Amino Acids
DOI 10.1007/s00726-017-2433-2

ORIGINAL ARTICLE

Lac-L-TTA, a novel lactose-based amino acid–sugar conjugate for anti-metastatic applications

Giovanni N. Roviello[1] · Roberta Iannitti[1] · Rosanna Palumbo[1] · Hayarpi Simonyan[3] · Caterina Vicidomini[1] · Valentina Roviello[2]

Received: 12 January 2017 / Accepted: 29 April 2017

Abstract Here we describe the synthesis, chromatographic purification, MS and NMR characterization of a new lactosyl-derivative, i.e. a lactosyl thiophenyl-substituted triazolyl-thione L-alanine (Lac-L-TTA). This amino acid–sugar conjugate was prepared by solution synthesis in analogy to the natural fructosyl-amino acids. Furthermore, we investigated the inhibition of PC-3 prostate cancer cell colony formation by this lactose derivative in comparison with the less polar fructose-based derivative, Fru-L-TTA. This let us to compare the properties of the artificial derivative, object of the present work, with the monosaccharide-based counterpart and to obtain a preliminary information on the influence of polarity on such biological activity. A significantly higher anticancer effect of Lac-L-TTA with respect to the fructose analogue emerged from our study suggesting that the anti-metastatic potential of fructosyl-amino acids can be enhanced by increasing the polarity of the compounds, for example by introducing disaccharide moieties in place of fructose.

Keywords Glycated amino acid · Anti-metastatic · Fructosyl-amino acid · Lactose

Handling Editor: G. J. Peters.

✉ Giovanni N. Roviello
giovanni.roviello@cnr.it

[1] CNR, Istituto di Biostrutture e Bioimmagini (Via Mezzocannone Site and Headquarters), 80134 Naples, Italy

[2] Centro Regionale di Competenza (CRdC) Tecnologie, Via Nuova Agnano, 11, 80125 Naples, Italy

[3] Institute of Pharmacy, Yerevan State University, 1 A. Manoogian Str., 0025 Yerevan, Armenia

Introduction

Amino acid–sugar conjugates (Campo et al. 2007; Kumar and Ramesh 2007) are hybrid compounds that combine the structural characteristics of amino acids with features typically found in simple carbohydrates. These glycosylated amino acids present amino-polyol carboxylic polyfunctionalized structures which can serve for the preparation of glyco- or peptidomimetics and glycopeptides (Di Fabio et al. 2001; Gruner et al. 2002; Rakosi et al. 2011; Risseeuw et al. 2007; Russo et al. 2016). In particular, the importance of glycopeptides as antiviral, antibiotics or anti-tumour vaccines, explains the great attention paid to amino acid–sugar derivatives and to the synthetic strategies for the conjugation of natural or artificial amino acids to various carbohydrates.

To this scope, a useful synthetic procedure is the Amadori rearrangement (Gallas et al. 2012) that enables to obtain fructosyl-amino acids, i.e. the *N*-(1-deoxy-2-D-fructosyl)-amino acids. These molecules which are formed from D-glucose and amino acids in foods are crucial in conferring their characteristic flavour (Mottram 1994). Fructosyl-amino acids can be obtained artificially by conversion of glycosylamines to fructosyl-amino acids under acidic conditions, and have useful chemical characteristics like the stability and the ability to form complexes with transition metal ions (Horikawa et al. 2002).

Regarding their biomedical usefulness, in vitro fructosyl-amino acids provoke the inhibition of colony formation of metastatic human cancer cells as found, e.g. in case of fructosyl-L-phenylalanine (FruPhe) by Glinsky et al. (1996). Moreover, fructosyl-L-histidine (FruHis), a fructosyl-amino acid found in tomato products, showed a significant protective effect against DNA oxidative

Published online: 06 May 2017

degradation, synergized with lycopene against the proliferation of metastatic rat prostate cancer cells in vitro, and inhibited tumourigenesis in vivo (Mossine et al. 2008).

Apart the natural Amadori products, artificial fructosyl-amino acids can be prepared by conjugating sugars with non-natural amino acids like the thiophenyl-substituted triazolyl-thione L-alanine (L-TTA) of Saghyan et al. (2014).

