Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Synthesis of an acridine orange sulfonamide derivative with potent carbonic anhydrase IX inhibitory action

Marco Bragagni[a], Fabrizio Carta[a], Sameh M. Osman[b], Zeid AlOthman[b] and Claudiu T. Supuran[a,b]

[a]Dipartimento Neurofarba, Sezione di Scienze Farmaceutiche e Nutraceutiche, Università degli Studi di Firenze, Sesto Fiorentino, Florence Italy; [b]Department of Chemistry, College of Science, King Saud University, Riyadh, Saudi Arabia

ABSTRACT

Acridine orange (AO) a fluorescent cationic dye used for the management of human musculoskeletal sarcomas, due to its strong tumoricidal action and accumulation in the acidic environment typical of hypoxic tumors, was used for the preparation of a primary sulfonamide derivative. The rationale behind the drug design is the fact that hypoxic, acidic tumors overexpress carbonic anhydrase (CA, EC 4.2.1.1) isoforms, such as CA IX, which is involved in pH regulation, proliferation, cell migration and invasion, and this enzyme is strongly inhibited by primary sulfonamides. The AO-sulfonamide derivative was indeed a potent, low nanomolar CA IX inhibitor whereas its inhibition of the cytosolic isoforms CA I and II was in the micromolar range. A second transmembrane, tumor-associated isoform, CA XII, was also effectively inhibited by the AO-sulfonamide derivative, making this compound an interesting theranostic agent for the management of hypoxic tumors.

Introduction

Acridine orange (**AO**) is a heterocyclic derivative used as a nucleic acid-selective fluorescent cationic dye useful for cell cycle determination, as it interacts with DNA and RNA by intercalation within the double helix or by electrostatic attractions to the negatively charged phosphate groups, respectively. It also enters acidic compartments such as lysosomes, becoming protonated and sequestered inside that region of the cell/tissue. In such low pH conditions, the dye emits orange light when excited by blue light, being used to identify engulfed apoptotic cells[1–3].

AO
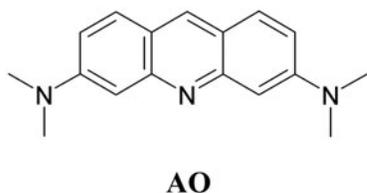
In the last years, Kusuzaki's and Baldini's groups found that AO accumulates in the musculoskeletal sarcomas[1–6]. After illumination of the tumors loaded with **AO** with visible light (or irradiation with low-dose X-rays), the dye rapidly exerted a selective killing of the cancer cells[1–6]. Thus, **AO** in combination with surgery and photodynamic (PD) or radiodynamic (RD) therapies has been proposed as an alternative approach for the management of human musculoskeletal sarcomas, due to its strong tumoricidal action following excitation with a light source at 466 nm, with promising results being obtained mainly in Japan[1–6].

Osteosarcomas[7] as many other tumor types were shown to overexpress the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1)[8–15] involved in several processes related to tumorigenesis, tumor progression and metastases formation[8,9]. CAs are highly effective catalysts for the hydration of carbon dioxide with the formation of bicarbonate and protons, being widespread in all life kingdoms, with seven genetically distinct families known to date[15–21]. By catalyzing the reversible CO_2 hydration to bicarbonate and protons, the CAs are involved in many physiological processes connected with electrolyte secretion[22–24], pH regulation[25–29], tumorigenesis[30–33], etc., and their inhibition leads to pharmacological effects[34–40]. Indeed, sulfonamide CA inhibitors (CAIs) are clinically used as diuretics, antiglaucoma, anticonvulsant, antiobesity and antitumor agents[34–44]. Many drug design strategies are presently available for designing effective and isoform-selective such agents[45–50], but the primary sulfonamides remain among the most investigated CAIs due to their high affinity for many CA isoforms of pharmacologic interest, rather convenient pharmacology and ease of preparation[45–50].

Here, we report a study in which we designed a compound which might combine the affinity of AO for the tumors and the fact that many of them overexpress CA isoforms involved in tumorigenesis (e.g. CA IX and XII)[9,19]. The designed compound incorporates both **AO** and sulfonamide moieties, which have affinity for the CAs. The **AO**-sulfonamide agent reported here could represent a theranostic agent for the management of hypoxic tumors.

Material and methods

Chemistry

Anhydrous solvents and all reagents were purchased from Sigma-Aldrich (Milan, Italy). All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen

CONTACT Claudiu T. Supuran claudiu.supuran@unifi.it Dipartimento Neurofarba, Sezione di Scienze Farmaceutiche e Nutraceutiche, Università degli Studi di Firenze, Via U. Schiff 6, Sesto Fiorentino, Florence 50019, Italy

atmosphere using dried glassware and syringes techniques to transfer solutions. Nuclear magnetic resonance (^{1}H 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; triplet; q, quadruplet; dd, double of doublet. The assignment of exchangeable protons (OH and NH) was confirmed by the addition of D$_2$O.

