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δ^1-Pyrroline-5-carboxylate reductase as a new target for therapeutics: inhibition of the enzyme from *Streptococcus pyogenes* and effects in vivo

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Abstract Compounds able to interfere with amino acid biosynthesis have the potential to inhibit cell growth. In both prokaryotic and eukaryotic microorganisms, unless an ornithine cyclodeaminase is present, the activity of δ^1-pyrroline-5-carboxylate (P5C) reductase is mandatory to proline production, and the enzyme inhibition should result in amino acid starvation, blocking in turn protein synthesis. The ability of some substituted derivatives of aminomethylenebisphosphonic acid and its analogues to interfere with the activity of the enzyme from the human pathogen *Streptococcus pyogenes* was investigated. Several compounds were able to suppress activity in the micromolar range of concentrations, with a mechanism of uncompetitive type with respect to the substrate P5C and non-competitive with respect to the electron donor NAD(P)H. The actual occurrence of enzyme inhibition in vivo was supported by the effects of the most active derivatives upon bacterial growth and free amino acid content.

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Abbreviations

IC_{50}	Concentration causing 50% inhibition of enzyme activity
LC_{50}	Concentration causing 50% inhibition of growth rate
OCD	Ornithine cyclodeaminase
P5C	δ^1-Pyrroline-5-carboxylic acid

Introduction

The development of bacterial resistance to current antibiotic therapies is an increasing threat for human health (Chen et al. 2009). Many of therapeutics that are currently in use came from a small set of molecular scaffolds, whose functional lifetime has been extended by synthetic tailoring. Because of the emergence and diffusion of multi-drug resistance, the discovery of new scaffolds is mandatory (Fischbach and Walsh 2009). As an alternative, new antibiotic targets should be identified (Pathania and Brown 2008). In this perspective, although they were exploited to date mainly as active principles for weed control (Tan et al. 2006), inhibitors of enzymes that catalyse key reactions in amino acid metabolism could represent promising new leads for the control of pathogenic microorganisms. In several instances, the inhibition of selected enzymes in amino acid biosynthesis has been indeed found to exert remarkable activity against bacteria (Harth and Horwitz 2003; Hutton et al. 2007; Liu et al. 2008; Ziebart et al. 2010).

From this point of view, little attention has been paid to date to proline synthesis. Proline plays an important role in protein structure, uniquely contributing to protein folding and stability (Ge and Pan 2009). Moreover, in a wide variety of microorganisms, a rapid and reversible increase in the intracellular concentration of free proline has been shown in response to either osmotic or temperature stress, implying a role in stress tolerance and osmoregulation (Empadinhas and Da Costa 2008; Takagi 2008). The ability of changing cellular osmolarity seems essential to cope with fluctuating external water potential, salinity and temperature, and survive in harsh environments (Höper et al. 2005). Some evidence also suggested that the ability of metabolising proline might function as a virulence factor for certain pathogenic bacteria (Nakajima et al 2008). In other cases, the same may occur indirectly: if unable to produce compatible osmolytes, the bacterial cell cannot achieve osmoadaptation in body fluids. As a consequence, the expression of certain virulence determinants (such as the pyelonephritis-associated pilus in *Escherichia coli*; Culham et al. 1998) is affected.

The development of effective inhibitors of proline synthesis may be hampered by the occurrence in bacteria of redundant biosynthetic pathways. Proline biosynthesis can in fact proceed from either glutamate or arginine, usually the glutamate route being the main pathway (Cunin et al. 1986; Aral and Kamoun 1997). Moreover, a stereospecific and irreversible conversion of L-ornithine to L-proline may be accomplished in a single step by the enzyme ornithine cyclodeaminase [OCD, EC 4.3.1.12] (Goodman et al. 2004). In the presence of two pathways, the inhibition of either of the enzymes that catalyze the rate-limiting steps would be ineffective, because proline starvation would not be achieved and the cell would be allowed to recover. However, because OCD has been identified only in a small set of soil and plant-associated bacteria, and the glutamate and the ornithine pathways share the last reaction catalyzed by a δ^1-pyrroline-5-carboxylate (P5C) reductase [EC 1.5.1.2], this goal might be achieved through the development of specific inhibitors for the latter enzyme. Since this dual anabolic route is shared also by plants (Lehmann et al. 2010), P5C reductase inhibitors might find application in crop protection as well. We previously synthesized and screened several derivatives of aminomethylenebisphosphonic acid (compound 01) for the ability to inhibit plant P5C reductase (Forlani et al. 2007). A group of phenyl derivatives were found effective in the micromolar to millimolar range, and indeed showed phytotoxic properties (Forlani et al. 2008a). Here, we report on the effectiveness of some derivatives and analogues of compound 01 against the bacterial P5C reductase, assessed at both the enzyme and the whole cell level.