This is a non-natural amino acid sharing structural features with the amino acids present in FruHis and FruPhe, i.e. carrying an aromatic side chain, but it differs from His and Phe for its two-ring side chain. Our interest in this kind of substrates is justified by the biological relevance of structures that bear heterocyclic rings or nucleobases connected to amino acid-containing moieties (Esposito et al. Esposito et al. 2015; Roviello et al. 2006, 2007, 2010a, b, 2011a, b, 2016a, b; Teta et al. 2013).

In a recent study (Roviello et al. 2017) we have demonstrated that L-TTA underwent the Amadori rearrangement and, that, the corresponding artificial Amadori product (Fru-L-TTA, Fig. 1) was endowed with some biological properties already described in the literature for the natural fructosyl-amino acids, which are of great interest for developing new pharmacological treatments of cancer (Glinsky et al. 1996).

As a prosecution of this recent research, we wanted to investigate the effect due to the addition of a galactose residue to the Fru-L-TTA, on the anti-metastatic activity of the resulting derivative Lac-L-TTA, whose structure is represented in Fig. 1.

Materials and methods

Chemicals

D-Lactose and acetonitrile were from J.T.Baker. Sodium metabisulfite was purchased from Carlo Erba. Methanol, acetic acid and deuterated methanol were from Sigma-Aldrich. TFA and acetonitrile for HPLC chromatography were purchased from Romil. The aromatic amino acid (L-TTA) used for the synthesis of Fru-L-TTA was prepared using the procedure of Saghyan et al. (2014).

Synthesis of (2S)-3-[4-propyl-3-(2-thienyl)-5-thioxo-1,2,4-triazol-1-yl]-2-[[2,3,4-trihydroxy-5-[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]oxy-tetrahydropyran-2-yl]methylamino]propanoic acid (Lac-L-TTA)

Lac-L-TTA was synthesized under the same experimental conditions reported for the preparation of Fru-L-TTA (see Roviello et al. 2017) starting from 720 mg of D-lactose added to 0.6 mmol of the L-amino acid, 15 mg of sodium metabisulfite, and 70 μL of glacial acetic, refluxing the mixture over 6 h. After chromatography on silica gel the desired product was recovered in a 29% yield. TLC, $CH_3CN:H_2O = 80:20$; Rf 0.3 (under the same conditions Rf of the less polar Fru-L-TTA was 0.5). HRMS MALDI-TOF (Fig. 2a) *m/z* 637.1835 (found), 637.1849 (expected for $[C_{24}H_{36}N_4O_{12}S_2 + H]^+$); 659.1616 (found), 659.1669 (expected for $[C_{24}H_{36}N_4O_{12}S_2$

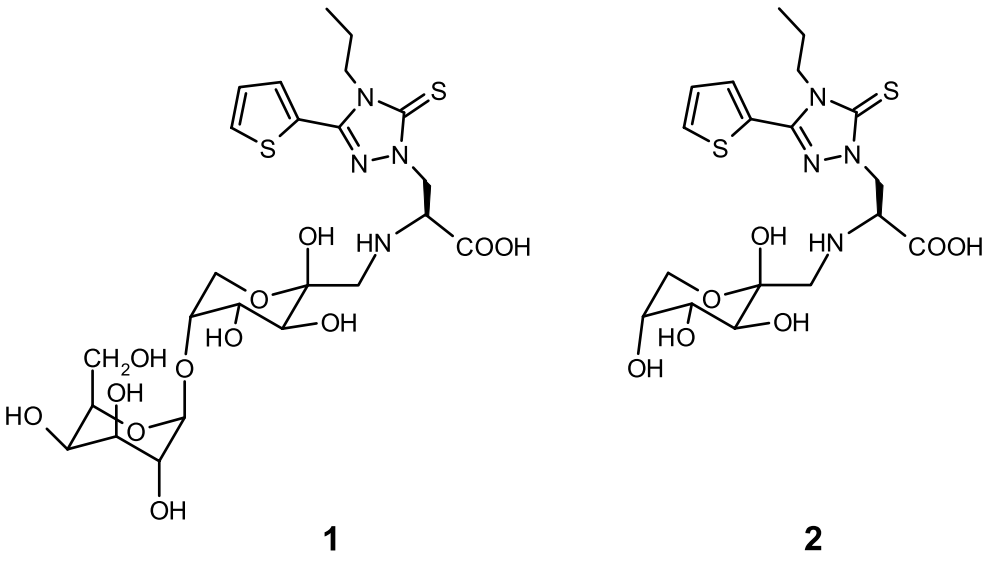


Fig. 1 Structural representation of compounds Lac-L-TTA (*1*) and Fru-L-TTA (*2*)

