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Screening of NOS Activity and Selectivity of newly Synthesized Acetamidines using RP-HPLC

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Abstract

Nitric Oxide Synthase (NOS) inhibitors could play a powerful role in inflammatory and neurodegenerative diseases. In this work, novel acetamidine derivatives of NOS were synthesized and the inhibitor activity was evalued. To screen the activity and selectivity, the L-citrulline residue, after the enzymatic NOS assay, was derivatized with o-phthaldialdehyde/N-acetyl cysteine (OPA/NAC) and then evaluated by RP-HPLC method with fluorescence detection.

All compounds did not affect the activity of endothelial and neuronal isoforms, while nine of them possessed a percentage of iNOS activity at 10 μM lower than 50%, and were selected for IC_{50} evaluation. Among them, a compound emerged as a very potent (IC_{50} of 53 nM) and selective iNOS inhibitor.

Keywords

High-performance liquid chromatography; Nitric Oxide Synthase inhibitor; acetamidine; fluorescence detection; OPA/NAC derivatization

1. Introduction

Nitric oxide (NO), an inorganic free radical, possesses a pivotal role in cell and neuronal signaling, blood pressure regulation, and the immune response [1]. NO is biosynthesized by a family of enzymes, named nitric oxide synthase (NOS) that oxidized L-arginine in L-citrulline. The biosynthesis of NO requires the presence of molecular oxygen, nicotinamide adenine dinucleotide phosphate (NADPH), and other cofactors like heme, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH_4). NOS is an homodimeric enzyme and each monomer can be divided into C-terminal reductase domain and N-terminal oxygenase domain; the electron transfer starts from NADPH in C-terminal domain of one monomer and, through FAD and FMN, reach the N-terminal domain of the other monomer, which binds L-arginine, BH_4 and heme. The oxidative reaction consists of two steps: the first forms the intermediate product N^{ω}-L-arginine, an the second forms L-citrulline and NO [2]. Three NOS isoforms have been identified in mammals: the constitutively expressed neuronal (nNOS) and endothelial (eNOS) and an inducible one (iNOS). The constitutive isozymes produce little amount of NO for short period of time and the production is Ca^{2+}-dependent, while the inducible isoform is Ca^{2+}-independent and produces large amount of NO for long period of time [3]. Even though NO mediates several physiological functions, a number of pathologies, including septic shock, arthritis, diabetes, ischemia-reperfusion injury, pain, and various neurodegenerative diseases, are associated with the overproduction of NO by nNOS and iNOS. In contrast, NO produced by eNOS maintains normal blood pressure and flow, and the inhibition leads to unwanted effects such as enhanced white cell and platelet activation, hypertension and increased atherogenesis. For this reason, only specific and selective inhibition of iNOS and/or nNOS provides a promising strategy in developing therapeutics for the treatment of inflammation, pain and neurodegenerative disorders [4-5].

NOS activity can be evaluated by the quantification of NO or citrulline levels. The most common NO detection assays include the colorimetric Griess assay, based on the reduction

of NO to nitrite and the detection of the chromophoric diazo compound, and the spectrophotometric oxyhemoglobin assay, based on the conversion via NO of oxyhemoglobin to methemoglobin that exert diverse optical spectra. The reaction catalyzed by NOS possesses a 1:1 stoichiometry, that is one mol of the substrate L-arginine produces one mol of NO and one mol of L-citrulline. For this reason, the detection of L- citrulline is equivalent to NO measurement. The most commonly used citrulline assays are the radiometric detection of ^{3}H- or ^{14}C-labeled L-citrulline formed by radiolabeled L-arginine, and, in the last years, emerged the reverse-phase high-performance liquid chromatography with fluorescence detection of citrulline [6-7].

In this paper the synthesis of novel acetamidine NOS inhibitors and the evaluation of the inhibitory activity toward inducible, endothelial, and neuronal NOS isozymes is described.

The aim of this study is to identify a potent and selective NOS inhibitor containing an acetamidine function that can be host in the active site of the enzyme where the guanidino group of the natural substrate, L-arginine, makes an hydrogen bond interaction with a glutamate residue. The acetamidine can react with the aminoacid residue and block the enzimatic reaction that conduct to NO production.

Our research group is involved in the synthesis and biological evaluation of different N-substituted acetamidines, structurally related to the leading scaffold 1400W (**1**, **Figure 1**), a potent and selective iNOS inhibitor with a IC_{50} of 0.08 μM [7-12].