Materials and methods

Enzyme purification

Escherichia coli BL21(DE3) pLysS cells, made competent by the calcium chloride method, were transformed with the pMCSG7 vector bearing the M1 GAS *Streptococcus pyogenes* P5C reductase gene (Nocek et al. 2005). Transformants were selected at 37°C on LB plates containing 100 mg l^{-1} ampicillin and 25 mg l^{-1} chloramphenicol. Freshly grown cultures in liquid LB medium (0.6 OD_{600}) were induced at 24°C with 1 mM IPTG. Cells were harvested by centrifugation 4 h after induction, and stored at −20°C. Pellets (about 2 g) were thawed and extracted in a mortar with 2 g g^{-1} alumina. All the subsequent operations were carried out at 4°C. The homogenate was resuspended in 20 ml g^{-1} of 50 mM Na phosphate buffer, pH 7.5, containing 200 mM NaCl and 0.5 mM DTT. Following clarification at 4,000g for 5 min, the extract was centrifuged at 18,000g for 15 min. The supernatant was immediately loaded at a constant flow of 10 ml h^{-1} onto a His-Select™ Nickel Affinity Gel (Sigma P6611) column (0.5 cm diameter, 2 ml bed-volume) equilibrated with extraction buffer. After extensive washing, the column was eluted stepwise with buffer containing increasing concentrations of imidazole, harvesting 1-ml fractions. The presence and the purity of the heterologous protein were determined by polyacrylamide gel electrophoresis under denaturing conditions. Pure fractions were combined, adjusted to a protein concentration of 0.5 mg ml^{-1}, filter sterilized (0.2 μm) and stored on ice. Under these conditions, the enzyme was remarkably stable, with more than 90% of the initial activity still retained after 6 month storage.

Enzyme assay

The physiological, forward reaction of P5C reductase was measured by following the P5C-dependent oxidation of NAD(P)H. Unless otherwise specified, the assay mixture contained 100 mM HEPES-KOH buffer, pH 7.5, 1 mM $MgCl_2$, 1 mM L-P5C and 0.4 mM NADH, in a final volume of 1 ml. A limiting amount of enzyme (0.60 nkat under standard assay conditions, corresponding to 25 ng protein, freshly water-diluted from the pure enzyme preparation) was added to the pre-warmed mixture, and the decrease in absorbance at 340 nm was determined at 37°C for up to 5 min by monitoring the sample at 30-s intervals against blanks from which P5C had been omitted. The activity was determined from the initial linear rate, with the assumption of an extinction coefficient of 6,220 M^{-1} cm^{-1}. DL-P5C was synthesized by the periodate oxidation of δ-allo-hydroxylysine, purified by cation-exchange chromatography

Fig. 1 Derivatives of compound 01 evaluated as possible inhibitors of P5C reductase from *Streptococcus pyogenes*
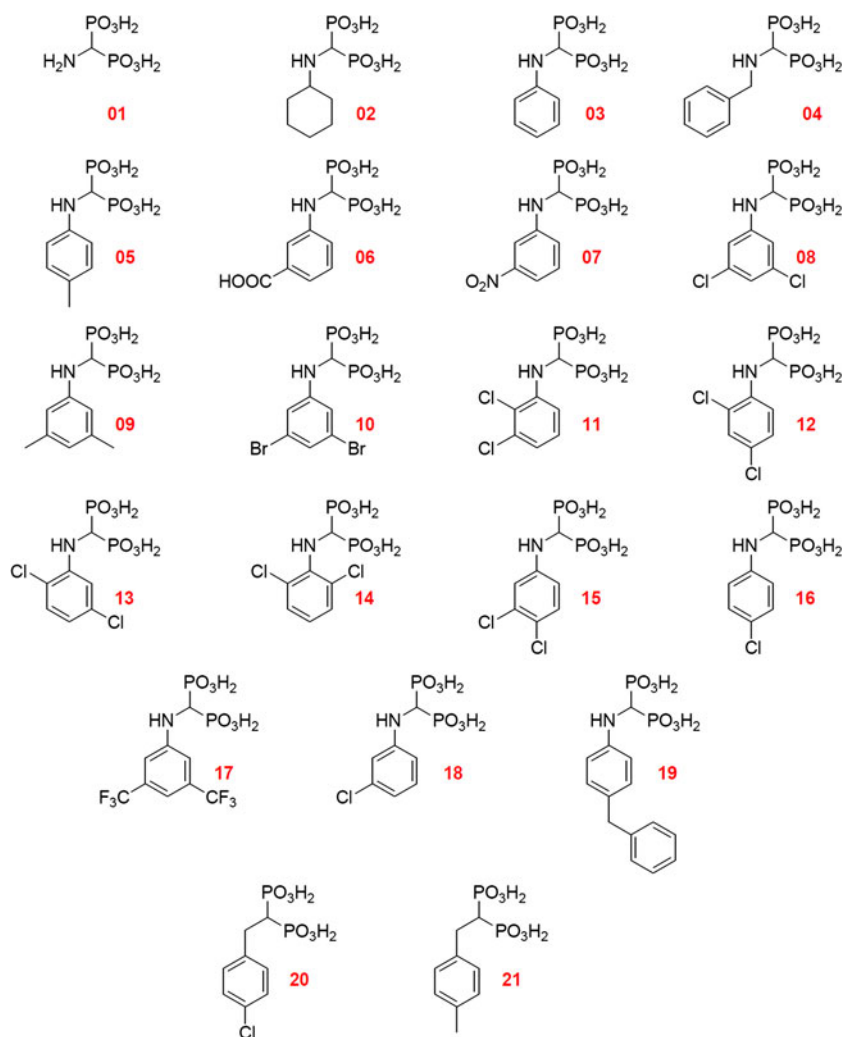
on a Dowex AG50 (200–400 mesh) column, and quantified by either the *o*-aminobenzaldehyde or the ninhydrin method, as described (Williams and Frank 1975). Final preparations in HCl 1 M were stored in the dark at 4°C. Proper dilutions were neutralized with the same volume of KOH 1 M just before being added to the reaction mixture. The indicated final concentration always refers to the L-isomer. The protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