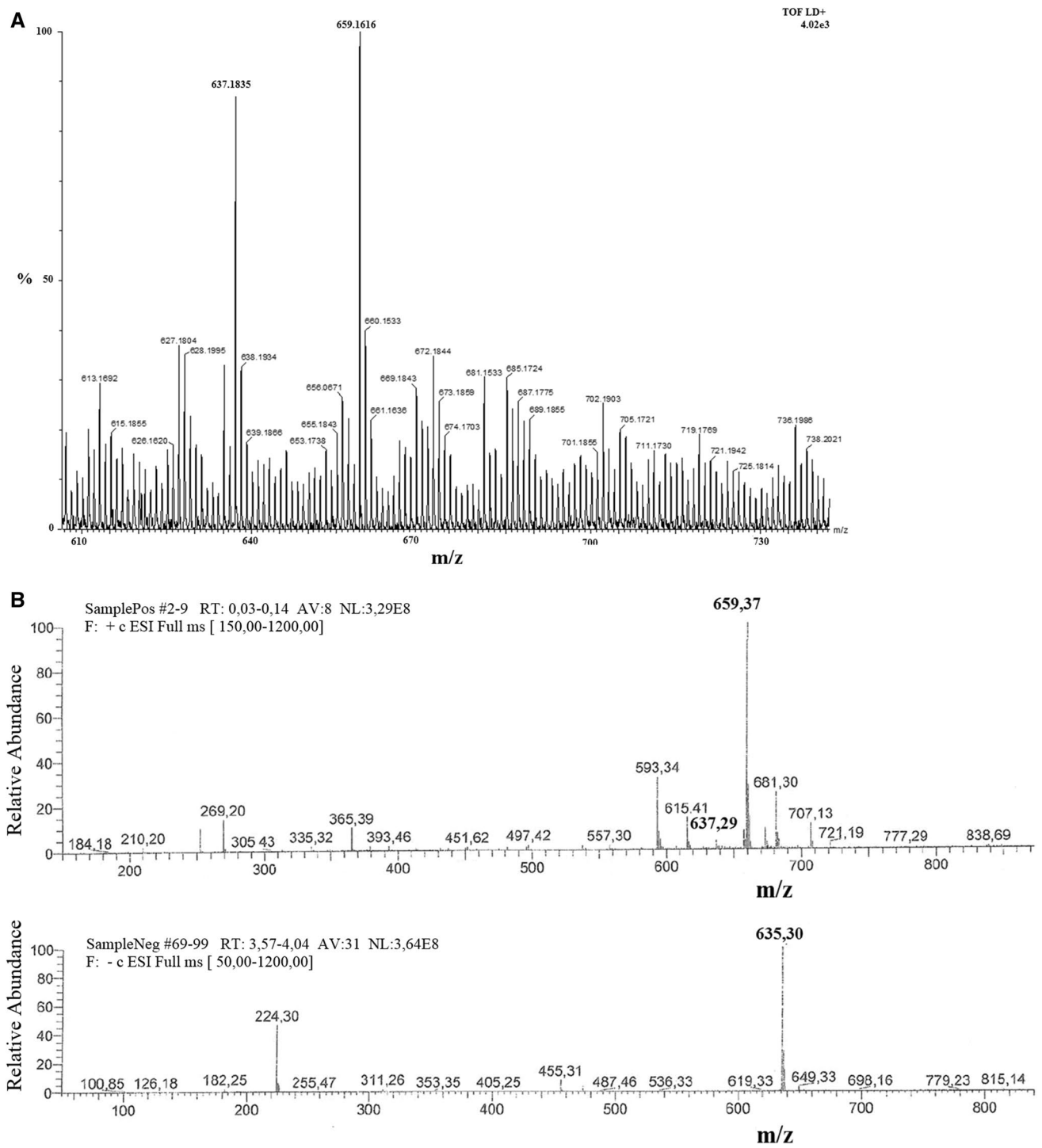


Fig. 2 HRMS (**a** MALDI-TOF) and ESI-MS (**b** positive and negative ions) characterization of Lac-L-TTA