The compounds reported in this study are related to 1400W and possess the amino group of the lead linked to an indole ring throughout an amide linker (**2-5**, **Figure 1**), or benzylmethylamino portion is substitued with an indole, quinoline or isoquinoline ring (**6-18**, **Figure 1**). The *in vitro* activity and selectivity for iNOS and/or nNOS vs eNOS was evaluated measuring the L-citrulline level using the reversed-phase high-performance liquid chromatography method with fluorescence detection based on the precolumn derivatization of citrulline with *o*-phthaldialdehyde/*N*-acetyl cysteine.

2 Materials and methods

2.1 Chemicals and reagents

All chemicals, and HPLC grade solvents were supplied from Sigma–Aldrich (St. Louis, MO, USA). Recombinant murine iNOS, recombinant human nNOS, and recombinant bovine eNOS were purchased from Vinci-Biochem s.r.l. (Vinci, Florence, Italy). HPLC-grade water was obtained by passage through an Elga Purelab water purification system (Elga Labwater,UK). A centrifuge EBA21 (Hettich, Germany) was used.

2.2 Standard solutions

Stock solutions of compounds **2-18** (1000 µM) were prepared in 40 mM HEPES buffer pH = 7.4 and then diluted with the appropriate volume of HEPES buffer pH = 7.4 to the appropriate concentration range of 0.01–10 µM. Stock solution of methanolic OPA contained 0.256 g OPA in 50 mL methanol. Stock solution of each NOS isoform (100 µg/mL) was prepared in 40 mM HEPES buffer pH = 7.4 and stored at –80 °C.

2.3 Methods

2.3.1 Chemistry

The acetamidine function was obtained by the reaction of an amine group with *S*-2-naphthylmethyl thioacetimidate (**19**), which is synthesized as previously described [8].

Compouds **2**-**5** were prepared according to the general **scheme 1**. BOC-protected *m*-xylylenediamine was treated with **19** and the consecutive deletion of the BOC group with trifluroacetic acid gave the lead compound **1**, that was then reacted, in presence of BOP and DIPA, with the appropriate indolyl alkyl carboxylic acid to give compounds **2**-**5**.

Compounds **6-10** and **12** were prepared according to the general **scheme 2**. The appropriate carboxylic acid was transformed in amide then reducted with lithium aluminium hydride in the corresponding amine. The final reaction with **19** generated the acetamidino group. Acetamidines **11** and **13-18** were obtained treating the corresponding amine, commercially available, with **19** according to the last reaction of the general **scheme 2**.

2.3.2 Chemical stability

The chemical stability analyses of compounds **2-18** in a simulated non-enzymatic gastric fluid, 0.01 M hydrochloric acid buffer (pH 2.0), in a simulated non-enzymatic intestinal fluid, a 0.01 M phosphate buffer (pH 7.4), and in a 0.1 mM sodium hydroxide solution (pH 9.0), were performed by HPLC with UV-Vis detection. 0.1 mL of compound stock solution (1 mg/mL in HPLC grade water) was added to 1.9 mL aqueous buffer solution, in screw-capped vials at 37 ± 0.5 °C. Samples (5 μL) were injected in the HPLC system at appropriate intervals for 72 h and analyzed.

2.3.3 Apparatus and chromatographic conditions for chemical stability

HPLC analyses were performed using a Waters (Milford, MA, USA) system composed of a P600 model pump, a 2487 Dual λ Absorbance detector, and a 7725i model sample injector (Rheodyne, Cotati, CA, USA) equipped with a 5μL loop. The analyses were performed on an XTerra MS C18 column (250 x 4.6 mm id, 5 μm particle size; Waters, Milford, MA, USA), equipped with an XTerra MS C18 guard column (Waters, Milford, MA, USA). A column thermostat oven module Igloo-Cil (Cil Cluzeau Info Labo, France) was used. Chromatograms were recorded on a Fujitsu Siemens Esprimo computer and data were processed by the Millenium32 software (Waters). The column was eluted at a flow rate of 0.7 mL/min with a mobile phase composed of 8 mM sodium borate (pH = 10) and CH_3OH (60:40), with 0.1% v/v trifluoroacetic acid. The mobile phase was daily prepared, filtered

through a 0.45 μm WTP membrane (Whatman,Maidstone, UK), and degassed before use. Column temperature was kept constant at 20 °C. The injection volume was 5 μL. The UV detector wavelength was set at 254 nm.