Synthesis of 3,6-bis(dimethylamino)-9-acridanthione 1[51]
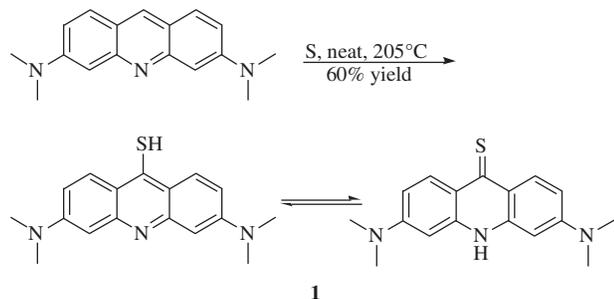
Acridine orange (3.0 g, 1.0 eq) and sulfur (0.43 g, 1.2 eq) were accurately mixed in a mortar and transferred to a pear shaped flask, which was preheated at 205 °C in a sand bath. The flask was maintained open at this temperature for 30 min. The dark purple residue formed was cooled down to r.t. and re-crystallized from DMF to afford the title compound 1 as a dark brown solid.

3,6-Bis(dimethylamino)-9-acridanthione 1: 60% yield; ^{1}H NMR: δ_H (400 MHz, DMSO-d_6) 3.11 (s, 12H), 6.42 (s, 2H), 6.89 (d, $J = 9.6$ Hz, 2H), 8.67 (d, $J = 9.6$ Hz, 2H), 11.68 (s, 1H). ^{13}C NMR: δ_C (100 MHz, DMSO-d_6) 40.5, 94.2, 111.0, 120.9, 132.1, 139.1, 153.6. m/z (ESI positive) 298.13 [M + H]$^+$.

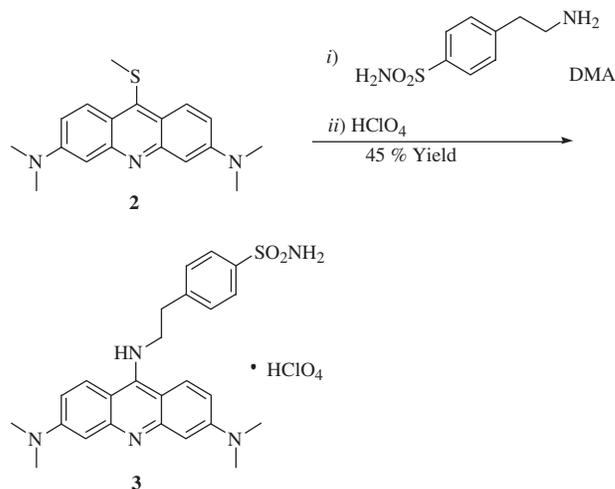Synthesis of 3,6-bis(dimethylamino)-9-(methylthio)acridine 2[52]
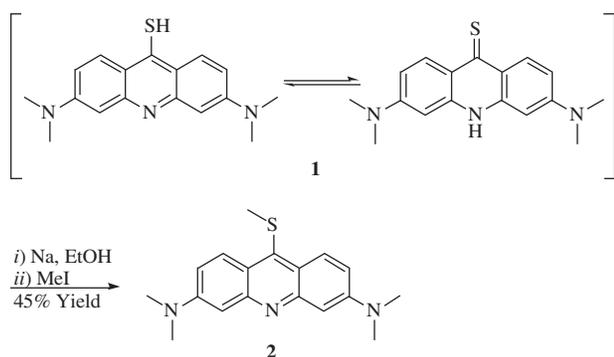
Sodium (0.38 g, 1.3 eq) was dissolved at r.t. in anhydrous ethanol (20 ml) under a nitrogen atmosphere, followed by the addition of 3,6-bis(dimethylamino)-9-acridanthione 1 (2 g, 1.0 eq). The reaction mixture was stirred under reflux for 30 min, cooled down to 50 °C and methyl iodide (1.15 g, 1.2 eq) was added drop-wise. The reaction mixture was left stirring o.n. at r.t. and filtrated. The filtrated solution was boiled, treated with decolorizing charcoal, filtered and heated again to boiling. Hot distilled water (5 ml) was added drop-wise under stirring. The solution was cooled down and maintained at 4 °C o.n. The red crystals which precipitated were recuperated by filtration and dried under vacuo.

