Phenyl-substituted aminomethylene-bisphosphonates inhibit human PSC reductase and show antiproliferative activity against proline-hyperproducing tumour cells

Giuseppe Forlani, Giuseppe Sabbioni, Daniele Ragno, Davide Petrollino and Monica Borgatti

ABSTRACT
In certain cancers, such as breast, prostate and some lung and skin cancers, the gene for the enzyme catalysing the second and last step in proline synthesis, δ-l-pyrroline-5-carboxylate (PSC) reductase, has been found upregulated. This leads to a higher proline content that exacerbates the effects of the so-called proline-PSC cycle, with tumour cells effectively using this method to increase cell survival. If a method of reducing or inhibiting PSC reductase could be discovered, it would provide new means of treating cancer. To address this point, the effect of some phenyl-substituted derivatives of aminomethylene-bisphosphonic acid, previously found to interfere with the catalytic activity of plant and bacterial PSC reductases, was evaluated in vitro on the human isoform 1 (PYCR1), expressed in E. coli and affinity purified. The 3.5-dibromo-mphenyl- and 3.5-dichlorophenyl-derivatives showed a remarkable effectiveness, with IC50 values lower than 1 μM and a mechanism of competitive type against both PSC and NADPH. The actual occurrence in vivo of enzyme inhibition was assessed on myelogenous erythroleukemic K562 and epithelial breast cancer MDA-MB-231 cell lines, whose growth was progressively impaired by concentrations of the dibromo derivative ranging from 10-6 to 10-3 M. Interestingly, growth inhibition was not relieved by the exogenous supply of proline, suggesting that the effect relies on the interference with the proline-PSC cycle, and not on proline starvation.

Introduction
Tumour development implies a deep reprogramming of cell metabolism, often inducing some additional or differential metabolic dependencies. These can be exploited for the identification of new therapeutic strategies based on substances able to target specific requirements of malignant cells. Among the changes in the metabolic fluxes undergone by tumoral cells to satisfy a larger demand for carbon skeletons, ATP and reducing power, increasing rates of the so-called proline-PSC cycle seem to play a significant role in several cancer types. Although some other mechanisms have been also hypothesised, such as an increase of collagen synthesis, enhanced, for instance during nutrient stress or metastasis formation, the main contribution of proline metabolism to tumorigenesis seems to rely upon the consequent augmented redox cycling and maintenance of pyridine nucleotides. Proline is synthesised from glutamate or ornithine in short pathways sharing the last step, the NAD(P)H-dependent reduction of the common intermediate δ-l-pyrroline-5-carboxylic acid (PSC) by PSC reductase (EC 1.5.1.2). PSC is also formed during the mitochondrial degradation of proline to glutamate, which involves two oxidative steps catalysed in sequence by proline dehydrogenase (ProDH; EC 1.5.5.2) and PSC dehydrogenase (PSCDH, also known as aldehyde dehydrogenase 4; EC 1.2.1.88). The former is believed to feed electrons directly to the respiratory ubiquinone pool, whereas the latter uses NAD+ as the electron acceptor. The occurrence of a shortcut in which the PSC released by ProDH is not further oxidised by PSCDH, but is reduced back to proline by PSC reductase, has been early hypothesised. Such apparently futile proline-PSC cycle (Figure 1) may provide the cell with a mechanism for transferring reducing equivalents from the cytosol to the mitochondrial matrix and, to fuel the respiratory chain. Moreover, ProDH activity may alternatively lead to ROS production, which can trigger in turn the apoptotic mechanism, or increased ATP synthesis for protective autophagy. Although in plants the occurrence of the proline-PSC cycle is still a matter of debate due to the physical separation of PSC reductase and ProDH, its role in human cell is now well established. Consistently, high levels of expression of both ProDH and PSC reductase have been reported in a series of cases in which cell metabolism needs to be enhanced, for instance during nutrient stress or metastasis formation.

Being highly expressed in several cancer types and possibly linked to tumour cell survival, the activity of PSC reductase represents a potential target for the development of new therapeutic approaches. In fact, recent studies on breast and prostate cancer cell lines demonstrated that PSC reductase knockdown was effective in inducing a reduction in tumour size in vivo and an increase in cell cycle arrest and apoptosis in vitro, respectively. These results would open interesting perspectives through gene

ARTICLE HISTORY
Received 1 February 2021
Revised 9 April 2021
Accepted 15 April 2021