2.3.4 Enzymatic assay conditions

To perform the enzymatic assay, 10 μL of NOS stock solution was added to 80 μL of HEPES buffer pH = 7.4, containing 0.1 mM $CaCl_2$ for iNOS or 1 mM $CaCl_2$ for eNOS and nNOS, 1 mM DTT, 0.5 mg/mL BSA, 10 μM FMN, 10 μM FAD, 30 μM BH_4, 10 μg/mL CaM, and 11μM Larginine. To measure enzyme inhibition, a 10 μL solution of inhibitor (0.01–10 μM) was added to the enzyme assay solution, followed by a pre-incubation time of 15 min at room temperature. The reaction was initiated by addition of 10 μL of NADPH 7.5 mM (iNOS) or 10 mM (eNOS and nNOS) and was carried out at 37°C for 20 min. The reaction was stopped by adding 500 μL of ice-cold CH_3CN and the mixture was brought to dryness under vacuum and eventually stored at –20°C, before the fluorescence derivatization. All assays were performed in triplicate.

2.3.5 Fluorescence derivatization

The OPA/NAC reagent was prepared with the molar ratios of 1:3, reacting for 90 min, 5 mL of methanolic OPA solution and 20 mL of 0.2 M borate buffer containing 0.1 g of NAC. Final pH = 9.3 ± 0.05. The OPA/NAC solution was stored at 4°C and saved for no longer than seven days.

600 μL of HPLC-grade water was added to the residue of the enzymatic assay and centrifuged at 6000 rpm for 20 min. The fluorescence reaction is realized stirring 190 μL of supernatant and 60 μL of OPA/NAC solution for 5 min.

2.3.6 Apparatus and chromatographic conditions for the biological assay

HPLC analyses were performed using a Waters (Milford, MA, USA) system composed of a P600 model pump, a 2475 multi-fluorescence detector, and a 7725i model sample injector (Rheodyne, Cotati, CA, USA) equipped with a 5 µL loop. The analyses were performed on an XTerra MS C8 column (250 × 4.6 mm id, 5 µm particle size; Waters, Milford, MA, USA), equipped with an XTerra MS C8 guard column (Waters, Milford, MA, USA). A column thermostat oven module Igloo-Cil (Cil Cluzeau Info Labo, France) was used. Chromatograms were recorded on a Fujitsu Siemens Esprimo computer and data were processed by the Empower Pro software (Waters, MA, USA). The column was eluted at a flow rate of 0.7 mL/min with linear gradients of buffers A (5%in CH_3CN in 15 mM sodium borate with 0.1% v/v trifluoroacetic acid, pH = 9.4) and B (50% in CH_3CN in 8 mM sodium borate with 0.1% v/v trifluoroacetic acid, pH = 9.4). The solvent gradient was: 0–20% B at 0–10 min, B to 25% at 10–15 min, then to 40% at 15–20 min and to 70% at 20–28 min. This composition was maintained until t = 35 min, before being reduced to the initial 0% B composition.

The mobile phase was prepared daily, filtered through a 0.45 µm WTP membrane (Whatman, Maidstone, UK), sonicated and degassed before use. Column temperature was kept constant at 20°C. The injection volume was 5 µL. The fluorescence intensity in the column eluate was monitored at 335 nm (excitation) and 439 nm (emission).

2.3.7 HPLC method evaluation

The RP-HPLC method applied in this study was previously validated as referred in ref. 7.

For linearity, a 0.999 correlation coefficient of the straight regression line was obtained in the range of 0.001–100 µM of derivatized citrulline standard solutions. The LOQ was 183 nmol/L, while LOD was 60 nmol/L.

3. Result and discussion

3. 1 Chemistry

Compounds described in this paper can be divided into two series: the first series contains compounds **2-5** (**Figure 1**) differing from the lead compound, the N-[3-(aminomethyl)benzyl]acetamidine (1400 W, **1**, **Figure 1**) for the presence of an indole ring connected through a methylene chain to the *N*- aminomethyl group throughout an amide linker, while in the other series, compounds **6-18** (**Figure 1**), the acetamidino group is connected, directly or by a linker, in different positions to an indole (**6-13**), quinoline (**14-16**) or isoquinoline (**17-18**) moiety.

3.2 Screening of the NOS inhibitor activity

The biological activity and selectivity of the new acetamidine derivatives **2-18** as selective inhibitors of NOS isozyme have been evaluated by means of *in vitro* assay using recombinant enzyme of murine iNOS, human nNOS, and bovine eNOS.