Enzyme inhibition and kinetic analysis

N-Substituted derivatives and analogues of compound 01 (Fig. 1) were synthesized and purified as described previously (Forlani et al. 2008a, and supporting information available therein). P5C reductase inhibition was evaluated by adding to the reaction mixture an appropriate water dilution of a 1–5 mM freshly prepared solution of a given compound, brought to pH 7.5–8.0 with KOH, to obtain a

final concentration of 0.2 mM. Results were expressed as percent of the activity measured in parallel untreated controls, and are mean ± SE over at least three independent replications. Active compounds were further tested in the micromolar range, from 0.1 to 100 μM. At least three measurements were performed for each dose. The concentration causing 50% inhibition of P5C reductase activity (IC_{50}) was estimated utilizing the linear regression equation of enzyme activity values, expressed as a percentage of untreated controls, plotted against the logarithm of inhibitor concentration. At least three concentrations in the rectilinear part of the resulting sigmoid curve were considered. Confidence limits were computed according to Snedecor and Cochran (1989). IC_{50} values and confidence intervals were confirmed also by the probit method (Finney 1971).

For kinetic evaluations, the enzyme was assayed in the presence of increasing concentrations of a given compound at varying those of the substrates. The concentration for the variable substrate ranged from 80 to 268 μM NADH and

from 125 to 500 μM L-P5C. At least eight doses were evaluated for each substrate, at no less than triplicate. In the case of P5C (uncompetitive inhibition), K_I values were estimated from Lineweaver–Burk plots of activity, on the basis of the corresponding lowering in the apparent K_M value; three inhibitor concentrations, ranging from 0.5 to 1.5-fold the IC_{50} value, were tested. For NADH, three inhibitor concentrations, ranging between 0.6 and 1.8-fold the IC_{50} value, were tested. However, because of a non-competitive type of inhibition, K_I values were estimated from Dixon plots of activity by evaluating the effect of six inhibitor levels, ranging from 0.2 to 1.0-fold the IC_{50} value, in the presence of four substrate concentrations. To confirm the inhibitory mechanism, NADPH was also tested as the variable substrate, at concentrations ranging from 12 to 144 μM. All reported data are presented as means $\pm$ SEM (standard error of the mean) over results obtained with different inhibitor or substrate concentrations, respectively.

Antimicrobial activity determination

Streptococcus pyogenes Rosenbach, ATCC 19615 strain, was maintained on Trypticase Soy (Oxoid) agar plates containing 5% defibrinated horse blood. A freshly grown single colony was transferred into 10 ml of liquid medium in a 50 ml flask that was incubated overnight at 37°C under shaking (80 rpm). Because of the pathogenic potential, in all instances bacterial suspensions were handled under a biohazard laminar flow hood, taking care to avoid the formation of aerosols leading to environmental contamination. Personnel also wore latex gloves and disposable face mask as an additional safeguard. All contaminated materials/glassware were treated with bleach before being autoclaved and discarded/washed, respectively. The suspension was used to inoculate 15 ml test tubes containing 3 ml of Roche Susceptibility Test medium (Hall et al. 1984) in which proline had been omitted, to an initial density of 0.05 OD_{600}. Growth at 37°C was followed as the increase of absorbance at 600 nm, measured at 1-h intervals with a Novaspec Plus spectrophotometer (Amersham Biosciences) equipped with a tube adapter. Data were used to calculate growth constants (k), and the effect of the presence of a given compound upon bacterial growth was expressed as percent reduction of k. Each treatment was carried out at least in triplicate. The final biomass was evaluated as wet weight increase. The concentration causing 50% inhibition of bacterial growth rate or the final biomass (LC_{50}) was estimated utilizing the linear regression equation of growth values, expressed as a percentage of that for untreated controls, plotted against the logarithm of the inhibitor concentration. Cell viability was assessed by serial dilution with physiological saline and viable count on standard medium. In all cases, absorbance values were consistent with cell density, as estimated by vital count.

