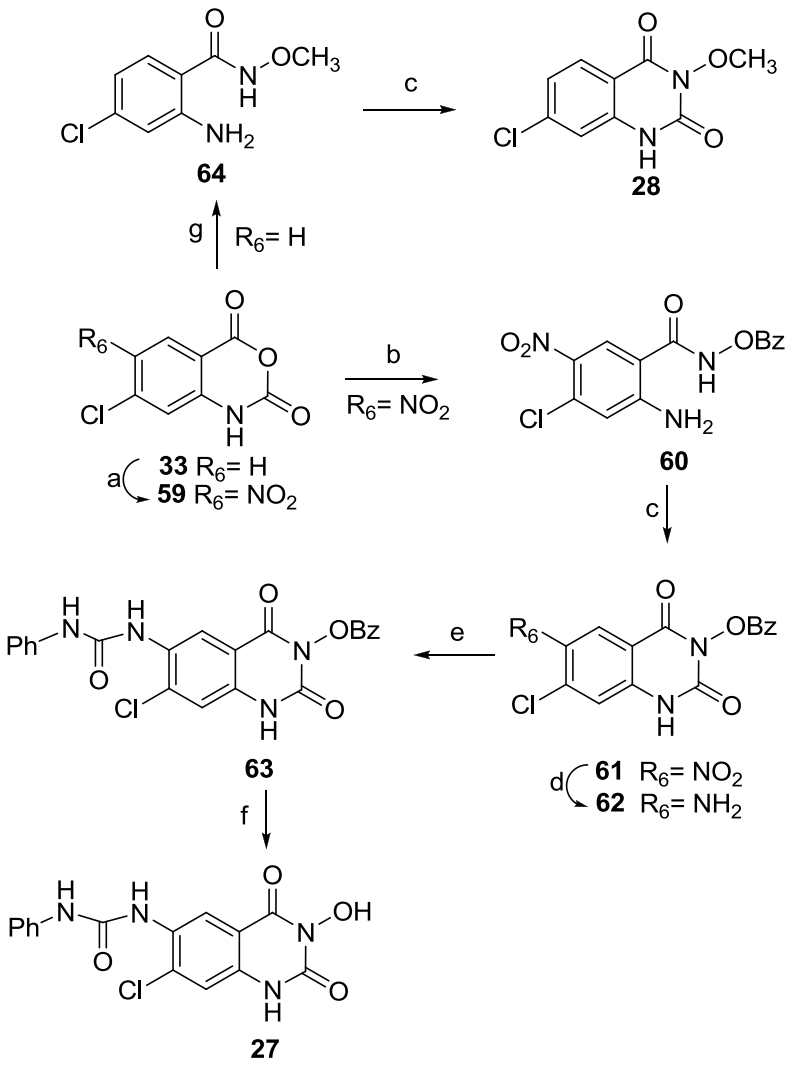
Scheme 1. (a) See ref 14; (b) O-benzylhydroxylamine hydrochloride, NEt_3, EtOH, reflux ; (c) triphosgene, NEt_3, rt; (d) 48% HBr, glacial acetic acid, reflux; (e) $SnCl_2$, EtOH, reflux.



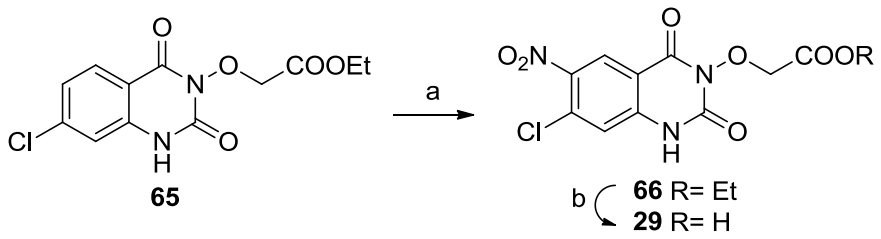
Scheme 2. (a) Diformylhydrazine, Me_3SiCl, anhydrous pyridine, 100 °C; (b) succinic anhydride, NaOAc, glacial AcOH, 60°C; (c) i) phthalic anhydride, NaOAc, glacial AcOH, 60°C, ii) 2.5 N NaOH, rt, iii) 6N HCl; (d) R_1NCO, anhydrous THF, reflux; (e) H_2, Pd/C, Parr apparatus, 30-35 psi.



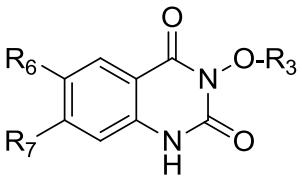
Scheme 3. (a) See ref 14; (b) Ac_2O, reflux; (c) 90% HNO_3, from -10 to 0 °C; (d) i) 2.5 N NaOH, rt, ii) 6N HCl; (e) $SnCl_2$, EtOH, reflux; (f) see ref 15; (g) HCHO, glyoxal, NH_4OAc, 50 °C.