The *in vitro* assay consists in the preparation of an enzymatic reaction, incubated for a period of time, in the absence (control) or in the presence of the inhibitor, and the activation of the enzymatic process with NADPH. The reaction was thermostated for 30 minutes at 37°C and then stopped. The inhibitor activity of each compound was evaluated by measuring the L-citrulline level, equal to NO production, and, for this reason, the enzymatic mixture was derivatized with OPA/NAC, and the citrulline fluorescent derivative was quantified with RP-HPLC. The introduction of 0.1% v/v of TFA as ion-pair in the mobile phase improved the chromatographic peak shape.

Since, for a therapeutic point of view, is a very powerful tool the inhibition of iNOS and/or nNOS without the involvement of the endothelial isoform, for a first biological screening 10 μM concentration of each compound in the presence of one NOS isoform was used.

Figure 2 shows the percentage of residual NOS activity in the presence of each compound compared to the control (absence of inhibitor) and blank (absence of NADPH, which starts the enzymatic reaction). The percentage of activity of endothelial and neuronal NOS are, for all acetamidine derivatives, superior to 95.4%, stating that these compounds do not affect these isoforms and can be considered as selective iNOS inhibitors. In the case of iNOS, on the contrary, all compounds show a decrease of NOS activity. In particular, in the series of 1400W derivatives, compounds **2**-**5**, the percentage of activity raise with the methyl chain length increasing between the indole ring and the amide; in fact, compound **2**, containing only a methylene shows 21.9 % of activity and compound **3**, containing two methylene chain, possesses 34% of activity. The chain of three or four methyl, compounds **3** and **4**, generate a loss of activity (53.9 and 86.6 % of activity, respectively).

In the indole series, **6**-**13**, the acetamidino group is directly liked or through methyl chain in different position of the indole ring. Compound **6**, in which the acetaminidino function is linked to C_2 of the indole via a methylene linker, possesses 50.6% of activity, and 10 μM can be considered as the IC_{50} value. Compounds **7**-**10**, differing from each other only for the chain length that connect the acetamidine to C_3, possess percentage of activity depending on the chain length: longer is the chain, lesser is the percentage of activity, until a length of three carbon atoms. Compound **8**-**10** show, respectively, 22.7%, 15.5%, and 20.5% of activity. The best value is obtained with compound **9**. Compound **11**, in which the acetaminidino function is directly linked to C_5, possesses 81.4% of activity indicating that this modification did not improve the inhibitory activity of this compound compared to the lead. Little modification to the structure of **11**, that is the introduction of a methylene spacer between the acetamidine and C_5, compound **12**, or the switch of the acetamidine to C_6, compound **13**, decrease the percentage of activity to 18.2% and 13.6 %, respectively, making them good inhibitor for further investigations.

The quinoline derivatives, **14**-**15**, which contain the acetamidine directly linked to C_3 and C_5, respectively, show 39.4 and 40.2 % value of percentage of activity. For the isoquinoline derivatives, **16**-**18**, the percentage of activity is, instead, higher than 56.8% for all compounds, containing the acetamidine in C_3, C_5, or C_8.

3.3 iNOS IC_{50} calculation

With the aim to estimate the iNOS IC_{50} value, the *in vitro* assay was realized in the presence of five different concentrations of the inhibitor (0.01, 0.1, 0.5, 1, and 10 μM). Compounds **2**-**3**, **8**-**10**, **12**-**15**, with a percentage of iNOS activity at 10μM under 50%, were selected for the IC_{50} calculation. In **Table 1** are shown the IC_{50} obtained for the selected compounds.

Compounds **2**-**3**, the 1400W derivatives, possess an IC_{50} value of 0.888 and 1.838 μM, respectively.

In the indole serie, the best IC_{50} value of 53 nM was obtained by compound **12**, in which the acetamidine moiety is liked to C_5 by a methyl chain, followed by compound **9** (IC_{50} of 0.241 μM) and compound **13** (IC_{50} of 0.446 μM). The substitution of the benzyl amino moiety of the lead **1** with an indole ring seems to be very promising.

In the series of quinoline derivatives, the IC_{50} values were higher than 2 μM.

3.4 Chemical stability

Compounds **2**-**18** were analyzed for the chemical stability in aqueous buffer at pH 2.0 with hydrochloric acid solution to simulate a non-enzymatic gastric fluid, at pH 7.4 with phosphate buffer to simulate a non-enzymatic intestinal fluid, and at pH 9.0 with sodium hydroxide solution. Compounds were injected in the HPLC system with UV detection at precise time (0, 1, 2, 4, 8, 24, 48, and 72 hours) and all of them resulted stable.