KEYWORDS
Antiproliferative activity; PSC reductase inhibitors; proline-PSC cycle; proline-overproducing tumours

https://doi.org/10.1080/14756366.2021.1919890

JOURNAL OF ENZYME INHIBITION AND MEDICINAL CHEMISTRY
2021, VOL. 36, NO. 1, 1248 – 1257

© 2021 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
therapy, when approved. In the meantime, an amenable approach would be represented by the identification of specific and effective inhibitors of P5CS reductase, to be used as antiproliferative agents. A previous screening of a commercially available library of pharmacologically active compounds pointed out pargyline as a fragment-like hit, with a modest IC50 of 200 μM, but an encouraging ligand efficiency. The analysis of a number of analogues allowed identifying 4-bromopargyline as the most effective inhibiting ligand efficiency. The analysis of a number of analogues pointed out pargylin as a pharmaceutically active compounds. A previous screening of a commercially available library of inhibitors of P5CS reductase, to be used as antiproliferative therapy, when approved. In the meantime, an amenable approach would be represented by the identification of specific and effective inhibitors of P5CS reductase, to be used as antiproliferative agents. A previous screening of a commercially available library of pharmacologically active compounds pointed out pargyline as a fragment-like hit, with a modest IC50 of 200 μM, but an encouraging ligand efficiency. The analysis of a number of analogues allowed identifying 4-bromopargyline as the most effective inhibitor, showing an IC50 value of 9 μM. However, the treatment of cancer cells with the compound resulted in mild effects, with 30–40% reduction of breast cancer cell proliferation at 10 μM, and an unclear dose-activity relationship on intracellular free proline at higher levels.

Inhibitors of P5CS reductase have been previously exploited as potential herbicides. A preliminary screening in vitro pointed out the ability of some aminomethylene bisphosphonic acids to interfere with the activity of the plant enzyme in the micromolar range. Synthesis and analysis of several active derivatives allowed defining the steric and electronic requirements for maintenance or enhancement of the inhibitory properties, and identifying 3,5-dibromophenylaminomethylene bisphosphonic acid as the most effective analogue. The ability of the latter to inhibit plant growth, and cause the accumulation of P5CS levels in treated seedlings, proved that the inhibition of P5CS reductase takes place in vivo. Moreover, the same compounds were found even more active in vitro against P5CS reductase of the human pathogen Streptococcus pyogenes, although concentrations 2 orders of magnitude higher than IC50 were required to inhibit bacterial growth. Based on this rationale, we investigated the effectiveness of aminomethylene-bisphosphonic acids in inhibiting human P5CS reductase and reducing proliferation of some selected tumour cells lines.

Methods

Substrates, reagents and inhibitors

Unless specified otherwise, all compounds were purchased from Sigma-Aldrich, and were of analytical grade. Δ1-pyrroline-5-carboxylic acid was synthesised by the periodate oxidation of Δ-allo-hydroxylysine (Sigma H0377) and purified by cation-exchange chromatography onto a Dowex AG50W-X4 (200–400 mesh) column, as described. DL-P5CS solutions in 1 M HCl were stored at 4 °C in the dark, and brought to neutral pH just before the assay using proper aliquots of a 1 M Tris base solution. Pargyline (N-methyl-N-(2-propynyl)benzylamine hydrochloride) was from Sigma (P8013), whereas 4-bromopargyline was a generous gift from prof. Reuven Agami (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Phenyl-substituted aminomethylene-bisphosphonic acids were synthesised as described. For 3,5-dibromophenylaminomethylene-bisphosphonic acid (Br2PAMBPA) a slightly different procedure was adopted, as it follows.

Procedure for the synthesis of 3,5-dibromophenylaminomethylene-bisphosphonic acid

3,5-Dibromoaniline (3.01 g, 12.00 mmol), triethyl orthoformate (2.40 mL, 14.40 mmol) and diethyl phosphite (6.18 mL, 48.00 mmol) were vigorously stirred and heated at 120 °C for 16 h. After cooling, volatiles were evaporated under reduced pressure. Trituration of the solid with n-hexane followed by filtration furnished the pure ester tetraethyl (3,5-dibromophenylaminomethylene)bisphosphonate (4.38 g, 68% yield) which was further dissolved in 85% H3PO4 in D2O. Peak assignments were aided by gradient-HMQC experiments.

Figure 1. The proline-P5C cycle. Abbreviations that are not listed in the text: OAT: ornithine-Δ-amino transferase; αKG: α-ketoglutarate; Glu: glutamate; P5CS: P5C synthetase; uQ: oxidised ubiquinone; uQH2: reduced ubiquinone.
Purification of human PSC reductase