Scheme 4. (a) 96-98% H_2SO_4, 70% HNO_3, 0 °C, see ref. 18; (b) O-benzylhydroxylamine hydrochloride, NEt_3, EtOH, reflux; (c) triphosgene, NEt_3, rt; (d) $SnCl_2$, EtOH, reflux; (e) PhNCO, anhydrous THF, reflux; (f) H_2, Pd/C, DMF, Parr apparatus, 30 psi; (g) O-methylhydroxylamine hydrochloride, NEt_3, EtOH, reflux.



Scheme 5. (a) 90 % HNO_3, from -10 to 0 °C; (b) i) 2.5 % NaOH, rt, ii) 6 N HCl.



1-5, 7-29

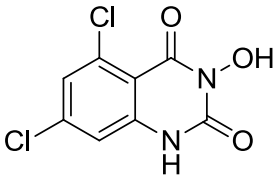


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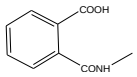
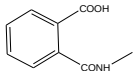
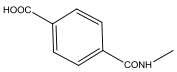
Table 1. Inhibition data of derivatives **1-29** and Acetazolamide (AZA), as reference compound, against hCA I, II, IX and XII isoforms, by a stopped-flow CO_2 hydrase assay.[21]

				K_i (nM)[a]			
	R_6	R_7	R_3	hCA I	hCA II	hCA IX	hCA XII
1	H	H	H	>10000	>10000	480	104.2
2	Cl	H	H	>10000	>10000	48.2	546
3	NO_2	H	H	>10000	>10000	4.6	6.1
4	H	Cl	H	>10000	>10000	69.7	3.4
5	Cl	Cl	H	>10000	>10000	8.2	7.5
6	-	-	-	>10000	>10000	7.7	5.8
7	H	CF_3	H	>10000	>10000	8.2	6.7
8	NH_2	H	H	>10000	>10000	272.6	723.6
9		H	H	>10000	>10000	69.5	445
10	$COOH(CH_2)_2CONH$	H	H	>10000	>10000	64.8	892
11		H	H	>10000	>10000	62.5	745
12	$PhCH_2NHCONH$	H	H	>10000	>10000	26.7	961
13	PhNHCONH	H	H	>10000	>10000	300.6	732.4
14	NO_2	Cl	H	>10000	>10000	7.9	8.0
15	NO_2	CF_3	H	>10000	>10000	9.7	8.2
16	NH_2	Cl	H	>10000	>10000	43.1	736.3
17		Cl	H	>10000	>10000	33.3	6.6
18		Cl	H	>10000	>10000	8.3	10.1
19		Cl	H	>10000	>10000	8.3	51.4

20		Cl	H	>10000	>10000	74.9	920
21		CF_3	H	>10000	>10000	22.1	8.9
22	PhCONH	Cl	H	>10000	>10000	58.8	101
23		Cl	H	>10000	>10000	8.1	8.7
24		Cl	H	>10000	>10000	7.9	9.6
25	$COOH(CH_2)_2CONH$	Cl	H	>10000	>10000	7.0	8.9
26	$PhCH_2NHCONH$	Cl	H	>10000	>10000	6.9	13.1
27	PhNHCONH	Cl	H	>10000	>10000	42.8	722.7
28	H	Cl	CH_3	>10000	>10000	208.2	>10000
29	NO_2	Cl	CH_2COOH	>10000	>10000	18.0	10.6
	Benzoydroxamic acid[b]			83100	179000	45900	9510
	AZA			250	12.1	25.4	5.6

[a]Mean from three different assays, and errors were within 10% of the reported values, by a stopped-flow, CO_2 hydrase assay.[21] TOGLIERE!!! [b]Ref.11a.

Table 2. Effect of some selected hCA inhibitors and Acetazolamide (AZA), as reference compound, on HT-29 cell viability.