Conclusion

In the present study, the synthesis and the biological evaluation of novel acetamidine derivatives as Nitric Oxide Synthase inhibitor were described. The calculation of NOS activity and selectivity was realized monitoring the L-citrulline production during the enzymatic assay by means of RP-HPLC fluorescence detection of L-citrulline derivatized with OPA/NAC reagent before the chromatographic injection.

In this series of acetamidine emerged five compounds with submicromolar inhibitor activity on iNOS that did not posses any inhibitor activity on nNOS and eNOS at 10 μM; thus they can be considered selective iNOS inhibitors. In particular, compound **12**, containing the acetamidino group linked by a methyl chain to C_5 of the indole ring, show a very good potency (IC_{50} of 53 nM) and selectivity.

References

[1] R. Bruckdorfer, The basic about nitric oxide, Mol. Aspects Med. 26 (2005) 3-31.

[2] W.K. Alderton, C.E. Cooper, R.G. Knowles, Nitric Oxide Synthases: structure, function and inhibition, Biochem. J. 357 (2001) 593-615.

[3] U. Forstermann, W.C. Sessa, Nitric Oxide Synthases: regulation and function, Eur Heart J 33 (2012), 829-837.

[4] T.L. Poulos, H. Li, Structural basis for isoform-selective inhibition in nitric oxide synthase, Acc. Chem. Res. 46 (2013) 390-398.

[5] P. Mukherjee, M.A. Cinelli, S. Kang, R.B. Silverman, Development of nitric oxide synthase inhibitors for neurodegenerative and neuropathic pain, Chem. Soc. Rev. 43 (2014) 6814-6838.

[6] J.M. Hevel, M.A. Marletta, Nitric oxide synthase assay, Methods Enzymol. 233 (1994) 250-258.

[7] C. Maccallini, M. Di Matteo, A. Ammazzalorso, A. D'Angelo, B. De Filippis, S. Di Silvestre, M. Fantacuzzi, L. Giampietro, A. Pandolfi, R. Amoroso, Reversed-phase high-performance liquid chromatography method with fluorescence detection to screen nitric oxide synthases inhibitors, J. Sep. Sci. 37 (2014) 1380–1385.

[8] C. Maccallini, A. Patruno, N. Bešker, J.I. Alì, A. Ammazzalorso, B. De Filippis, S. Franceschelli, L. Giampietro, M. Pesce, M. Reale, M.L. Tricca, N. Re, M. Felaco, R.

Amoroso, Synthesis, biological evaluation, and docking studies of N-substituted acetamidines as selective inhibitors of inducible nitric oxide synthase, J. Med. Chem. 52 (2009) 1481–1485.

[9] C. Maccallini, A. Patruno, F. Lannutti, A. Ammazzalorso, B. De Filippis, M. Fantacuzzi, S. Franceschelli, L. Giampietro, S. Masella, M. Felaco, N. Re, R. Amoroso, N-Substituted acetamidines and 2-methylimidazole derivatives as selective inhibitors of neuronal nitric oxide synthase, Bioorg. Med. Chem. Lett. 20 (2010) 6495-6499.

[10] M. Fantacuzzi, C. Maccallini, F. Lannutti, A. Patruno, S. Masella, M. Pesce, L. Speranza, A. Ammazzalorso, B. De Filippis, L. Giampietro, N. Re, R. Amoroso, Selective inhibition of iNOS by benzyl- and dibenzyl derivatives of N-(3-aminobenzyl)acetamidine, ChemMedChem 6 (2011) 1203-1206.

[11] C. Maccallini, A. Patruno, A. Ammazzalorso, B. De Filippis, M. Fantacuzzi, S. Franceschelli, L. Giampietro, S. Masella, M. L. Tricca, R. Amoroso, Selective inhibition of inducible nitric oxide synthase by derivatives of acetamidine, Med. Chem. 8 (2012) 991-995.

[12] C. Maccallini, M. Montagnani, R. Paciotti, A. Ammazzalorso, B. De Filippis, M. Di Matteo, S. Di Silvestre, M. Fantacuzzi, L. Giampietro, M.A. Potenza, N. Re, A. Pandolfi, R. Amoroso, Selective acetamidine-based nitric oxide synthase inhibitors: synthesis, docking, and biological studies", ACS Med. Chem. Lett. 6 (2015) 635−640

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