The gene coding for P5C reductase 1 (PYCR1, gene ID 5831), cloned into the expression vector pET28a13, was kindly provided by prof. Zihe Rao (Tsinghua University, China). For heterologous expression, E. coli BL21(DE3) pLysS cells (Invitrogen) were made from the plasmid containing the recombinant vector and selected on kanamycin-containing LB plates. About 6 h after inducing gene expression in liquid LB medium at 24°C, the cultures were harvested by centrifugation and stored at −20°C. Aliquots (about 1 g cell material) were lysed in an ice-cold mortar with 2 g l−1 alumina and resuspended in 20 mL l−1 extraction buffer (50 mM Na phosphate buffer, pH 7.5, containing 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) captoethanol, and denatured 5 min at 100°C. The His-tagged protein was purified from clarified extracts by affinity chromatography onto a His-TrapFF column (1 mL, GE Healthcare 17–5319-01). Stepwise elution was achieved by increasing concentrations of imidazole in extraction buffer. Discontinuous SDS–polyacrylamide gel electrophoresis was performed at 8 mA with a 5% stacking and a 12% separating gel, using a Minigel system (BioRad). Samples were mixed with the same volume of 125 mM Tris–HCl buffer (pH 6.8) containing 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) β-mercaptoethanol, and denatured 5 min at 100°C. Proteins were visualised by soaking gels in Quick Coomassie Stain (CliniSciences) overnight.

PSC reductase assay

Enzyme activity was measured at 30°C as the PSC-dependent oxidation of NAD(P)H. Assays were performed in 96-microwell plates in a final volume of 0.2 mL. The assay mixture contained 4 mM DL-PSC and 0.5 mM of either NADH or NADPH in 50 mM Tris–HCl buffer, pH 7.5. Parallel blanks were performed in which PSC had been omitted. The decrease of absorbance was measured for 5 min at 0.5-min intervals using a Lededet plate reader (Labexim, Lengau, Austria) equipped with a LED plugin at 340 nm. Residual NAD(P)H content was estimated on the basis of calibration curves obtained under the same conditions. Activity was calculated by linear regression of data using Prism 6 for Windows, version 6.03 (GraphPad Software, San Diego, CA); one unit of activity was defined as the amount of enzyme that catalyses the PSC-dependent oxidation of 1 nmol NAD(P)H s−1 (nkat). Proteins were measured by the Coomassie Brilliant Blue method15, using bovine serum albumin as the standard. To evaluate substrate affinity, invariable substrates were fixed at the same levels as in the standard assay. For variable substrates, L-PSC ranged from 400 to 2000 μM, while the concentration of NADH and NADPH ranged from 125 to 500 μM. To evaluate the inhibition brought about by pargylines and aminomethylene-bisphosphonates, freshly prepared 10 mM stock solution in water and 100 mM Tris–HCl buffer pH 7.5, respectively, were diluted with water and added to the reaction mixture before enzyme addition. Parallel control samples were carried out in the absence of any inhibitor. Activity was calculated as described above. Mean activity value in controls was set as 100%, and activity in treated samples was expressed as percent of such value. Percent values were plotted against the logarithm of inhibitor concentration, and data were interpolated using the equation \( Y = 100/(1 + 10^{((\log IC_{50})-X)\times HillSlope}) \). Assays were performed in triplicate (technical replications). Each experiment was repeated at least twice with different enzyme preparations (biological replicates). \( K_I \) and \( V_{max} \) values, as well as the concentrations causing 50% inhibition (IC50), \( K_I \) values and their confidence intervals were estimated by non-linear regression analysis using Prism 6.

Human cell lines and growth conditions

Human myelogenous erythroleukemic K562 cells36 were cultured in a humidified atmosphere of 5% CO2/air either in complete RPMI 1640 medium (Lonza, Verviers, Belgium) or in a modified medium without L-proline (United States Biological, Massachusetts, USA), both supplemented with 10% foetal bovine serum (FBS; Biowest, Nuaillé, France), 100 U mL−1 penicillin and 100 μg mL−1 streptomycin. Human epithelial breast cancer MDA-MB-231 cells37 were cultured under the same conditions in DMEM medium (Lonza, Verviers, Belgium) supplemented with 10% FBS, 100 U mL−1 penicillin, 10 μg mL−1 streptomycin and 2 mM L-glutamine.

Antiproliferative assays

K562 cells in the late exponential phase of growth were seeded in 1 mL medium without FBS in 24-well plates to an initial density of about 20,000 cells mL−1. Treatments with Br2PAMBPA in the range 1–200 μM were carried out 5 h after seeding. Cell growth was monitored at increasing time after the treatment by determining cell density on 50–μL aliquots with a Z1 Coulter Counter (Coulter Electronics), considering a dimensional range between 8 and 20 μm. MDA-MB-231 cells in the late exponential phase of growth (90% confluence) were washed with sterile PBS, incubated with sterile trypsin-EDTA solution (Sigma-Aldrich, Saint Louis, USA) for 2 min, resuspended in fresh DMEM medium and centrifuged at 1000 g at room temperature for 5 min. Pellets were resuspended in sterile PBS and spun again to remove any residue of trypsin. Cells were finally resuspended in DMEM medium without FBS, and seeded in 24-well plates in a final volume of 1 mL to an initial density of about 20,000 cells mL−1. Treatments with Br2PAMBPA in the range 1–200 μM were carried out 5 h after seeding, and 2% FBS was added to each well 24 h thereafter. Cell growth was monitored at increasing time after the treatment by destructive harvest. For both cell lines each treatment was carried out at least in triplication, and each experiment was performed at least twice.