3,6-Bis(dimethylamino)-9-(methylthio)acridine 2: 45% yield; ^{1}H NMR: δ_H (400 MHz, DMSO-d_6) 2.48 (s, 3H), 3.13 (s, 12H), 6.90 (d, $J = 2.8$ Hz, 2H), 7.37 (dd, $J = 9.6$ 2.8 Hz, 2H), 8.41 (d, $J = 9.6$ Hz, 2H).

^{13}C NMR: δ_C (100 MHz, DMSO-d_6) 21.3, 41.1, 104.2, 117.8, 120.9, 128.4, 143.3, 151.9, 152.5. m/z (ESI positive) 312.15 [M + H]$^+$.

Synthesis of 4-(2-((3,6-bis(dimethylamino)acridin-9-yl)amino)ethyl)benzenesulfonamide perchlorate 3.

3,6-Bis(dimethylamino)-9-(methylthio)acridine 2 (0.50 g, 1.0 eq) and 4-(2-aminoethyl)benzenesulfonamide (0.96 g, 3 eq) were dissolved in anhydrous DMA (20 ml) at 140 °C. The reaction mixture was stirred at the same temperature for 2 h under a nitrogen atmosphere, cooled down to r.t. and a 6 M aqueous solution of perchloric acid (0.48 g, 3.0 eq) was added. The mixture was maintained at 0 °C for 1 h and the dark red crystals obtained were collected by centrifugation, washed with water and dried under vacuo.

4-(2-((3,6-Bis(dimethylamino)acridin-9-yl)amino)ethyl)benzenesulfonamide perchlorate 3: 45% yield; ^{1}H NMR: δ_H (400 MHz, DMSO-d_6) 3.16 (s, 12H), 3.25 (t, $J = 7.6$ Hz, 2H), 4.21 (q, $J = 7.6$ Hz, 2H), 6.52 (d, $J = 2.8$ Hz, 2H), 7.02 (dd, $J = 9.6$ 2.8 Hz, 2H), 7.34 (s, 2H), 7.50 (d, $J = 8.4$ Hz, 2H), 7.75 (d, $J = 8.4$ Hz, 2H), 8.22 (d, $J = 9.6$ Hz, 2H), 8.46 (t, 1H), 12.2 (s, 1H). ^{13}C NMR: δ_C (100 MHz, DMSO-d_6) 36.1, 40.3, 50.5, 95.0, 104.5, 112.1, 126.8, 128.0, 130.3, 143.2, 143.5, 143.6, 154.3, 155.2. m/z (ESI positive) 464.20 [M − ClO$_4$]$^+$.

Synthesis of N,N,N′,N′-tetramethyl-N″-phenethyl-acridine-3,6,9-triamine 4.
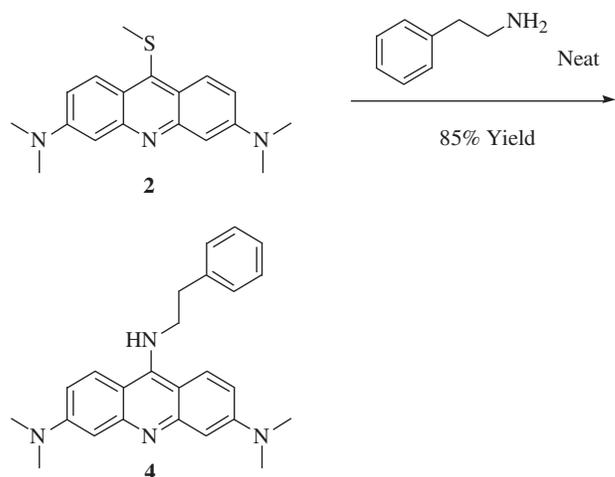
3,6-Bis(dimethylamino)-9-(methylthio)acridine 2 (0.4 g, 1.0 eq) was introduced in a pear shaped flask followed by

phenylethylamine (0.47 g, 3.0 eq) at r.t. Then the flask was transferred to a sand bath, pre-heated at 140 °C, and left open at this temperature for 1.5 h. The dark residue was cooled-down to r.t., crushed with a spatula, repeatedly washed with water and dried under *vacuo*.

N,N,N',N'-tetramethyl-*N''*-phenethyl-acridine-3,6,9-triamine **4**: 85% yield; ^{1}H NMR: δ_H (400 MHz, DMSO-d$_6$) 3.04 (t, $J = 7.6$ Hz), 3.06 (s, 12H), 3.93 (q, $J = 7.6$ Hz, 2H), 6.64 (d, 2H), 6.92 (dd, $J = 9.6$ 2.8 Hz, 2H), 7.29 (m, 5H), 8.05 (d, $J = 9.6$ Hz, 2H). ^{13}C NMR: δ_C (100 MHz, DMSO-d$_6$) 36.4, 40.5, 51.0, 95.2, 104.6, 112.0, 127.6, 127.9, 129.6, 129.8, 139.4, 143.4, 154.2, 155.1. *m/z* (ESI positive) 385.23 [M + H]$^+$.