+ Na]$^+$). LC–ESI-MS (Fig. 2b; positive ions) *m/z* 637.27 (found), 637.70 (expected for [$C_{24}H_{36}N_4O_{12}S_2 + H]^+$); 659.37 (found), 659.68 (expected for [$C_{24}H_{36}N_4O_{12}S_2$ + Na]$^+$); (negative ions) *m/z* 635.30 (found), 635.68 (expected for [$C_{24}H_{36}N_4O_{12}S_2 - H]^-$).

^{1}H NMR δ_H (500 MHz, CD_3OD, δ, ppm,): 0.95 (3H, t, $J = 7.0$ Hz, CH_3); 1.79 (2H, m, N–$CH_2C\mathit{H}_2CH_3$); 4.23 (2H, t, J = 8.0 Hz, N–$C\mathit{H}_2CH_2CH_3$); 3.44–4.36 (14H, m, sugar); 4.51–4.63 (3H, m, –CH_2CH_α); 7.24–7.74 (3H, m, thiophen).

Apparatus

^{1}H NMR spectra were recorded (at 25 °C) on a Varian 500 MHz spectrometer. Chemical shifts (δ) are given in parts per million (ppm), while proton chemical shifts were referenced to residual CHD_2OD (δ = 3.3 ppm, quin) signals.

We performed the column chromatography over silica gel (63–200 μm, Merck) and TLC analyses on precoated Kiesel gel 60F 254 plates (Merck); the compound was detected by UV visualization and with the aid of the following TLC stains: (1) *p*-anisaldehyde/H_2SO_4 (5% each) in 90% ethanol (Sigma-Aldrich) solution followed by heating at 90 °C; (2) iodine.

Cell culture

Human prostate cancer cells (PC-3) were maintained in RPMI 1640 medium (Gibco) supplemented with 10% foetal bovine serum (Gibco), 1% L-glutamine (Lonza) at 37 °C, in a 5% CO_2 humidified atmosphere and harvested at ~90% confluence.