		Cell viability %			
		48 h incubation		72 h incubation	
Compound	Concentration (μM)	Normoxia	Hypoxia	Normoxia	Hypoxia
AZA	0	100.00 ± 3.48	100.00 ± 1.82	100.00 ± 3.26	100.00 ± 4.16
	30	96.78 ± 9.59	107.60 ± 5.81	97.61 ± 7.22	106.52 ± 4.91
	100	100.66 ± 5.54	101.68 ± 4.36	95.84 ± 3.35	110.79 ± 3.27
	300	101.02 ± 10.98	85.72 ± 4.66	93.19 ± 5.16	84.14 ± 1.20*
3	0	100.00 ± 8.85	100.00 ± 7.25	100.00 ± 5.19	100.00 ± 5.20
	30	101.32 ± 3.34	98.77 ± 6.09	95.58 ± 3.86	94.68 ± 6.33
	100	95.55 ± 2.73	99.55 ± 3.76	94.13 ± 3.99	93.68 ± 4.47
	300	95.11 ± 2.78	96.69 ± 5.65	94.26 ± 5.08	87.26 ± 8.49
5	0	100.00 ± 3.48	100.00 ± 1.82	100.00 ± 3.26	100.00 ± 4.16
	30	93.43 ± 4.79	105.94 ± 4.91	107.41 ± 4.79	93.28 ± 5.40
	100	90.99 ± 4.52	76.04 ± 3.26*	73.53 ± 4.52*	78.11 ± 6.47*
	300	9.44 ± 0.36***	14.83 ± 1.20***	20.59 ± 0.36*	24.51 ± 4.27*
6	0	100.00 ± 8.85	100.00 ± 7.25	100.00 ± 5.19	100.00 ± 5.20
	30	92.59 ± 5.29	101.06 ± 2.34	94.17 ± 8.48	93.00 ± 3.55
	100	93.85 ± 4.18	102.10 ± 3.11	95.92 ± 5.10	87.03 ± 3.55
	300	90.96 ± 3.63	103.77 ± 2.00	94.93 ± 6.13	78.89 ± 5.67*
12	0	100.00 ± 2.56	100.00 ± 3.88	100.00 ± 3.48	100.00 ± 2.57
	30	57.42 ± 3.72***	50.60 ± 2.20***	32.42 ± 1.06***	33.42 ± 2.88***
	100	56.89 ± 1.97***	45.98 ± 4.82***	51.7 ± 2.49***	40.24 ± 3.06***
	300	85.60 ± 2.82*	78.16 ± 4.67**	61.26 ± 3.46***	59.86 ± 4.24***
14	0	100.00 ± 2.56	100.00 ± 3.88	100.00 ± 3.48	100.00 ± 2.57
	30	58.25 ± 2.62***	45.68 ± 2.06***	36.78 ± 2.7***	41.63 ± 3.31***
	100	72.51 ± 4.03***	64.61 ± 4.12***	61.24 ± 2.82***	55.90 ± 2.64***
	300	77.59 ± 2.21***	70.80 ± 5.69***	70.30 ± 2.88***	63.45 ± 2.98***
15	0	100.00 ± 2.56	100.00 ± 3.88	100.00 ± 3.48	100.00 ± 2.57
	30	53.89 ± 1.2***	45.21 ± 3.23**	41.55 ± 1.84***	38.63 ± 1.30***
	100	57.70 ± 1.2***	46.79 ± 4.62***	45.26 ± 1.12***	38.17 ± 2.22***

	300	45.10 ± 0.6***	50.25 ± 3.37***	49.7 ± 0.5***	40.29 ± 1.73***
19	0	100.00 ± 2.56	100.00 ± 3.88	100.00 ± 3.48	100.00 ± 2.57
	30	48.45 ± 1.24***	50.62 ± 3.24***	36.37 ± 2.03***	20.65 ± 3.09***
	100	56.17 ± 1.90***	60.12 ± 2.63***	57.34 ± 3.69***	45.66 ± 3.47***
	300	106.02 ± 3.00	88.92 ± 5.42	68.31 ± 3.10***	65.20 ± 4.71***

HT-29 cells were treated with increasing concentrations of hCA Inhibitors in comparison with AZA. Incubation was allowed for 48 or 72 h in normoxic or hypoxic conditions. Cell viability was measured by MTT assay. 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).