Carbonic anhydrase assay

A stopped-flow method[53] has been used for assaying the CA catalyzed CO_2 hydration activity with Phenol red as indicator, working at the absorbance maximum of 557 nm, following the initial rates of the CA-catalyzed CO_2 hydration reaction for 10–100 s. 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 (0.01 mM) were prepared in distilled-deionized water with 5% DMSO and dilutions up to 0.1 nM were done thereafter with the assay buffer. The Inhibition constant (K_I) was obtained by considering the classical Michaelis–Menten equation which has been fitted by non-linear least squares by using PRISM 3. All CA isozymes used in the experiments were purified recombinant proteins obtained as reported earlier by our group[54–64].

Results and discussion

The rationale of this work was to design a hybrid molecule which may show enhanced affinity for tumor cells due to the presence of both **AO** and sulfonamide moieties in its molecule. In addition, these hybrid compounds may retain the fluorescent properties of **AO**, and thus could be useful for PD and/or RD therapies, but these aspects are not investigated in this paper.

The synthetic procedure for obtaining the hybrid is shown in Scheme 1. Acridine was reacted with elemental sulfur, leading to the thiol/thione derivative **1**, which was methylated at the sulfur atom with methyl iodide, leading to the key methylthio-intermediate **2**[51,52].

Reaction of **2** with amines, such as phenethylamine or 4-amino-ethylbenzensulfonamide, led to the heterocyclic amines **3** and **4**, one incorporating the primary sulfonamide moiety (compound **3**) and the other one possessing exactly the same scaffold as **3**, but without the sulfonamide group (compound **4**), Scheme 1. Derivative **4** is in fact useful as a negative control in the enzyme inhibition experiments reported here (see later in the paper). Since all the purification procedures used to isolate the derivative **3** as the free base were unsuccessful, we converted the *in situ* formed free-base **3** (TLC monitoring) to its corresponding perchlorate salt, which precipitated at 0 °C within 1 h to afford the desired compound in good yield and excellent purity. The use of perchlorate salts for the isolation as well as purification of small molecule
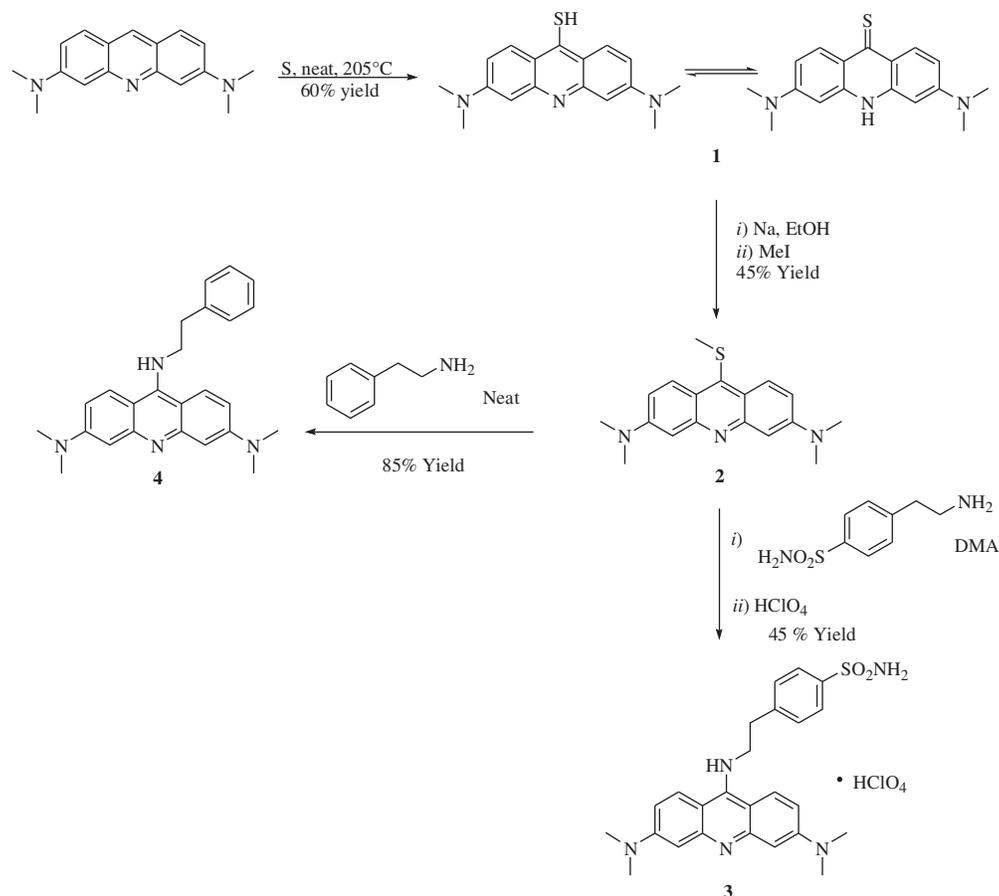
Scheme 1. General synthetic procedure for 4-(2-((3,6-bis(dimethylamino)acridin-9-yl)amino)ethyl)benzenesulfonamide perchlorate **3** and *N,N,N',N'*-tetramethyl-*N''*-phenethyl-acridine-3,6,9-triamine **4**.