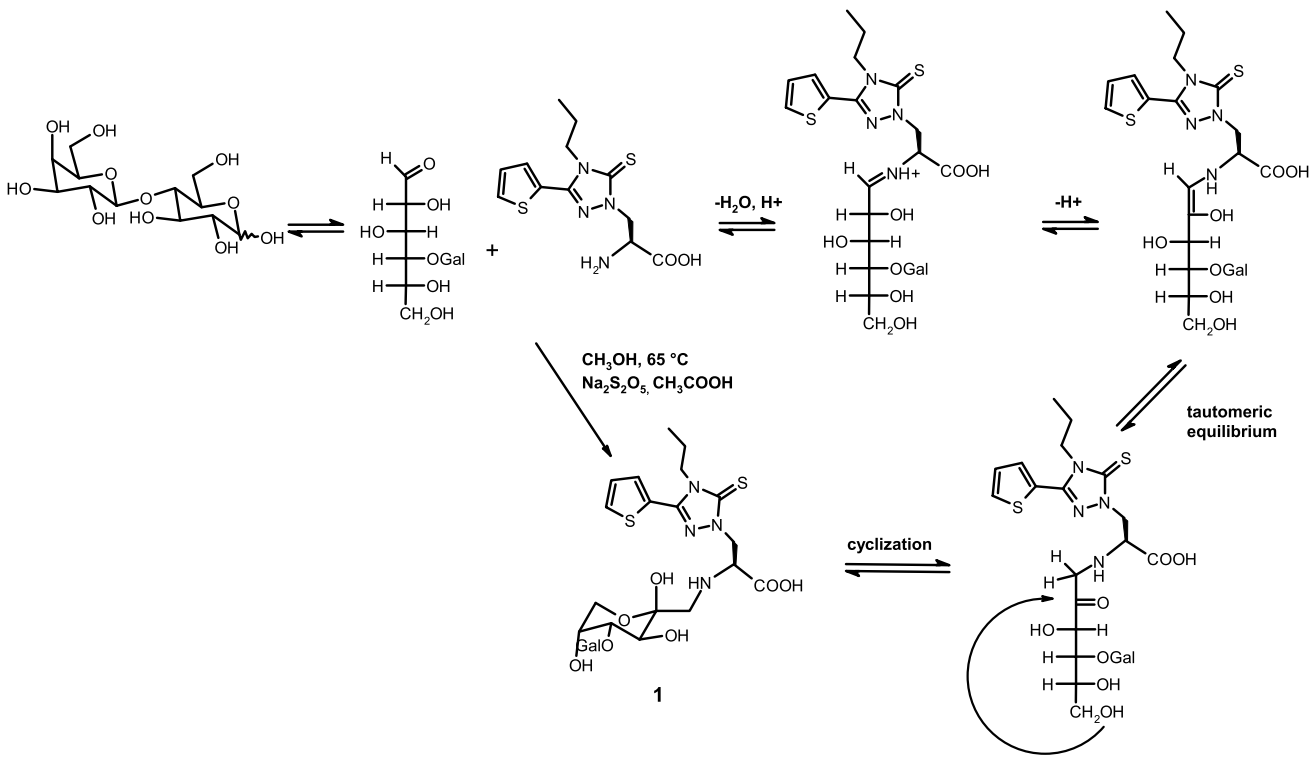
Clonogenic assay

Inhibition of colony formation was determined by means of a clonogenic assay. More in detail, 350 cancer cells were seeded in six-well plates and incubated overnight to allow cell adhesion. Subsequently, culture medium was removed and cells were incubated over 14 days with different concentrations of lactose derivative in triplicate. After two weeks we determined the colony formation by crystal violet assay. In particular, the culture medium was removed and the cells were fixed with 10% formalin solution (Sigma-Aldrich) for 15 min at dark, and stained with 0.1% v/w crystal violet (Sigma-Aldrich); after 30 min the cells were washed twice with double distilled water and let to dry. Colonies of diameter greater than 50 μm were counted. Cells incubated with medium alone were used as control.

Results and discussion

Synthesis of Lac-L-TTA

We prepared Lac-L-TTA by refluxing the aromatic amino acid L-TTA [described by Saghyan et al. (2014)] in methanol in the presence of an excess of D-Lactose under acidic conditions. This corresponds to the procedure of the Amadori reaction, whose mechanism we applied here for the first time to the thiophenyl-substituted triazolyl-thione L-alanine and the natural disaccharide as depicted in the following Scheme 1.



Scheme 1 Synthesis of Lac-L-TTA and interpretation of the mechanism

After chromatographic purification, we analysed samples of Lac-L-TTA by mass spectrometry and NMR that confirmed the identity of the desired product.

The MS profile of Lac-L-TTA comprises not only the $[M + H]^+$ peak (Fig. 2) but also that corresponding to the dominant $[M + Na]^+$ at 659 m/z. A minor peak at 593 *m/z* (Fig. 2b) can be explained in terms of CO_2 loss, i.e. with the formation of the $[M - CO_2 + H]^+$ cation. In negative ion mode the ESI-MS analysis afforded the $[M - H]^-$ peak at 635 m/z, which, jointly with the HRMS $[M + Na]^+$ and $[M + H]^+$ peaks (Fig. 2a), confirmed the structure of the amino acid–sugar conjugate.

Interestingly, no conversion of this product of the Amadori reaction to dione or ene dione derivatives (Estendorfer et al. 1990) was observed in our case, differently from our previous report on Fru-L-TTA (Roviello et al. 2017). We observed this difference probably because the hydroxyl on the C-4 is employed in the linkage with galactose, which prevents the initial dehydration required in the mechanism for the obtainment of the ene dione derivative (Roviello et al. 2017). The yield of this reaction (29%) was lower than that corresponding to the synthesis of Fru-L-TTA (49%). This can be due to the different polarities of the sugars employed in the two cases, with the more polar lactose being less prone to undergo the Amadori rearrangement in the organic solvent used for the reaction. Moreover, the higher steric hindrance of lactose with respect to glucose is another aspect that should be considered in the interpretation of the different reactivities.