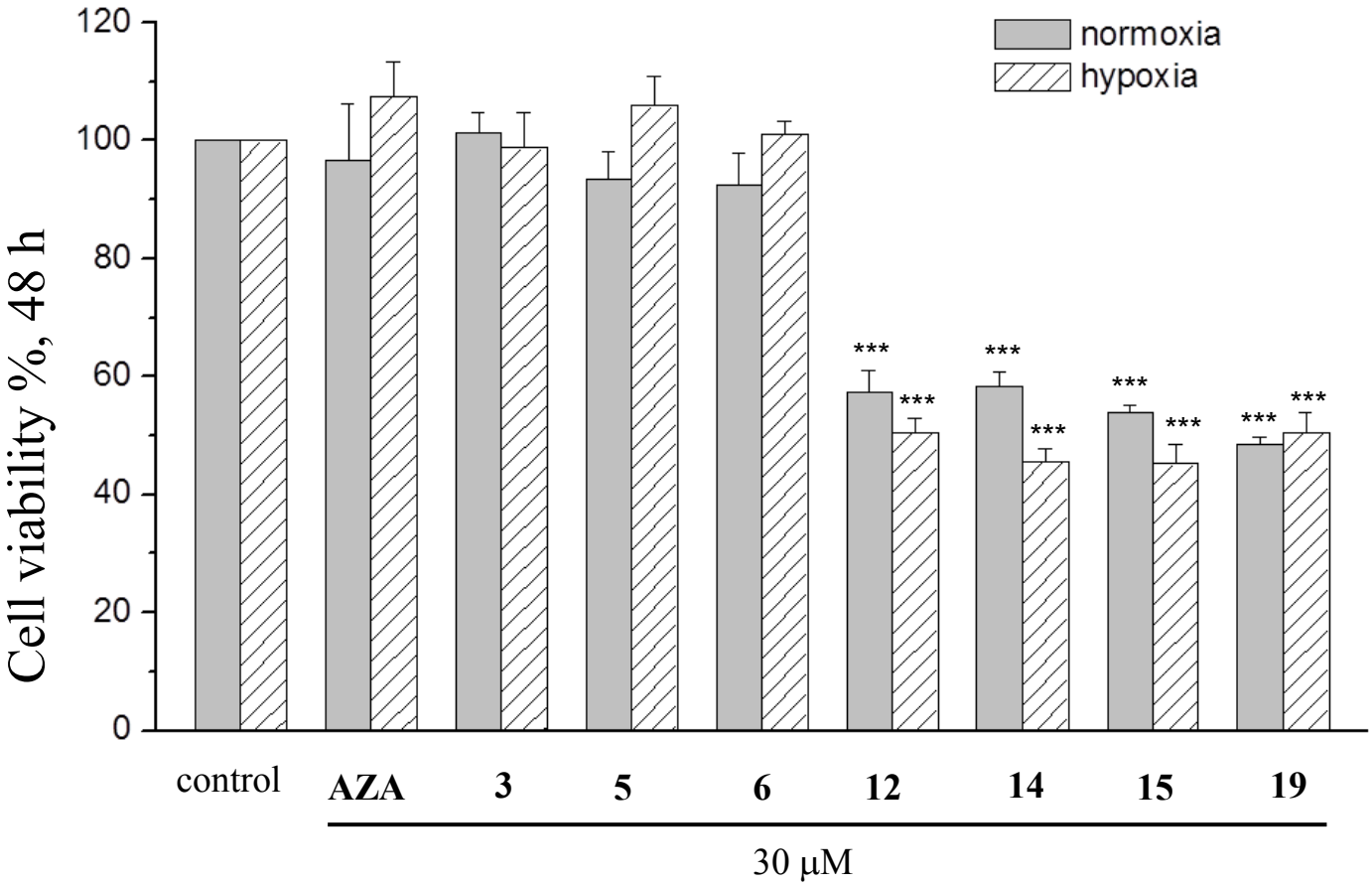
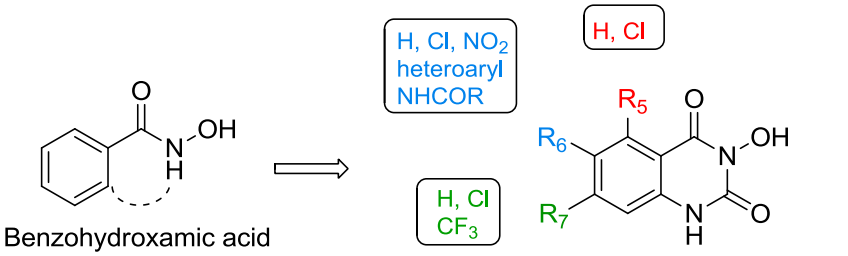


Figure 1. HT-29 cells ($1 \cdot 10^4$/well) were treated with compounds **3**, **5**, **6**, **12**, **14**, **15**, and **19** (30 μM) in comparison to **AZA** (30 μM) as control. Incubation was allowed for 48 h in in normoxic (20% O_2) and hypoxic conditions (0.1% O_2). Cell viability was measured by the reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an index of mitochondrial compartment functionality. Formazan crystals produced by the reaction were dissolved in DMSO and the absorbance measured at 550 nm. Control condition was arbitrarily set as 100% and values are expressed as the mean ± S.E.M. of three experiments. ***P<0.001 in comparison to control (0 μM).

Table of Graphic Content



New highly potent
hCA IX and XII inhibitors