Amino acid extraction, separation and quantification

For amino acid analysis, bacterial cells were harvested by centrifugation for 10 min at 4,000g. Pellets were resuspended with 2 ml g^{-1} of a 3% (w/v) solution of 5-sulphosalicylic acid, and extracted by sonication with a UP50H ultrasonic processor (Hielscher, Germany) equipped with a Sonotrode MS1 tip, with 6 cycles of 30-s sonication at 70% of maximum power and 2-min cooling on ice. Cell debris was removed by centrifugation at 12,000g at 4°C, and samples (20 μl) were mixed with the same volume of o-phthaldialdehyde solution (0.5 M in 0.5 M sodium borate buffer, pH 10.0, containing 0.5 M β-mercaptoethanol and 10% [v/v] methanol). After exactly 60 s, 20 μl of derivatized samples were injected onto a 4.6 × 250 mm Zorbax ODS column (Rockland Technologies, Newport, DE), and elution proceeded as previously described (Mazzucotelli et al. 2006), monitoring the eluate at 340 nm. This procedure allowed complete resolution of equimolar mixtures of derivatizable amino acids, with detection limit of about 0.1 nmol. Proline and total amino acid content were quantified by the acid ninhydrin method, as described (Williams and Frank 1975). Results were expressed as μmol g^{-1} cells, and are mean $\pm$ SD over at least three replications.

Results and discussion

Phenyl derivatives of compound 01 are potent inhibitors of *S. pyogenes* P5C reductase

Within the frame of a project aimed at evaluating P5C reductase inhibitors as possible new leads for antibiotics, *Streptococcus pyogenes* was chosen as the experimental system to take advantage from both the availability of detailed information about the structure of the enzyme (Nocek et al. 2005) and the absence in this species of a gene coding for an OCD. A blast search, performed on the complete genome sequence of the M1 strain (Ferretti et al. 2001; http://www.genome.ou.edu/strep.html) using the *Pseudomonas putida* KT2440 gene coding for the cyclase as the query, yielded in fact no result. To reduce manipulation of this human pathogen, the P5C reductase gene was cloned (Nocek et al. 2005) and expressed in *E. coli*. The protein was purified to electrophoretic homogeneity, and the activity characterized throughout in order to ensure proper assay conditions (Petrollino D and Forlani G,

Table 1 Effect of phenyl derivatives and analogues of compound 01 upon the catalytic rate of *S. pyogenes* P5C reductase

Compound	Enzyme inhibition at 0.2 mM[a]	IC$_{50}$ (μM)[b]
01	3.0 ± 2.1	ND
02	8.1 ± 1.5	ND
03	3.0 ± 0.4	ND
04	3.0 ± 2.2	ND
05	33.4 ± 1.5	>1 mM
06	21.6 ± 1.5	>1 mM
07	3.2 ± 0.3	ND
08	91.1 ± 0.3	0.22 ± 0.02
09	70.5 ± 6.0	25.6 ± 9.6
10	98.4 ± 0.1	0.22 ± 0.01
11	95.4 ± 1.3	0.87 ± 0.10
12	52.0 ± 1.2	12.0 ± 6.1
13	63.9 ± 2.2	2.7 ± 0.8
14	96.2 ± 0.1	0.88 ± 0.07
15	92.5 ± 0.5	0.39 ± 0.02
16	61.5 ± 1.1	1.4 ± 0.3
17	71.2 ± 1.0	0.78 ± 0.09
18	71.2 ± 2.1	0.90 ± 0.13
19	94.5 ± 0.1	0.41 ± 0.05
20	33.8 ± 0.7	>1 mM
21	11.5 ± 3.9	ND

[a] P5C-dependent NADH oxidation was measured at 37°C for 5 min in the presence of 0.2 mM of a given compound. Results were expressed as percent of the activity measured in parallel, untreated controls. At least three replications were run for each phosphonate, and six for the control. Data are presented as percent inhibition, and are means ± SE over replications

[b] Active compounds (percent inhibition at 0.2 mM > 20%) were further tested in the micromolar range, from 0.1 to 100 μM. The concentrations causing 50% inhibition (IC$_{50}$) were estimated utilizing the linear regression equation of enzyme activity values, expressed as a percentage of untreated controls, plotted against the logarithm of inhibitor concentration. *ND* not determined

manuscript in preparation). When the activity of the enzyme was measured in the presence of various derivatives of compound 01 (Fig. 1) at 0.2 mM, a differential effect was evident (Table 1). Several substituted phenyl derivatives were able to completely suppress activity, while others were substantially ineffective. Active compounds were further tested in the micromolar range, allowing us to calculate the concentrations able to inhibit by 50% the enzymatic activity (Table 1). On the whole, bisphosphonates showed a strikingly higher effectiveness against the bacterial enzyme, with IC$_{50}$ values about two orders of magnitude lower than those previously found for plant P5C reductase (Fig. S1 in supplemental materials; Forlani et al. 2008a). This is not surprising since the inhibitors, because of the unavailability of the three-dimensional structure of