Table 1. hCA I, II; IX and XII inhibition data with compounds **3**, **4**, **AAZ** and **AO**, by a stopped-flow CO_2 hydrase assay[53].

Compound	K_I (nM)[a]			
	hCA I	hCA II	hCA IX	hCA XII
3	7680	1650	9.1	4.9
4	>50,000	>50,000	>50,000	>50,000
AAZ	250	12	25.1	5.6
AO	>50,000	>50,000	>50,000	>50,000

[a]Errors in the range of 5% of the reported values, from three different determinations (data not shown).

compounds as CAIs is well reported, also in consideration that the inorganic counterion does not have any effect on the CAs activity[7–9].

Carbonic anhydrase inhibition

We assessed the CA inhibitory activity of compounds **3** and **4**, using the clinically used drug acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide, **AAZ**) as positive control, for the inhibition of four human (h) isoforms, hCA I and II (cytosolic, widely distributed enzymes) as well as hCA IX and XII (transmembrane, tumor-associated enzymes) – Table 1.

Data of Table 1 show that **AO** and its non-sulfonamide derivative **4**, did not inhibit any CA isoform investigated here, whereas sulfonamides **3** and **AAZ** acted as inhibitors. The **AO**-sulfonamide hybrid **3** was a micromolar inhibitor of the cytosolic isoforms hCA I and II, with inhibition constants of 1.65–7.68 μM, whereas the transmembrane, tumor-associated isoforms hCA IX and XII were much more effectively inhibited, with inhibition constants of 4.9–9.1 nM. Acetazolamide was a medium potency hCA I inhibitor and a highly effective one for the remaining three isoforms, with inhibition constants of 5.6–25.1 nM (Table 1). These data show that the **AO**-sulfonamide hybrid **3** is a tumor-associated CA isoforms selective inhibitor, making it a valuable candidate for theranostic applications in the field of hypoxic tumors.

Conclusions

We report here the synthesis and enzyme inhibition data of acridine orange, a fluorescent cationic dye used for the management of human musculoskeletal sarcomas, as well as those of a new compound based on the **AO** scaffold on which a sulfonamide zinc-binding moiety was introduced by using an original procedure. Due to the strong tumoricidal action and accumulation in the acidic environment (typical of hypoxic tumors) of **AO**, we designed the hybrid in such a way as to incorporate an additional functionality which may lead to interaction with hypoxic tumors, many of which overexpress CA IX and XII. Such enzymes are in fact involved in pH regulation, proliferation, cell migration and invasion in many cancer types. The reported **AO**-sulfonamide derivative was indeed a potent, low nanomolar CA IX inhibitor whereas its inhibition of the cytosolic isoforms CA I and II was in the micromolar range. A second transmembrane, tumor-associated isoform, CA XII, was also effectively inhibited by the **AO**-sulfonamide derivative, making this compound an interesting theranostic agent for the management of hypoxic tumors.