Cell viability assay

Cell viability was assessed by the Trypan Blue exclusion test38. Stained cells were counted in a Bürker chamber. Viable and nonviable cells were recorded separately, and the results of three independent counts were pooled. Data were expressed as percent of alive cells.
Measurement of intracellular free proline and P5C levels

For proline and P5C quantification, treatments were carried out in T-25 flasks containing 10 mL medium. Cells were harvested by centrifugation at 1000 \( g \) for 5 min at RT. Each pellet was resuspended in 1.4 mL of sterile PBS and spun again to remove the medium. Washed pellets were transferred on ice, resuspended in a minimal volume of a 3% (w/v) solution of 5-sulfosalicylic acid and subjected to various freeze-thaw cycles to induce rupture of the phospholipid membrane. Cell debris were sedimented by centrifugation at 12,000 \( g \) for 5 min at RT, and the supernatants were analysed by the acid ninhydrin method of Williams and Frank, as described\(^{33}\).

Results

Effect of phenyl-substituted aminomethylenebisphosphonates on the activity of human P5C reductase 1

In order to investigate whether some aminomethylenebisphosphonic acids that had been previously identified as effective inhibitors of plant\(^{28-31}\) and bacterial\(^{32}\) P5C reductase have the potential to inhibit also the human enzyme, P5C reductase-1 was purified through heterologous expression in \( E. \) coli, and the recombinant His-tagged protein was affinity purified by a stepwise gradient of imidazole (panel A). Homogeneity of purified preparations was checked by SDS-PAGE (panel B). Number of lanes in the gel refer to the fractions in panel A: E, crude extract; MM, molecular markers (Fermentas SM0431). Specific activity of the enzyme was measured at varying either substrate, using NADPH (panel C) or NADH (panel D) as the electron donor. Reported data are mean ± SE over three technical replicates. The whole analysis was repeated with three different enzyme preparations, and similar data were obtained. Both Michaelis-Menten constants and \( V_{\text{MAX}} \) values pointed at a higher catalytic efficiency with NADH.

Figure 2. Purification and kinetic characterisation of human P5C reductase. The gene for P5C reductase 1 (PYCR1, gene ID 5831) was heterologously expressed in \( E. \) coli, and the recombinant His-tagged protein was affinity purified by a stepwise gradient of imidazole (panel A). Homogeneity of purified preparations was checked by SDS-PAGE (panel B). Number of lanes in the gel refer to the fractions in panel A: E, crude extract; MM, molecular markers (Fermentas SM0431). Specific activity of the enzyme was measured at varying either substrate, using NADPH (panel C) or NADH (panel D) as the electron donor. Reported data are mean ± SE over three technical replicates. The whole analysis was repeated with three different enzyme preparations, and similar data were obtained. Both Michaelis-Menten constants and \( V_{\text{MAX}} \) values pointed at a higher catalytic efficiency with NADH.
of human P5C reductase\textsuperscript{27} was at first assessed. With NADPH as the electron donor, pargyline and 4-bromopargyline confirmed their efficacy (Figure 3(A,B)), with IC\textsubscript{50} values (81 and 9 \textmu M, respectively) that were compatible with those (198 and 9 \textmu M) found hitherto\textsuperscript{27}. A strikingly lower effectiveness was on the contrary evident if NADH was used instead, with IC\textsubscript{50} values more than one order of magnitude higher (Figure 3(A,B)). When the same experiment was performed with phenyl-substituted aminomethylenebisphosphonates, a remarkably different picture was obtained. Both the 3,5-dichlorophenyl- and the 3,5-dibromo-phenyl-derivative almost completely suppressed the activity of human P5C reductase at concentrations as low as 1 \textmu M, and very similar IC\textsubscript{50} values were found irrespectively of the pyridine dinucleotide used (Figure 3(C,D)). The effect of some other analogues with a different substitution pattern in the phenyl ring\textsuperscript{32} was also determined. Although several of them were found active in the micromolar range (data not presented), no one showed higher effectiveness than Br\textsubscript{2}PAMBPA, which was therefore used for further determinations.