the plant enzyme, had been designed based on the results of a computer-aided docking analysis performed with just the crystal structure of *S. pyogenes* P5C reductase (Forlani et al. 2007). As to the scaffold, aminobisphosphonates showed higher effectiveness than the corresponding amino- and hydroxyphosphonates (Table S1 in supplemental materials). In addition, the replacement of the phenyl moiety with various pyridyl rings was found detrimental (Table S2 in supplemental materials). Concerning substituents in the phenyl rings, the presence of two halogen moieties yielded the highest inhibitory rates. This is consistent with previous data on the plant enzyme (Forlani et al. 2008a). However, in this case some other analogues showed equipotency, such as compounds 16 and 18, bearing a single chlorine, or compound 19, with a benzyl residue. The latter case is remarkable, since it shows that there is an additional cavity accommodating this bulky group.

The mode of action of the most effective inhibitors strengthens the possibility that inhibition may occur and be effective in vivo

The ability to interfere with the catalytic mechanism of a given enzyme in vitro does not necessarily imply that the treatment with the inhibitor would cause a block of the corresponding metabolic pathway in vivo. This is true for instance in the case of reversible inhibitors acting competitively. The initial suppression of enzyme activity leads to an increase of the intracellular level of the substrate(s), which in turn can relieve the inhibitory effect (Cascante et al. 2002). In order to obtain a better insight into the inhibitory properties of bisphosphonates, a proper kinetic analysis was therefore performed. Results, summarized in Fig. 2, showed that, analogously to the plant enzyme, *S. pyogenes* P5C reductase is inhibited by compound 08 by a mechanism of noncompetitive type with respect to either NADH or NADPH, and of uncompetitive type with respect to P5C. Similar data were obtained with the other most effective derivative, compound 19 (Fig. S2 in supplemental materials). Replotting of the data allowed calculation of inhibition constants (Table 2) that in all cases were below 1 μM. This set of results has some remarkable implications. Because inhibitors bind only to the P5C-enzyme complex, substrate binding in the P5C reductase active site could be of sequential character [P5C binds before NAD(P)H], as previously hypothesized only on the basis of the crystal structure of the protein (Nocek et al. 2005). This would be an unusual feature, since NAD-dependent reductases usually bind the dinucleotide co-substrate first. It confirms that enzyme inhibition occurs also when NADPH is the electron donor, ruling out the possibility that (because of the higher

Fig. 2 Kinetic analysis of the inhibition of *S. pyogenes* P5C reductase by compound 08. The enzyme was incubated with a fixed and saturating amount of P5C in the presence of increasing inhibitor concentrations, as indicated, at varying NADH (**a**) or NADPH (**b**) level. Lines converging to the *x*-axis in Lineweaver–Burk plot accounted for an inhibition of non competitive type. A similar analysis was carried out at varying P5C concentration, using either NADH (**c**) or NADPH (**d**) as the electron donor. Parallel lines in Lineweaver–Burk plot pointed to an inhibition of uncompetitive type. Unvariable substrate concentration was fixed at 1, 0.4 and 0.2 mM for L-P5C, NADH and NADPH, respectively
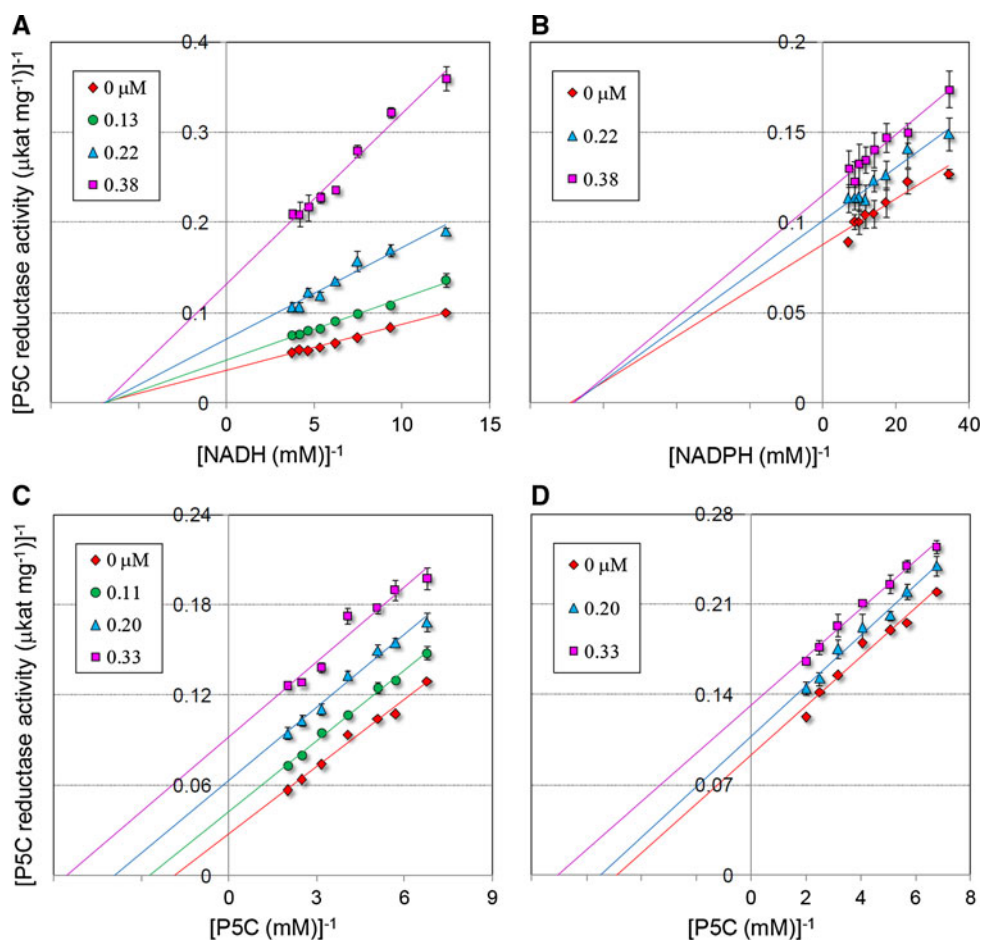