Acknowledgements

Disclosure statement

Funding

References

1. Kusuzaki K, Murata H, Matsubara T, et al. Review. Acridine orange could be an innovative anticancer agent under photon energy. In Vivo 2007;21:205–14.
2. Matsubara T, Kusuzaki K, Matsumine A, et al. Acridine orange used for photodynamic therapy accumulates in malignant musculoskeletal tumors depending on pH gradient. Anticancer Res 2006;26:187–93.
3. Nakamura T, Kusuzaki K, Matsubara T, et al. A new limb salvage surgery in cases of high-grade soft tissue sarcoma using photodynamic surgery, followed by photo- and radiodynamic therapy with acridine orange. J Surg Oncol 2008;97:523–8.
4. Matsubara T, Kusuzaki K, Matsumine A, et al. Photodynamic therapy with acridine orange in musculoskeletal sarcomas. J Bone Joint Surg Br 2010;92:760–2.
5. Kusuzaki K, Hosogi S, Ashihara E, et al. Translational research of photodynamic therapy with acridine orange which targets cancer acidity. Curr Pharm Des 2012;18:1414–20.
6. Fotia C, Avnet S, Kusuzaki K, et al. Acridine orange is an effective anti-cancer drug that affects mitochondrial function in osteosarcoma cells. Curr Pharm Des 2015;21:4088–94.
7. Perut F, Carta F, Bonuccelli G, et al. Carbonic anhydrase IX inhibition is an effective strategy for osteosarcoma treatment. Expert Opin Ther Targets 2015;19:1593–605.
8. Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat Rev Drug Discov 2008;7:168–81.
9. Neri D, Supuran CT. Interfering with pH regulation in tumours as a therapeutic strategy. Nat Rev Drug Discov 2011;10:767–77.
10. Capasso C, Supuran CT. An overview of the alpha-, beta- and gamma-carbonic anhydrases from Bacteria: can bacterial carbonic anhydrases shed new light on evolution of bacteria? J Enzyme Inhib Med Chem 2015;30:325–32.
11. Supuran CT. Structure-based drug discovery of carbonic anhydrase inhibitors. J Enzyme Inhib Med Chem 2012;27:759–72.
12. Supuran CT. Carbonic anhydrase inhibitors. Bioorg Med Chem Lett 2010;20:3467–74.
13. Supuran CT. Bacterial carbonic anhydrases as drug targets: toward novel antibiotics? Front Pharmacol 2011;2:34.
14. Del Prete S, Vullo D, De Luca V, et al. Biochemical characterization of recombinant beta-carbonic anhydrase (PgiCAb) identified in the genome of the oral pathogenic bacterium *Porphyromonas gingivalis*. J Enzyme Inhib Med Chem 2015;30:366–70.

15. Supuran CT. Carbonic anhydrases: from biomedical applications of the inhibitors and activators to biotechnological use for CO(2) capture. J Enzyme Inhib Med Chem 2013;28:229–30.

16. Capasso C, Supuran CT. Anti-infective carbonic anhydrase inhibitors: a patent and literature review. Expert Opin Ther Pat 2013;23:693–704.

17. Capasso C, Supuran CT. Sulfa and trimethoprim-like drugs – antimetabolites acting as carbonic anhydrase, dihydropteroate synthase and dihydrofolate reductase inhibitors. J Enzyme Inhib Med Chem 2014;29:379–87.

18. Supuran CT. Structure and function of carbonic anhydrases. Biochem J 2016; 473:2023–32.

19. Lou Y, McDonald PC, Oloumi A, et al. Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. Cancer Res 2011;71:3364–76.

20. Casini A, Scozzafava A, Mincione F, et al. Carbonic anhydrase inhibitors: water-soluble 4-sulfamoylphenylthioureas as topical intraocular pressure-lowering agents with long-lasting effects. J Med Chem 2000;43:4884–92.

21. Alterio V, Di Fiore A, D'Ambrosio K, et al. Multiple binding modes of inhibitors to carbonic anhydrases: how to design specific drugs targeting 15 different isoforms? Chem Rev 2012;112:4421–68.

22. Carta F, Aggarwal M, Maresca A, et al. Dithiocarbamates strongly inhibit carbonic anhydrases and show antiglaucoma action in vivo. J Med Chem 2012;55:1721–30.

23. Scozzafava A, Menabuoni L, Mincione F, Supuran CT. Carbonic anhydrase inhibitors. A general approach for the preparation of water solublesulfonamides incorporating polyamino-polycarboxylate tails and of their metal complexes possessing long lasting, topical intraocular pressure lowering properties. J Med Chem 2002;45:1466–76.

24. Fabrizi F, Mincione F, Somma T, et al. A new approach to antiglaucoma drugs: carbonic anhydrase inhibitors with or without NO donating moieties. Mechanism of action and preliminary pharmacology. J Enzyme Inhib Med Chem 2012;27:138–47.

25. Švastová E, Hulíková A, Rafajová M, et al. Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH. FEBS Lett 2004;577:439–45.

26. Dubois L, Lieuwes NG, Maresca A, et al. Imaging of CA IX with fluorescent labelledsulfonamides distinguishes hypoxic and (re)-oxygenated cells in a xenograft tumor model. Radiother Oncol 2009;92:423–8.

