N-Nitrosulfonamides as carbonic anhydrase inhibitors: a promising chemotype for targeting Chagas disease and leishmaniasis.

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Chemistry

Anhydrous solvents and all reagents were purchased from Sigma-Aldrich, Alfa Aesar and Fluorochem. 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, ^{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; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets. The assignment of exchangeable protons (OH and NH) was confirmed by the addition of D_2O. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates. Flash chromatography purifications were performed on Merck Silica gel 60 (230-400 mesh ASTM) as the stationary phase and methanol/dichloromethane were used as eluents. Melting points (m.p.) of Ag salts were measured in open capillary tubes with a Gallenkamp MPD350.BM3.5 apparatus and are all >300°C. The solvents used in MS measures were acetone, acetonitrile (Chromasolv grade), purchased from Sigma-Aldrich (Milan - Italy), and mQ water 18 MΩ, obtained from Millipore's Simplicity system (Milan-Italy). The mass spectra were obtained using a Varian 1200L triple quadrupole system (Palo Alto, CA, USA) equipped by Electrospray Source (ESI) operating in both positive and negative ions. Stock solutions of analytes were prepared in acetone at 1.0 mg mL^{-1} and stored at 4°C. Working solutions of each analyte were freshly prepared by diluting stock solutions in a mixture of mQ H_2O/ACN 1/1 (*v/v)* up to a concentration of 1.0 µg mL^{-1} The mass spectra of each analyte were acquired by introducing, via syringe pump at 10 /L min^{-1}, of the working solution. Raw-data were collected and processed by Varian Workstation Vers. 6.8 software.

General synthetic procedure of N-nitrobenzenesulfonamides 9-16.

Procedure 1. NH_4NO_3 (1.1 equiv) was added portion wise to a solution of aromatic sulfonamides **1-7** (0.25g, 1.0 equiv) in 95% H_2SO_4 (1 mL) at 0°C. The suspension was stirred at the same temperature for 15 min, then quenched with slush (8 mL) and the formed precipitate was filtered-off and washed with cold water. The collected powder was

recrystallized with water to afford the title compound **9-15**. The characterization of derivatives **9-11**, **14**, **15** was previously reported.[20]

Procedure 2. NH_4NO_3 (1.1 equiv) was added portionwise to a solution of aromatic sulfonamides **8**, **17**, **19** (0.25 g, 1.0 equiv) in 95% H_2SO_4 (1 mL) at 0°C. The suspension was stirred at the same temperature for 15 min, then slowly quenched with $NH_4OH_{(aq.)}$ until pH=8 was obtained. The yellow solution was concentrated under *vacuum*. MeOH (15 mL) was added and the formed suspension was filtered-off. The organic solvent was concentrated under *vacuum* and the obtained residue was triturated with Et_2O to afford the title compound **16**, **18**, **20**.

2-(Dimethylamino)-N-nitrobenzenesulfonamide (**12**).

Compound **12** was obtained according the general procedure 1 earlier reported. White solid. 71 % yield; δ_H (400 MHz, DMSO-d_6): 8.14 (d, J = 8.3 Hz, 1H), 7.90 (m, 2H, Ar), 7.72 (t, J = 7.6 Hz, 1H, Ar), 3.36 (s, 6H, 2 x CH_3). δ_C (100 MHz, DMSO-d_6): 143.11, 135.98, 133.65, 131.61, 131.56, 122.91, 48.43; ESI-MS (m/z) $[M-H]^-$: calculated for $C_8H_{10}N_3O_4S$ 244.0, found 244.0

3-(Dimethylamino)-N-nitrobenzenesulfonamide **(13)**.

Compound **13** was obtained according the general procedure 1 earlier reported. White solid. 75 % yield; δ_H (400 MHz, DMSO-d_6): 8.17 (d, J = 8.1 Hz, 1H, Ar), 7.91 (m, 2H, Ar), 7.73 (t, J = 7.4 Hz, 1H, Ar), 3.37 (s, 6H, 2 x CH_3). δ_C (100 MHz, DMSO-d_6): 143.02, 136.00, 133.65, 131.70, 131.56, 122.95, 48.46; ESI-MS (m/z) $[M-H]^-$: calculated for $C_8H_{10}N_3O_4S$ 244.0; found 244.0