Table 2 Inhibition constants of *Streptococcus pyogenes* P5C reductase activity for two of the most active phenyl derivatives of compound 01

	Compound 08 (μM)	Compound 19 (μM)
K_I against L-P5C$^a_{(NADH)}$	0.67 ± 0.04	0.98 ± 0.07
K_I against L-P5C$^a_{(NADPH)}$	0.81 ± 0.01	ND
K_I against NADH^b	0.11 ± 0.01	0.37 ± 0.00
K_I against NADPH^b	0.68 ± 0.06	ND

ND not determined

[a] Uncompetitive inhibition, K_I value estimated from Lineweaver–Burk plots

[b] Noncompetitive inhibition, K_I value estimated from Dixon plots

steric hindrance) its binding may displace the inhibitor from the enzyme. It implies that an intracellular concentration of the inhibitor as low as 10 μM should bring about more than 90%-reduction of P5C reductase activity irrespectively from the increase of P5C level that would be caused as a consequence. All these elements concur in strengthening the possibility that these inhibitors may exert their effect also in vivo.

Aminomethylenebisphosphonic acids inhibit the growth of *S. pyogenes*, and cause an actual reduction of the intracellular pool of free proline

To substantiate this possibility, the growth of bacterial cultures was measured following the addition of increasing concentrations of the most active compounds to the culture medium. When supplied in the same range (10^{-6} to 10^{-5} M) in which they had been found to progressively reduce enzyme activity in vitro, they failed to exert cytostatic effects. However, if the concentration was increased over 100 μM, they showed a remarkable ability to inhibit cell proliferation (Fig. 3). The effect was proportional to the dose, and to the relative effectiveness shown by a given compound in vitro. Notably, for all compounds tested but mainly for compound 19 the treatment with low doses, which were unable to reduce growth, caused a remarkable and proportional lag before the attainment of the exponential phase. Such behaviour may be suggestive of the occurrence of detoxifying activiti(es) able to modify the inhibitor molecule. Once induced, they would be able to destroy low amounts of bisphosphonates, allowing the bacterial cell to recover. This would also explain the reason