**Kinetic analysis of the inhibition brought about by Br\textsubscript{2}PAMBPA on human P5C reductase 1**

Aminomethylenebisphosphonates had been found to inhibit plant\textsuperscript{28} and bacterial\textsuperscript{32} P5C reductases with a mechanism of non-competitive type with respect to NAD(P)H and of uncompetitive type with respect to P5C. To ascertain whether the same holds true for human P5C reductase, a kinetic analysis was performed. Results are summarised in Figure 4. With P5C as the variable substrate, lines converging to the Y-axis in Lineweaver-Burk plots were consistent with an inhibition of competitive type with either NADPH (panel A) or NADH (B) as the electron donor. A similar pattern was obtained at varying NADPH concentration (Figure 4(C)), whereas with NADH results were consistent with a mixed type mechanism of inhibition (Figure 4(D)). A mechanism of competitive type may be an unfavourable feature for an inhibitor, since substrate accumulation in vivo might lead to a partial reversal of the inhibitory effect. To take one step further, experiments were therefore carried out to verify whether P5C reductase inhibition by Br\textsubscript{2}PAMBPA takes place inside human cells, leading to proline starvation.

**Effect of Br\textsubscript{2}PAMBPA on the growth of human cancer cell lines**

When increasing concentrations of Br\textsubscript{2}PAMBPA were added to a modified RPMI medium not containing proline, the growth of human myelogenous leukaemia K562 cells was indeed found to be inhibited in the range from 2 to 200 \textmu M, with an effect that was proportional to the dose. Inhibition took place 24–36 h following the addition, and 50%-inhibition was achieved at about 20 \textmu M.
Interestingly, not only the growth rate was slowed down, but treated cells reached a plateau at a lower cell density than untreated controls (Figure 5(A)). Cell viability was significantly reduced at concentrations exceeding 50 μM, yet most cells were still alive at doses at which growth had been severely impaired (Figure 5(B)). Remarkably, when the intracellular levels of free proline were quantified, concentrations in treated cultures were not significantly different than in controls (Figure 5(B)), suggesting that the inhibitory effect was not related to proline starvation. Consistently, when the experiment was repeated in unmodified RPMI medium containing 20 mg L^{-1} (174 μM) Pro, the inhibition was not reversed. On the contrary, a slightly higher effect was evident, with 50% inhibition at about 7 μM (Figure 5(C)), and a more pronounced effect on cell viability (Figure 5(D)). Once again, when free proline content was expressed with respect to viable cells, no reduction was evident up to 50 μM, dose at which less than 50% reduction was evident in cells whose proliferation has been almost completely abolished. In all cases, no detectable (> 1 nmol [10^6 cells]^{-1}) levels of P5C were found.

To obtain further evidence, similar experiments were carried out with a breast cancer cell line (MDA-MB-231) in which the inhibition of P5C reductase 1 by small-hairpin RNA had been reported to significantly reduce growth and invasion capabilities45. However, in this case cell proliferation strictly required the addition of FBS to DMEM medium, and preliminary experiments carried out in standard medium showed only mild effects at Br2PAMBPA concentrations exceeding 50 μM (data not shown). Because such a failure may depend on the interaction between the inhibitor and some FBS components, preventing (or greatly reducing) its uptake by human cells46, cell cultures were treated with the bisphosphonate under serum-free conditions, and FBS was added 24 h thereafter. By using this protocol, confirmative results were obtained (Figure 6). Starting from 5 μM, both cell growth rate and maximal cell density were progressively reduced, and from 50 μM onward cell proliferation was completely abolished (Figure 6(A)). Almost identical patterns were obtained when the medium was supplemented with 20 mg L^{-1} Pro, condition under which the growth of untreated controls was slightly speeded up (Figure 6(B)).

Discussion

In human cells, three forms of PSC reductase are present that have specialised roles and different subcellular localizations44. The mitochondria-localized PYCR1 is believed to play a main role in the production of proline from glutamate for protein synthesis. PYCR1 has similar $K_{m}$s for NADPH and NADH, but a higher reaction rate with NADH, and is competitively inhibited by proline. During recent years, PYCR1 has attracted increasing interest

![Figure 4. Kinetic analysis of the inhibition of HsPYCR1 by Br2PAMBPA. The catalytic rate of the purified enzyme was measured at varying the concentration of a given substrate in the absence or in the presence of increasing levels of the inhibitor. Lines converging to the Y-axis in double reciprocal (Lineweaver-Burk) plots accounted for an inhibition of competitive type against P5C when both using NADPH (panel A) or NADH (panel B) as the electron donor. The same result was evident with respect to NADPH (panel C). On the contrary, with NADH as the variable substrate the pattern was suggestive of an inhibition of mixed-type (panel D). Data are mean over three technical replicates. The whole analysis was repeated three times, and similar patterns were obtained. The reported $K_i$ values are mean±SD over replications.](image-url)
because its upregulation has been found involved in tumour growth and metastatic progression of several types of cancers\textsuperscript{2–5}. A wide study of mRNA profiles from about two thousand tumours across 19 different cancer types showed PYCR1 as one of the most consistently overexpressed metabolic genes\textsuperscript{47}, and its down-regulation was found to reduce tumour growth and invasion.