*3-Amino-4-hydroxy-N,5-dinitrobenzenesulfonamide ammonium salt (***16***).*

Compound **18** was obtained according the general procedure 2 earlier reported. Brownish solid. 67 % yield; δ_H (400 MHz, DMSO-d_6): 7.71 (d, J = 2.4, 1H), 7.53 (d, J = 2.4, 1H), 7.25 (s, 4H, exchange with D_2O, NH_4^+); δ_C (100 MHz, DMSO-d_6): 145.81, 135.92, 135.54, 134.12, 121.67, 116.29; ESI-MS (m/z) $[M-H]^-$: calculated for $C_6H_5N_4O_7S$ 277.0, found 276.9.

5-Amino-N-nitro-1,3,4-thiadiazole-2-sulfonamide ammonium salt (**18**).

Compound **18** was obtained according the general procedure 2 earlier reported. Yellow solid. 77 % yield; δ_H (400 MHz, DMSO-d_6): 7.63 (s, 2H, exchange with D_2O, NH_2), 7.22 (s, 4H, exchange with D_2O, NH_4^+); δ_C (100 MHz, DMSO-d_6): 172.66, 158.98; ESI-MS (m/z) $[M-H]^-$: calculated for $C_2H_2N_5O_4S_2$ 224.0, found 223.9.

5-Imino-4-methyl-N-nitro-4,5-dihydro-1,3,4-thiadiazole-2-sulfonamide ammonium salt (**20**).

Compound **20** was obtained according the general procedure 2 earlier reported. Yellow solid. 77 % yield; δ_H (400 MHz, DMSO-d_6): 10.05 (s, 1H, exchange with D_2O, NH), 7.18 (s, 4H, exchange with D_2O, NH_4^+), 3.94 (s, 3H, CH_3); δ_C (100 MHz, DMSO-d_6): 168.22, 154.70, 38.72; ESI-MS (m/z) $[M-H]^-$: calculated for $C_3H_4N_5O_4S_2$ 238.0; found 237.9.

General synthetic procedure of Ag salts of N-nitrobenzenesulfonamides 21-30.

Procedure 3. Ag_2CO_3 (0.5 equiv) was added to a suspension of N-nitrobenzenesulfonamides **9-14** (0.2 g, 1.0 equiv) in H_2O (5 mL), and the mixture was stirred o.n. at rt protected from light. The reaction was heated to boiling point, filtered quickly and the recrystallized powder was collected by filtration to afford the title compound **21-26**.

Procedure 4. $AgNO_3$ (1.1 equiv) was added to a solution of N-nitrobenzenesulfonamides **15**, **16**, **18**, **20** (0.2 g, 1.0 equiv) and NaOH (0.9 equiv) in H_2O (5 mL) and the formed precipitate was filtered-off and recrystallized from water to afford the title compound **27-30.**

2-Amino-N-nitrobenzenesulfonamide silver salt (**21**).

Compound **21** was obtained according the general procedure 3 earlier reported. Grey solid. 72 % yield; δ_H (400 MHz, DMSO-d_6): 7.51 (d, J = 7.9 Hz, 1H, Ar), 7.17 (t, J = 7.5 Hz, 1H, Ar), 6.70 (d, J = 8.2 Hz, 1H, Ar), 6.55 (t, J = 7.5 Hz, 1H, Ar), 5.81 (bs, 2H, exchange with D_2O, NH_2); δ_C (100 MHz, DMSO-d_6): 146.80, 132.66, 130.66, 121.40, 116.28, 114.45;

ESI-MS (m/z) $[M-H]^-$: calculated for $C_2H_2N_5O_4S_2$ 224.0, found 223.9; $[M^+]$: calculated for Ag 106.9, found 106.9.

3-Amino-N-nitrobenzenesulfonamide silver salt (**22**).

Compound **22** was obtained according the general procedure 3 earlier reported. Grey solid. 74 % yield; δ_H (400 MHz, DMSO-d_6): 7.45 (d, J = 8.8 Hz, 2H, Ar), 6.55 (d, J = 8.8 Hz, 2H, Ar), 5.84 (bs, 2H, exchange with D_2O, NH_2); δ_C (100 MHz, DMSO-d_6): 148.52, 142.77, 128.46, 116.49, 114.76, 112.86; ESI-MS (m/z) $[M-H]^-$: calculated for $C_2H_2N_5O_4S_2$ 224.0, found 223.9; $[M^+]$: calculated for Ag 106.9, found 106.9.