Fig. 3 Effect of increasing concentrations of P5C reductase inhibitors on *S. pyogenes* growth. Late-log grown cultures were used to inoculate parallel tubes containing 3 ml of minimal medium to an initial OD_{600} of 0.1. One hour after the inoculum, a proper amount of a given bisphosphonate was added. The growth was followed for the subsequent 12 h as the increase in absorbance (**a**, **d**). After logarithmic transformation of the data, growth constants were calculated over the rectilinear part of the curves, whereas the final biomass (fresh weight) was evaluated following centrifugation 24 h after the inoculum (**b**, **c**)
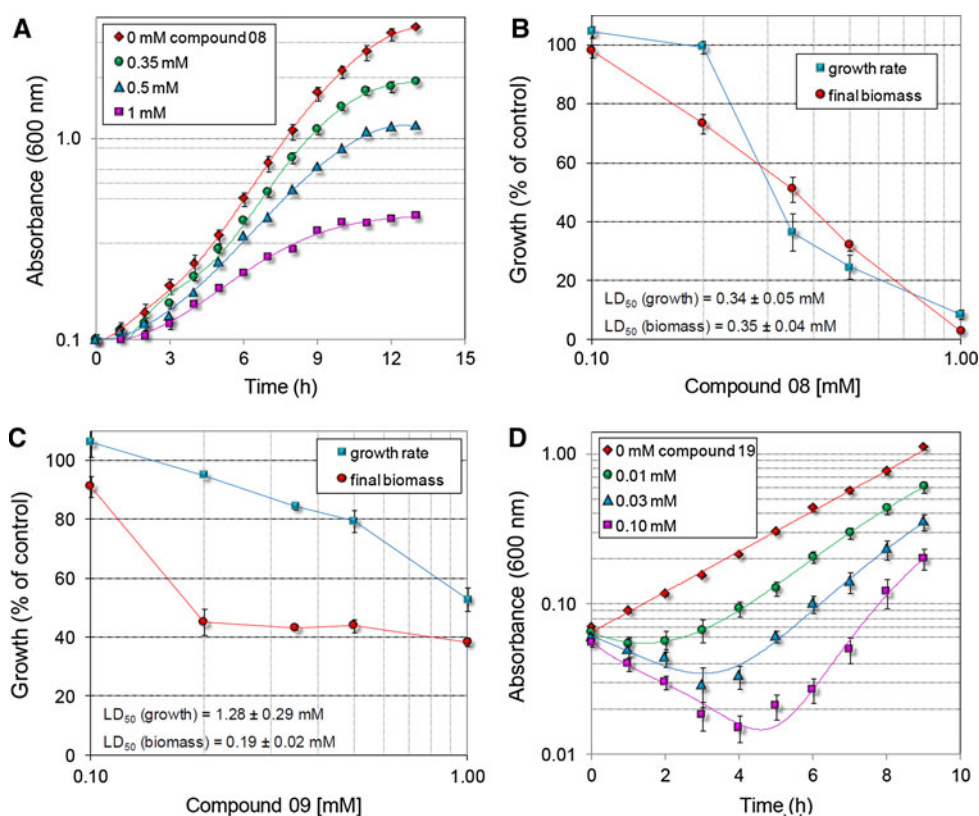
why higher concentrations are required in vivo than in vitro to suppress P5C reductase activity: growth inhibition would take place only when the concentration of the compounds overcomes the detoxifying potential of the bacterial cell. However, other reasons might explain the lower effectiveness in vivo. Because of the high polarity of the phosphonic moiety, these compounds are endowed with a remarkable negative charge that can strongly reduce their uptake into the cell. This hypothesis would be consistent with results obtained in the case of both the phosphonate herbicide glyphosate (Forlani et al. 2008b) and some anticorrosive polyphosphonates (Forlani et al. 2011), for which an almost negligible uptake rate at low external concentrations was found to limit metabolization by the bacterial cell.

Whatever the reason for the reduced efficacy in vivo, further evidence was required to substantiate an actual occurrence of proline starvation. With this aim, free amino acid content was determined in bacterial cultures treated with inhibitory levels of compound 08. Results, outlined in Table 3, indeed showed a reduction of free proline concentration inside the cell. In addition, the level of the metabolically related intermediate ornithine was severely affected. Therefore, a corresponding increase of free glutamate would be expected, which on the contrary lowered, but if the sum of glutamate and glutamine is considered instead, a mild increase was in fact evident at 500 μM. However,

significant fluctuations were noticed also for amino acids of the aspartate family, and for glycine. The metabolic basis of these effects is not plain, and could indicate the occurrence of further phosphonate targets in amino acid metabolism. Therefore, the existence of a causal connection between proline starvation and inhibition of cell proliferation is not certain. Above 500 μM, a general reduction of free amino acid content was found (data not shown), such that made difficult to distinguish specific effects. This notwithstanding, if the percent contribution of proline to total amino acid content was considered instead of its absolute concentration, a consistent pattern was evident (Fig. 4).

Conclusions and perspectives

To the best of our knowledge, this is the first paper considering the possibility to affect pathogen growth by proline starvation. This aim was pursued by inhibiting P5C reductase, the enzyme at the converging point of the two main metabolic routes leading to proline biosynthesis in bacteria. Several substituted phenyl derivatives of compound 01 were found to completely suppress enzyme activity in vitro at micromolar concentrations, and the mode of action of the most active compounds strengthened the possibility that a block of proline synthesis may occur in vivo as a consequence. Experiments performed with the human pathogen *S. pyogenes* indeed showed both a reduction of bacterial

Table 3 Free amino acid content in *S. pyogenes* cells treated with the most active inhibitor of P5C reductase, compound 08