27. Pacchiano F, Carta F, McDonald PC, et al. Ureido-substituted benzenesulfonamides potently inhibit carbonic anhydrase IX and show antimetastatic activity in a model of breast cancer metastasis. J Med Chem 2011;54:1896–902.

28. Winum JY, Scozzafava A, Montero JL, Supuran CT. Therapeutic potential of sulfamides as enzyme inhibitors. Med Res Rev 2006;26:767–92.

29. Pacchiano F, Aggarwal M, Avvaru BS, et al. Selective hydrophobic pocket binding observed within the carbonic anhydrase II active site accommodate different 4-substituted-ureido-benzenesulfonamides and correlate to inhibitor potency. Chem Commun (Camb) 2010;46:8371–3.

30. Carta F, Garaj V, Maresca A, et al. Sulfonamides incorporating 1,3,5-triazine moieties selectively and potently inhibit carbonic anhydrase transmembrane isoforms IX, XII and XIV over cytosolic isoforms I and II: solution and X-ray crystallographic studies. Bioorg Med Chem 2011;19:3105–19.

31. Garaj V, Puccetti L, Fasolis G, et al. Carbonic anhydrase inhibitors: synthesis and inhibition of cytosolic/tumor-associated carbonic anhydrase isozymes I, II and IX with sulfonamides incorporating 1,2,4-triazine moieties. Bioorg Med Chem Lett 2004;14:5427–33.

32. Garaj V, Puccetti L, Fasolis G, et al. Carbonic anhydrase inhibitors. Novel sulfonamides incorporating 1,3,5-triazine moieties as inhibitors of the cytosolic and tumor-associated carbonic anhydrase isozymes I, II and IX. Bioorg Med Chem Lett 2005;15:3102–8.

33. McDonald PC, Winum JY, Supuran CT, Dedhar S. Recent developments in targeting carbonic anhydrase IX for cancer therapeutics. Oncotarget 2012;3:84–97.

34. Carta F, Scozzafava A, Supuran CT. Sulfonamides (RSO$_2$NH$_2$): a patent review 2008–2012. Expert Opin Ther Pat 2012;22:747–58.

35. Masini E, Carta F, Scozzafava A, Supuran CT. Antiglaucoma carbonic anhydrase inhibitors: a patent review. Expert Opin Ther Pat 2013;23:705–16.

36. Monti SM, Supuran CT, De Simone G. Anticancer carbonic anhydrase inhibitors: a patent review (2008–2013). Expert Opin Ther Pat 2013;23:737–49.

37. Carta F, Supuran CT. Diuretics with carbonic anhydrase inhibitory action: a patent and literature review (2005–2013). Expert Opin Ther Pat 2013;23:681–91.

38. Supuran CT. Carbonic anhydrase inhibitors as emerging drugs for the treatment of obesity. Expert Opin Emerg Drugs 2012;17:11–15.

39. Scozzafava A, Supuran CT, Carta F. Antiobesity carbonic anhydrase inhibitors: a literature and patent review. Expert Opin Ther Pat 2013;23:725–35.

40. Supuran CT. The safety and clinical efficacy of acetazolamide for the treatment of idiopathic intracranial hypertension. Expert Rev Neurother 2015;15:851–6.

41. Supuran CT. Advances in structure-based drug discovery of carbonic anhydrase inhibitors. Expert Opin Drug Discov 2017;12:61–88.

42. De Simone G, Alterio V, Supuran CT. Exploiting the hydrophobic and hydrophilic binding sites for designing carbonic anhydrase inhibitors. Expert Opin Drug Discov 2013;8:793–810.

43. Scozzafava A, Menabuoni L, Mincione F, et al. Carbonic anhydrase inhibitors. Synthesis of water-soluble, topically effective, intraocular pressure-lowering aromatic/heterocyclic sulfonamides containing cationic or anionic moieties: is the tail more important than the ring? J Med Chem 1999;42:2641–50.

44. Borras J, Scozzafava A, Menabuoni L, et al. Carbonic anhydrase inhibitors. Part 73. Synthesis of water-soluble, topically effective intraocular pressure lowering aromatic/heterocyclic sulfonamides containing 8-quinoline-sulfonyl moieties: is the tail more important than the ring? Bioorg Med Chem 1999;7:2397–406.

45. Winum JY, Supuran CT. Recent advances in the discovery of zinc-binding motifs for the development of carbonic anhydrase inhibitors. J Enzyme Inhib Med Chem 2015;30:321–4.

46. Briganti F, Pierattelli R, Scozzafava A, Supuran CT. Carbonic anhydrase inhibitors. Part 37. Novel classes of carbonic anhydrase inhibitors and their interaction with the native and cobalt-substituted enzyme: kinetic and spectroscopic investigations. Eur J Med Chem 1996;31:1001–10.