4-Amino-N-nitrobenzenesulfonamide silver salt (**23**).

Compound **23** was obtained according the general procedure 3 earlier reported. Grey solid. 79 % yield; δ_H (400 MHz, DMSO-d_6): 7.45 (d, J = 8.8 Hz, 2H, Ar), 6.55 (d, J = 8.8 Hz, 2H, Ar), 5.84 (bs, 2H, exchange with D_2O, NH_2); δ_C (100 MHz, DMSO-d_6): 153.35, 131.26, 126.94, 113.10; ESI-MS (m/z) $[M-H]^-$: calculated for $C_2H_2N_5O_4S_2$ 224.0, found 223.9; $[M^+]$: calculated for Ag 106.9, found 106.9.

2-(Dimethylamino)-N-nitrobenzenesulfonamide silver salt (**24**).

Compound **24** was obtained according the general procedure 3 earlier reported. Grey solid. 71 % yield; δ_H (400 MHz, DMSO-d_6): 7.91 (d, J = 7.9 1H, Ar), 7.56 (t, J = 7.1 Hz, 1H, Ar), 7.45 (d, J = 7.8 Hz, 1H, Ar), 7.25 (t, J = 7.5 Hz, 1H, Ar), 2.71 (s, 6H, 2 x CH_3); δ_C (100 MHz, DMSO-d_6): 154.19, 136.75, 134.54, 132.29, 124.97, 124.17, 47.16; ESI-MS (m/z) $[M-H]^-$: calculated for $C_2H_2N_5O_4S_2$ 224.0, found 223.9; $[M^+]$: calculated for Ag 106.9, found 106.9.

3-(Dimethylamino)-N-nitrobenzenesulfonamide silver salt (**25**).

Compound **25** was obtained according the general procedure 3 earlier reported. Grey solid. 70 % yield; δ_H (400 MHz, DMSO-d_6): 7.90 (d, J = 7.8 Hz, 2H, Ar), 7.68 (m, 2H, Ar), 7.41 (m, 2H, Ar), 3.00 (s, 6H, 2 x CH_3); δ_C (100 MHz, DMSO-d_6): 151.32, 134.79, 134.65, 131.85, 124.72, 123.06, 47.58; ESI-MS (m/z) $[M-H]^-$: calculated for $C_2H_2N_5O_4S_2$ 224.0, found 223.9; $[M^+]$: calculated for Ag 106.9, found 106.9.

4-(Dimethylamino)-N-nitrobenzenesulfonamide silver salt (**26**).

Compound **26** was obtained according the general procedure 3 earlier reported. Grey solid. 73 % yield; δ_H (400 MHz, DMSO-d_6): 7.61 (d, *J* = 8.9 Hz, 2H, Ar), 6.71 (d, *J* = 8.9 Hz, 2H, Ar), 2.97 (s, 6H, 2 x CH_3); δ_C (100 MHz, DMSO-d_6): 159.24, 153.48, 131.03, 126.82, 111.39, 40.75; ESI-MS (m/z) $[M-H]^-$: calculated for $C_2H_2N_5O_4S_2$ 224.0, found 223.9; $[M^+]$: calculated for Ag 106.9, found 106.9.

4-(Aminomethyl)-N-nitrobenzenesulfonamide silver salt (**27**).

Compound **27** was obtained according the general procedure 4 earlier reported. Grey solid. 62 % yield; δ_H (400 MHz, DMSO-d_6): 7.75 (d, *J* = 8.3 Hz, 2H, Ar), 7.50 (d, *J* = 8.3 Hz, 2H, Ar), 4.10 (bs, 2H, exchange with D_2O, NH_2), 3.83 (s, 2H, CH_2); δ_C (100 MHz, DMSO-d_6): 145.40, 141.09, 128.57, 128.11, 47.27; ESI-MS (m/z) $[M-H]^-$: calculated for $C_2H_2N_5O_4S_2$ 224.0, found 223.9; $[M^+]$: calculated for Ag 106.9, found 106.9.

3-Amino-4-hydroxy-N,5-dinitrobenzenesulfonamide silver salt (**28**).