---

\textbf{Figure 5.} Effect of micromolar levels of Br\textsubscript{2}PAMBPA upon the growth of human myelogenous leukaemia K562 cells. The compound was added 5 h after seeding to a modified RPMI medium not containing proline, and the resulting growth was followed until untreated controls reached the early stationary phase (panel A). Cell viability and free proline content were measured by destructive harvest in parallel samples 96 h after the inoculum. Proline concentration was expressed with respect to either total or viable cells (panel B). The same set of determinations was carried out also in unmodified RPMI medium containing 20 mg L\textsuperscript{-1} Pro (panels C and D). In all cases results are means ± SD over biological triplicates. Statistical significance of differences between treated and control samples in panels B and D were determined by multiple t test using the Holm-Sidak method, with \( \alpha = 1.00\% \) (*).

\textbf{Figure 6.} Effect of micromolar levels of Br\textsubscript{2}PAMBPA upon the growth of human epithelial breast cancer MDA-MB-231 cells. The compound was added to DMEM medium 5 h after seeding, and 2% FBS was added 24 h thereafter. The resulting growth was followed by destructive harvest until untreated controls reached confluence (panel A). The same experiment was carried out also in DMEM medium supplemented with 20 mg L\textsuperscript{-1} Pro (panel B). In all cases results are means ± SE over six biological replicates.
capabilities, making cancer cells more susceptible to conventional chemotherapeutics. Therefore, PSC reductase represents nowadays an attractive potential target for cancer therapy.

Preliminary results towards the identification of specific inhibitors for human PSC reductase have been recently described, showing 4-bromopargyline as the most effective substance. However, PSC reductase had been previously exploited as a potential target for herbicides. A long-lasting research in our laboratory allowed identifying halogen-substituted derivatives of phenyl-aminomethylene-bisphosphonic acid as potent inhibitors of the plant enzyme. The same compounds were also found to efficiently inhibit the enzyme from a bacterial pathogen. Their evaluation on human PSC reductase in vitro, herein reported, showed an even higher effectiveness, with IC50 values two orders of magnitude lower than those for the plant enzyme, and tenfold lower than that for 4-bromopargyline. Moreover, the same inhibition patterns were found using either NADH or NADPH as the electron donor, whereas in our hands pargyline showed efficacy in the micromolar range only against the NADPH-dependent activity. The higher sensitivity of human PSC reductase corresponded to a different kinetic mechanism. A remarkable difference between the amino acid sequence of the mammal and the plant enzyme does indeed exist, resulting into significant variations in their three-dimensional structure that seem thus to involve the binding site for aminobisphosphonates and influence their interaction. A mechanism of competitive type may represent a drawback, in that substrate accumulation deriving from enzyme inhibition could relieve at least in part the effect of the inhibitors in vitro. Confirmative results in a cellular system are thus crucial to support the possibility of using aminomethylene-bisphosphonates to reduce PSC reductase activity inside the cell.

When increasing concentrations of the most active compound were added to the culture medium of two human cancer cell lines, antiproliferative effects were in fact found starting from levels about tenfold higher than IC50 values in vitro. This notwithstanding, cell growth was progressively reduced in the range from 2 to 20 μM Br2PAMBPA, and completely abolished at higher concentrations. Interestingly, even at levels at which proliferation was only slowed down, cell cultures did not attain the same maximal densities than untreated controls, but arrested growth at values that were inversely proportional to the dose. Results obtained with myelogenous leukaemia K562 cells, which grow in suspension to highest densities, were confirmed with substrate-adhering breast cancer MDA-MB-231 cells that were chosen as a validated system in which PYCR1 is overexpressed and its inhibition causes the reduction of growth and invasion capabilities. In the latter case, the addition of FBS to the medium was found to greatly reduce Br2PAMBPA effectiveness, most likely as a consequence of inhibitor binding to BSA or other serum components, which may prevent the bisphosphonate from entering the cell. This would represent a serious obstacle for in vivo treatments with the compound, yet the problem could be overcome by the adoption of suitable delivery systems.