aa	Untreated control		250 μM compound 08		500 μM compound 08	
	μmol (g fw)$^{-1}$	%	μmol (g fw)$^{-1}$	%	μmol (g fw)$^{-1}$	%
Asp	2.76 ± 0.42	12.0	1.45 ± 0.01	7.2	1.48 ± 0.04	6.9
Glu	5.99 ± 0.21	26.1	2.91 ± 0.14	14.4	3.97 ± 0.12	18.4
Asn	0.18 ± 0.02	0.8	0.22 ± 0.01	1.1	0.23 ± 0.02	1.1
Unknown	3.24 ± 0.06	14.1	2.25 ± 0.02	11.2	2.27 ± 0.06	10.5
Ser	0.38 ± 0.03	1.7	0.41 ± 0.02	2.0	0.33 ± 0.01	1.5
Gln	3.09 ± 0.19	13.4	4.56 ± 0.09	22.6	4.81 ± 0.24	22.4
His	0.92 ± 0.09	4.0	1.00 ± 0.02	5.0	0.93 ± 0.13	4.3
Arg	0.21 ± 0.06	0.9	0.33 ± 0.06	1.6	0.22 ± 0.03	1.0
Gly	0.59 ± 0.07	2.6	1.71 ± 0.04	8.5	1.73 ± 0.10	8.0
Thr	1.20 ± 0.16	5.2	1.66 ± 0.04	8.3	1.75 ± 0.08	8.1
Ala	0.12 ± 0.01	0.5	0.09 ± 0.02	0.4	0.10 ± 0.01	0.5
Tyr	0.47 ± 0.02	2.1	0.48 ± 0.02	2.4	0.48 ± 0.04	2.2
Trp + Met	0.32 ± 0.04	1.4	0.24 ± 0.06	1.2	0.36 ± 0.04	1.7
Val	0.70 ± 0.09	3.0	0.78 ± 0.03	3.9	0.68 ± 0.02	3.1
Phe	0.61 ± 0.05	2.7	0.67 ± 0.02	3.3	0.76 ± 0.05	3.5
Iso	0.24 ± 0.01	1.1	0.24 ± 0.00	1.2	0.26 ± 0.02	1.2
Leu	0.41 ± 0.02	1.8	0.39 ± 0.00	2.0	0.43 ± 0.03	2.0
Orn	0.22 ± 0.03	0.9	0.04 ± 0.01	0.2	0.06 ± 0.02	0.3
Lys	0.68 ± 0.08	3.0	0.30 ± 0.04	1.5	0.35 ± 0.03	1.6
Pro	0.63 ± 0.03	2.8	0.41 ± 0.02	2.0	0.34 ± 0.01	1.6
All	22.98 ± 0.86	100.0	20.10 ± 0.38	100.0	21.52 ± 1.50	100.0

Amino acid pools were quantified by reverse-phase HPLC following derivatization with *o*PDA; Pro and total amino acid content were measured by the ninhydrin method. Cys was undetectable. Mean values ± SE over 4 replicates are reported
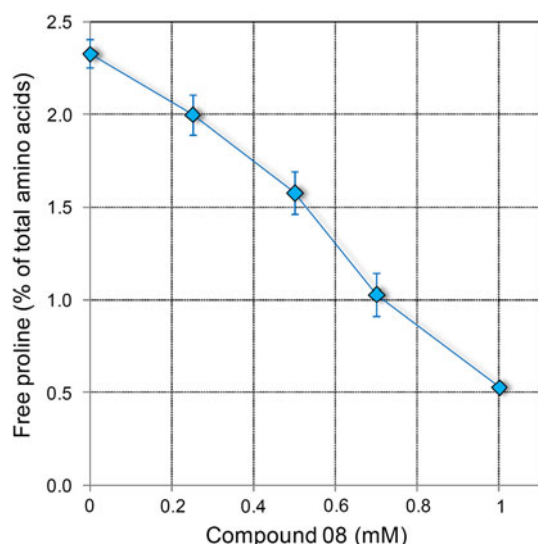

Fig. 4 Effect of the treatment with increasing concentrations of compounds 08 on free proline concentration in *S. pyogenes* cells. Data were expressed as percent of total amino acid content, and are mean ± SE over 5 replications obtained in two independent experiments

growth and a decrease of the intracellular level of free proline. Such effects were found at concentrations largely exceeding those able to inhibit the enzyme in vitro, most likely due to poor membrane permeability. Moreover, the intracellular level of other amino acids than proline was found to be reduced, therefore, suggesting that further targets may occur. It has in fact been reported that bisphosphonates inhibit the activity of several enzymes, including some known antibacterial targets, like 1-deoxy-D-xylulose-5-phosphate reductoisomerase [E.C. 1.1.1.267], undecaprenyl diphosphate synthase [E.C. 2.5.1.31], geranyltranstransferase [E.C. 2.5.1.10] and farnesyltranstransferase [E.C. 2.5.1.29] (Guo et al. 2007). The MEP/DOXP pathway is lacking in *S. pyogenes* (http://kegg.jp/kegg-bin/show_pathway?spg00900), but the latter enzymes are present, and their inhibition may concur in determining the observed cytostatic effects. Instead of being a drawback, the occurrence of multiple targets would be a positive feature for an antibiotic, as it lowers the risk of resistance selection. In any case, the present work clearly established the possibility of starving cells for proline through the treatment with P5C reductase inhibitors. A structure-activity relationship analysis is currently in progress based upon these results in order to identify new derivatives that may retain high effectiveness against the bacterial enzyme while coupling it with improved permeability across the biological membrane. The attainment of this goal would provide us with

new leads and increase the possibility of developing effective therapeutics targeting proline biosynthesis.

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