Compound **28** was obtained according the general procedure 4 earlier reported. Brownish solid. 42 % yield; δ_H (400 MHz, DMSO-d_6): 7.59 (d, J = 2.4, 1H, Ar), 7.38 (d, J = 2.4, 1H, Ar); δ_C (100 MHz, DMSO-d_6): 143.76, 141.18, 135.82, 133.48, 117.54, 112.47; ESI-MS (m/z) $[M-H]^-$: calculated for $C_2H_2N_5O_4S_2$ 224.0, found 223.9; $[M^+]$: calculated for Ag 106.9, found 106.9.

5-Amino-N-nitro-1,3,4-thiadiazole-2-sulfonamide silver salt (**29**).

Compound **29** was obtained according the general procedure 3 earlier reported. Grey solid. 58 % yield; δ_H (400 MHz, DMSO-d_6): 8.04 (bs, 2H, exchange with D_2O, NH_2); δ_C (100 MHz, DMSO-d_6): 173.60, 160.94; ESI-MS (m/z) $[M-H]^-$: calculated for $C_2H_2N_5O_4S_2$ 224.0, found 223.9; $[M^+]$: calculated for Ag 106.9, found 106.9.

5-Imino-4-methyl-N-nitro-4,5-dihydro-1,3,4-thiadiazole-2-sulfonamide silver salt (**30**).

Compound **30** was obtained according the general procedure 4 earlier reported. Grey solid. 51 % yield; δ_H (400 MHz, DMSO-d_6): 9.79 (bs, 1H, exchange with D_2O, NH_2), 3.75 (s, 3H,

CH3); δ_C (100 MHz, DMSO-d_6): 169.43, 157.49, 38.80; ESI-MS (m/z) $[M-H]^-$: calculated for $C_3H_4N_5O_4S_2$ 238.0; found 237.9; $[M^+]$: calculated for Ag 106.9, found 106.9.

Carbonic anhydrase inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity.[27] 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 Na_2SO_4 (for maintaining constant the ionic strength), 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 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 [31,32] and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier.[33,34]

Trypansoma cruzi and *Leishmania* parasites cultures

Epimastigote forms of the *T. cruzi* clone Dm28c [35], *T. cruzi* Y [36] strains obtained from the Laboratory of Cellular Ultrastructure), *Leishmania (L.) infantum* MHOM/BR/1974/PP75 and *L. amazonensis* IFLA/BR/1967/PH8 were donate by the *Leishmania* Type Culture Collection (LTCC) all parasites of Oswaldo Cruz Institute/Fiocruz (Rio de Janeiro, RJ, Brazil) were used. The parasites were maintained in PBHIL medium supplemented with 10% bovine serum (FBS) at 28 °C.[37]

RAW 264.7 Macrophage cell line cultures

RAW 264.7 murine macrophages were obtained from the National Institute of Metrology, Quality and Technology (Instituto Nacional de Metrologia, Qualidade e Tecnologia, INMETRO) and maintained in DMEM medium supplemented with 10% FBS at 37ºC in a 5% controlled CO_2 atmosphere. Cell maintenance was performed every 48-72 h, time necessary for cells to achieve confluent monolayers.

Inhibitory activity of inhibitor on epimastigotes of *Trypanosoma cruzi* and promastigotes of *Leishmania*

Trypanosoma cruzi Dm28c e Y and promastigotes of *Leishmania amazonensis and L. infantum*

The evaluation of anti-parasites activity was performed by successive microdilutions in 96 well plates (1.8x 10^6 parasites/well) incubated for 48 hours with the synthetic compounds in the PHBIL medium supplemented with 10% FBS in the following concentrations: 256, 128, 64, 32, 16, 8, 4, and 2 μM. The experiment controls were: negative control (culture medium without parasite) and positive culture (culture medium with parasite). Benznidazole and Amphotericin B were used as reference drugs of *T. cruzi* and *Leishmania* respectively and were progressively diluted with the parasite. The Minimum inhibitory concentration (MIC) for epimastigotes (*T. cruzi* DM28c and Y) and promastigotes (*L. amazonensis* and *L. infantum*) was performed using resazurin (125 μM) as an indicator of

cellular metabolic function. MIC was determined as the lowest concentration of the inhibitor capable of inhibiting in vitro growth of the parasites by spectrophotometric analysis at 490 and 595 [38] The determination of IC_{50} (concentration of drug which reduces epimastigotes number cells by 50%).