Interestingly, neither Br2PAMBPA treatment led to proline starvation, nor the exogenous supply of the amino acid decreased its effectiveness. These results might be consistent with the occurrence of other unrelated target(s) of bisphosphonates in cell metabolism. In fact, the same compounds were found in plants to inhibit also the activity of both cytosolic and plastidal forms of glutamine synthetase, with similar or even higher effectiveness than PSC reductase. However, the possibility that the observed antiproliferative effects may depend on the inhibition of glutamine synthetase is unlikely, since the human enzyme has been found substantially insensitive to these compounds (IC50 for 3.5-dichlorophenyl-AMPBA of 780 ± 30 μM). Moreover, also phytotoxic effects of these bisphosphonates were found unrelated to proline starvation, which in treated tissues on the contrary accumulated along with the putatively toxic intermediate PSC. Both in control and treated K562 cells PSC was undetectable, and free proline concentration was constant in the range 4–5 mmol (106 cells)−1, levels that were found conserved independently of the presence of exogenous proline in the culture medium, suggesting a strict homeostatic control. Only the treatment with highest Br2PAMBPA concentrations caused a slight but significant lowering in free proline content. Such results are consistent with those obtained with 4-bromopargyline, whose effect on the intracellular level of proline was not proportional to the dose.

On the whole, data strongly suggest that the antiproliferative activity of PSC reductase inhibitors is mediated by an interference with the proline-PSC cycle. In this half-biosynthetic, half-catabolic set of reactions, proline is oxidised by ProDH to PSC but the latter, instead of being further oxidised to glutamate in the mitochondrion by PSCDH, is reduced back to proline in the cytosol by PSC reductase (Figure 1). Without resulting in a net change of free proline level, this apparently futile cycle may allow the transfer of reducing equivalents from the cytosol to the mitochondrion, fueling the respiratory chain. An increased activity of this cycle can improve cancer cell survival, proliferation, and metastasis through a variety of mechanisms ranging from enhanced ATP production, nucleotide synthesis, anaplerosis, and redox homeostasis. Inhibition of PSC reductase is therefore expected to interfere with all these mechanisms, hampering cell growth even without impacting on proline availability for protein synthesis.

Further information is obviously required to strengthen the possibility of using Br2PAMBPA or other bisphosphonates for cancer therapy. Next steps will be a deeper insight on the molecular mechanisms fostering cell growth inhibition, the study of the effects against non tumoral cells, and the evaluation of the therapeutic potential in more sophisticated experimental systems. In any case, the availability of specific and effective inhibitors of PSC reductase could help elucidating the functions of the PSC-proline cycle in either physiological or pathological environments. Work is currently in progress with these aims in our laboratory.

Disclosure statement
The authors declare no conflict of interest. The authors alone are responsible for the content and writing of this article.

Funding
This research was supported by grants from the University of Ferrara [FAR 2019].

ORCID
Monica Borgatti http://orcid.org/0000-0001-8470-2496

References
1. Luengo A, Gui DY, Van der Heiden MG. Targeting metabolism for cancer therapy. Cell Chem Biol 2017;24:1161–80.
2. Ding Z, Ericksen RE, Escande-Beillard N, et al. Metabolic pathway analyses identify proline biosynthesis pathway as a promoter of liver tumorigenesis. J Hepatol 2020;72:725–35.

3. Xiao S, Li S, Yuan Z, Zhou L. Pyrrolidine-5-carboxylate reductase 1 (PYCR1) upregulation contributes to gastric cancer progression and indicates poor survival outcome. Ann Transl Med 2020;8:937.

4. D’Aniello C, Patriarca EJ, Phang JM, Minchiotti G. Proline metabolism in tumor growth and metastatic progression. Front Oncol 2020;10:776.

5. Phang JM. Proline metabolism in cell regulation and cancer biology: recent advances and hypotheses. Antioxid Redox Signal 2019;30:635–49.

6. Forlani G, Makarova KS, Ruszkowski M, et al. Evolution of plant \( \delta^1 \)-pyrroline-5-carboxylate reductases from phylogenetic and structural perspectives. Front Plant Sci 2015;6:567.

7. Liu W, Phang JM. Proline dehydrogenase (oxidase) in cancer. Biofactors 2012;38:398–406.

8. Korasick DA, Končitková R, Kopečná M, et al. Structural and biochemical characterization of aldehyde dehydrogenase 12, the last enzyme of proline catabolism in plants. J Mol Biol 2019;431:576–92.

9. Cabassa-Hourton C, Schertl P, Bordenave-Jacquemin M, et al. Proteomic and functional analysis of proline dehydrogenase 1 link proline catabolism to mitochondrial electron transport in Arabidopsis thaliana. Biochem J 2016;473:2623–34.

10. Phang JM. Transfer of reducing equivalents into mitochondria by the interconversions of proline and \( \delta^1 \)-pyrroline-5-carboxylate. Arch Biochem Biophys 1983;225:95–101.