47. Supuran CT, Carbonic anhydrase inhibitors. In: Puscas I, ed. Carbonic anhydrase and modulation of physiologic and pathologic processes in the organism. Timisoara: Helicon; 1994:29–111.

48. Clare BW, Supuran CT. Carbonic anhydrase activators. Part 3. Structure–activity correlations for a series of isozyme II activators. J Pharm Sci 1994;83:768–73.

49. Di Cesare Mannelli L, Micheli L, Carta F, et al. Carbonic anhydrase inhibition for the management of cerebral ischemia: in vivo evaluation of sulfonamide and coumarin inhibitors. J Enzyme Inhib Med Chem 2016;31:894–9.

50. Kalinin S, Supuran CT, Krasavin M. Multicomponent chemistry in the synthesis of carbonic anhydrase inhibitors. J Enzyme Inhib Med Chem 2016;31:185–99.

51. Elslager EF. 9-Substituted 3,6-bis(dimethylamino)acridines. J Med Chem 1962;27:4346–8.

52. Elslager EF, Haley NF, McLean R, et al. Inhibition of platelet aggregation. 2. 9-{[(Dialkylamino)alkyl]thio}-3-(dimethylamino)acridines and related acridine derivatives. J Med Chem 1971;14:782–8.

53. Khalifah RG. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. J Biol Chem 1971;246:2561–73.

54. Yamali C, Gul HI, Sakagami H, Supuran CT. Synthesis and bioactivities of halogen bearing phenolic chalcones and their corresponding bis Mannich bases. J Enzyme Inhib Med Chem 2016;31:125–31.

55. Mollica A, Locatelli M, Macedonio G, et al. Microwave-assisted extraction, HPLC analysis, and inhibitory effects on carbonic anhydrase I, II, VA, and VII isoforms of 14 blueberry Italian cultivars. J Enzyme Inhib Med Chem 2016;31:1–6.

56. Margheri F, Ceruso M, Carta F, et al. Overexpression of the transmembrane carbonic anhydrase isoforms IX and XII in the inflamed synovium. J Enzyme Inhib Med Chem 2016;31:60–3.

57. Mishra CB, Kumari S, Angeli A, et al. Design, synthesis and biological evaluation of N-(5-methyl-isoxazol-3-yl/1,3,4-thiadiazol-2-yl)-4-(3-substitutedphenylureido) benzenesulfonamides as human carbonic anhydrase isoenzymes I, II, VII and XII inhibitors. J Enzyme Inhib Med Chem 2016;31:174–9.

58. Diaz JR, Fernández Baldo M, Echeverría G, et al. A substituted sulfonamide and its Co (II), Cu (II), and Zn (II) complexes as potential antifungal agents. J Enzyme Inhib Med Chem 2016;31:51–62.

59. Supuran CT, Kalinin S, Tanç M, et al. Isoform-selective inhibitory profile of 2-imidazoline-substituted benzene sulfonamides against a panel of human carbonic anhydrases. J Enzyme Inhib Med Chem 2016;31:197–202.

60. Federici C, Lugini L, Marino ML, et al. Lansoprazole and carbonic anhydrase IX inhibitors sinergize against human melanoma cells. J Enzyme Inhib Med Chem 2016;31:119–25.

61. Chohan ZH, Scozzafava A, Supuran CT. Unsymmetrical 1,1′-disubstituted ferrocenes: synthesis of Co(ii), Cu(ii), Ni(ii) and Zn(ii) chelates of ferrocenyl -1-thiadiazolo-1′-tetrazole, -1-thiadiazolo-1′-triazole and -1-tetrazolo-1'-triazole with antimicrobial properties. J Enzyme Inhib Med Chem 2002;17:261–6.

62. Del Prete S, Vullo D, Fisher GM, et al. Discovery of a new family of carbonic anhydrases in the malaria pathogen Plasmodium falciparum – the η-carbonic anhydrases. Bioorg Med Chem Lett 2014;24:4389–96.

63. Supuran CT, Scozzafava A, Mastrolorenzo A. Bacterial proteases: current therapeutic use and future prospects for the development of new antibiotics. Expert Opin Ther Pat 2001;11:221–59.

64. Supuran CT, Barboiu M, Luca C, et al. Carbonic anhydrase activators. Part 14. Synthesis of mono- and bis-pyridinium salt derivatives of 2-amino-5-(2-aminoethyl)- and 2-amino-5-(3-aminopropyl)-1,3,4-thiadiazole, and their interaction with isozyme II. Eur J Med Chem 1996;31:597–606.