Cytotoxicity assay in macrophages

Cytotoxicity was performed using tetrazolium dye (MTT) colorimetric assay. RAW 264.7 macrophages cells were harvest after confluent monolayer achievement.[39] The cells were washed twice with PBS and a cellular suspension of 10^6 cells/ml was prepared in fresh DMEM culture medium. Aliquots of 100 ml of the cellular suspension were placed into polystyrene 96-well plates, and then incubated at 37 ^{0}C in a 5% CO_2 atmosphere for 6 h (in order to obtain an adherence of macrophages. After this period, the adherent cells were subjected to treatment with several concentrations of the drugs (2–256 μM), and then incubated for additional 48 h. Finally, 20 ml of a MTT solution (5 mg/ml) were added to each well and the plates incubated for 4 h. Macrophage viability was determined after formazan crystals solubilization with DMSO followed by the absorbance measurement at 570 nm using a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA).

Determination of selectivity index

A selectivity index (SI) was calculated and is defined as the RAW IC_{50} value divided by the *T. cruzi* IC_{50} value ($SI = CC_{50}/CI_{50}$), which expresses the safety index of the tested substance Benznidazole (Sigma-Aldrich, Milan, Italy) was kept as a positive control drug for the cytotoxicity assay on RAW 264.7.

Cytotoxicity assay with tripomastigotes forms of *Trypanosma cruzi*

Vero cells were subculture weakly by enzyme dissociation. Briefly, confluent cultures were dissociated with trypsin and EDTA solution (0,02%) and isolated cells seeded at a density of 8 x 10^5 cells/ 150 cm2 culture flask in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Cultures were maintained at 37 °C in 5% of CO_2 atmosphere. Vero cells (1.5 x 10^4) were seeded on 96-well white culture plates (Greiner Bio-One North America, Inc.) and, after 24 h of culture in RPMI 1640 medium supplemented with 10%

fetal bovine serum, the monolayers were treated for 72 h at 37 °C with a serial two-fold dilution of benznidazole (Bz) and silver N-nitrosulfonamides (1.95 – 500 μM). Cell viability, determined by quantitation of ATP in metabolic active cells, was measured with CellTiter-Glo kit (Promega Corporation, Madison, WI, USA). The luminescent signal was read on FlexStation 3 reader (Molecular Devices, Sunnyvale, CA, USA). CC_{50} value, the concentration of compound that reduced 50% of Vero cells viability, was determined by linear regression. Controls were performed in dimethyl sulfoxide (DMSO; ≤1%). Three independent assays were performed in duplicate.

***In vitro* trypanocidal activity assay: trypomastigote and amastigote forms**

Tissue culture-derived trypomastigotes were harvested from *T. cruzi*-infected Vero cells cultures at 4 days post infection (4 dpi). Briefly, Vero cells monolayers were infected with *T. cruzi*, Dm28c clone genetically modified to express luciferase (Dm28c-Luc), at a ratio of 10:1 parasites/host cell and, after 24h of infection, free trypomastigotes were washed out and replaced by RPMI 1640 medium supplemented with 10% FBS. At 4 dpi, trypomastigotes released by infected cells were harvested and used in experimental assays.[40]

The effect of silver N-nitrosulfonamides was evaluated against trypomastigote and intracellular amastigote forms of *T. cruzi*. Tissue culture-derived trypomastigotes (1×10^6 parasites/well), Dm28c clone genetically modified to express luciferase (Dm28c-Luc), were exposed for 24 h at 37 °C to a range concentration of Bnz and N-nitrosulfonamides derivatives (0.41 - 100 μM). For intracellular amastigotes, Vero cells cultures infected with *T. cruzi* (Dm28c-Luc clone) for 24 h were treated for 72 h at 37 °C with different concentrations of the inhibitors and Bz (0.41 - 100 μM). The viability of parasites, both trypomastigotes and intracellular amastigotes, was evaluated after addition of the enzyme substrate, luciferin (300 μg/mL), followed by luminescence measurement using FlexStation 3 reader. The concentration of compound capable of reducing the number of viable parasites by 50% (IC_{50} value) was calculated by linear regression. The final concentration of DMSO did not exceed 1%. Three independent assays were performed in duplicate.

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