11. Phang JM. The regulatory functions of proline and pyrroline-5-carboxylic acid. Curr Top Cell Regul 1985;25:91–132.

12. Launay A, Cabassa-Hourton C, Eubel H, et al. Proline oxidation fuels mitochondrial respiration during dark-induced leaf senescence in Arabidopsis thaliana. J Exp Bot 2019;70:6203–14.

13. Hancek CN, Liu W, Alvord WG, Phang JM. Co-regulation of mitochondrial respiration by proline dehydrogenase/oxidase and succinate. Amino Acids 2016;48:859–72.

14. Hu CA, Donald SP, Yu J, et al. Overexpression of proline oxidase induces proline-dependent and mitochondria-mediated apoptosis. Mol Cell Biochem 2007;295:85–92.

15. Huynh TYL, Zareba I, Basanowska W, et al. Understanding the role of key amino acids in regulation of proline dehydrogenase/proline oxidase (prodh/pxo)-dependent apoptosis/autophagy as an approach to targeted cancer therapy. Mol Cell Biochem 2020;466:35–44.

16. Winter G, Todd CD, Trovato M, et al. Physiological implications of arginine metabolism in plants. Front Plant Sci 2015;6:534.

17. Liu W, Hancock CN, Fischer JW, et al. Proline biosynthesis augments tumor cell growth and aerobic glycolysis: involvement of pyridine nucleotides. Sci Rep 2015;5:17206.

18. Phang JM, Pandhare J, Liu Y. The metabolism of proline as microenvironmental stress substrate. J Nutr 2008;138:2085–95.

19. Elia I, Funck D, Forlani G. \( \Delta^1 \)-pyrroline-5-carboxylate reductase from Arabidopsis thaliana: stimulation or inhibition by chloride ions and feed-back regulation by proline depend on whether NADPH or NADH acts as co-substrate. New Phytol 2014;202:911–9.
40. Forlani G, Bertazzini M, Zarattini M, et al. Functional properties and preliminary structural characterization of rice \( \delta \)-pyrroline-5-carboxylate reductase. Front Plant Sci 2015;6:565.
41. Ruszkowski MJ, Nocek B, Forlani G, Dauter Z. The structure of *Medicago truncatula* \( \delta \)-pyrroline-5-carboxylate reductase provides new insights into regulation of proline biosynthesis in plants. Front Plant Sci 2015;6:869.
42. Yeh GC, Harris SC, Phang JM. Pyrroline-5-carboxylate reductase in human erythrocytes. J Clin Invest 1981;67:1042–6.
43. Lorans G, Phang JM. Proline synthesis and redox regulation: differential functions of pyrroline-5-carboxylate reductase in human lymphoblastoid cell lines. Biochem Biophys Res Commun 1981;101:1018–25.
44. Christensen EM, Patel SM, Korasick DA, et al. Resolving the cofactor-binding site in the proline biosynthetic enzyme human pyrroline-5-carboxylate reductase 1. J Biol Chem 2017;292:7233–43.
45. Ding J, Kuo M-L, Su L, et al. Human mitochondrial pyrroline-5-carboxylate reductase 1 promotes invasiveness and impacts survival in breast cancers. Carcinogenesis 2017;38:519–31.
46. Zhang Y, Xu YY, Sun WJ, et al. FBS or BSA inhibits EGCG induced cell death through covalent binding and the reduction of intracellular ROS production. Biomed Res Int 2016;2016:5013409.
47. Nilsson R, Jain M, Madhusudhan N, et al. Metabolic enzyme expression highlights a key role for MTHFD2 and the mitochondrial folate pathway in cancer. Nat Commun 2014;5:3128.
48. Das RP, Singh BG, Kunwar A, et al. Tuning the binding, release and cytotoxicity of hydrophobic drug by Bovine Serum Albumin nanoparticles: influence of particle size. Colloids Surf B Biointer 2017;158:682–8.
49. Obojska A, Berlicki Ł, Kafarski P, et al. Herbicidal pyridyl derivatives of amino-methylene-bisphosphonic acid inhibit plant glutamine synthetase. J Agric Food Chem 2004;52:3337–44.
50. Occhipinti A, Berlicki Ł, Giberti S, et al. Effectiveness and mode of action of phosphonate inhibitors of plant glutamine synthetase. Pest Manag Sci 2010;66:51–8.
51. Kosikowska P, Bochno M, Macegoniuk K, et al. Bisphosphonic acids as effective inhibitors of *Mycobacterium tuberculosis* glutamine synthetase. J Enz Inh Med Chem 2016;31:931–8.
52. Hu C-A, Lin WW, Obie C, Valle D. Molecular enzymology of mammalian \( \Delta \)-pyrroline-5-carboxylate synthase. Alternative splice donor utilization generates isoforms with different sensitivity to ornithine inhibition. J Biol Chem 1999;274:6754–62.