Biological study

Among the several types of human cancers, prostate cancer is one of the most common, and represents one of the major causes of tumour-associated deaths worldwide (Jemal et al. 2011). There exist two main families of prostate cancers: one includes the prostatic adenocarcinoma, while the other group comprises small cell neuroendocrine carcinoma (SCNC). This latter is a highly aggressive malignancy able to provoke locally advanced malignancy or distant metastases (Tai et al. 2011). PC-3 cells are carcinoma cell lines, which were isolated from bone metastases causing very aggressive types of prostate cancer (Tai et al. 2011).

Metastasis, i.e. the morphological stage of the generalization of a tumour through the body, represents a major cause of tumour-related death (Mehlen and Puisieux 2006). Thus, the development of novel compounds endowed with anti-metastatic activity is highly desirable and, in this regard, the colony formation assay is a very discriminating tool for the evaluation of novel candidates as anti-metastatic drugs in vitro.

It is well established that natural Amadori products could interfere with the processes of carbohydrate-mediated intercellular recognition and adhesion, implied in the reduction of cell migration and colony formation, particularly in case of prostate cancer cells (Wang et al. 2009). Thus, we were interested in conducting in vitro assays to determine the potential also of artificial Amadori compounds, such as Lac-L-TTA, as anti-metastatic agents, also in comparison with the less polar Fru-L-TTA (Roviello et al. 2017).

More in detail, we performed our experiments with our amino acid–sugar conjugate on prostate cancer cells and found that, as shown in Fig. 3, Lac-L-TTA inhibited PC-3 colony formation of approximately 70% in 2 mM treated cells with respect to control. We also compared the above-reported effect of Lac-L-TTA with that shown by the less polar fructose-based derivative Fru-L-TTA, which showed an inhibition effect comparable only when cells were given a 4 mM concentration of this derivative (Fig. 3).

In other terms, we found that Lac-L-TTA showed an inhibitory effect on PC-3 colony formation higher than the fructose derivative, reducing significantly the colony number already at the lowest concentration employed in this assay. The mechanism at the basis of the greater activity of the more polar Lac-L-TTA with respect to Fru-L-TTA (Fig. 3) is an aspect that merits the due attention and our future efforts for its interpretation. In fact, it could be associated with the different molecular polarities and/or to a differential biomolecular recognition possibility associated with the lactose-derived substrate.

Interestingly, by comparing our findings with the already reported data found for natural Amadori compounds (Glinsky et al. 1996), we can conclude that Lac-L-TTA inhibited the colony formation at a higher extent with respect to several natural fructosyl-amino acids, and it was significantly more active than its corresponding fructose derivative

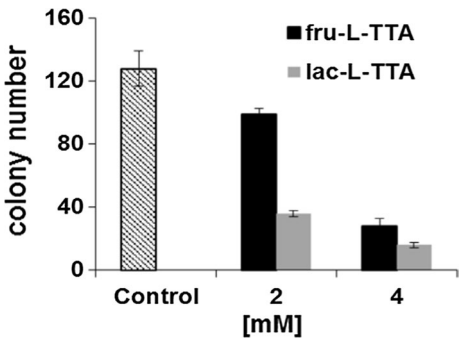


Fig. 3 Dose-dependent inhibition of PC-3 colony formation by Lac-L-TTA in comparison with Fru-L-TTA: PC-3 cells were seeded in six-well plates and treated with different concentrations of compounds (at 2 and 4 mM concentrations) over 2 weeks. Subsequently, crystal violet assay was performed and colonies greater than 50 μm were counted. Histogram of colony number: the values represent the means ± DS of triplicate data from three independent experiments

already studied by us (Roviello et al. 2017). In fact, the addition of a galactose residue to the Fru-L-TTA molecule resulted in a ~twofold increase in the inhibitory activities of Lac-L-TTA, as determined in the clonogenic growth assay with PC-3 cell lines (Fig. 3). Still, these data seem to suggest also the significance of β-galactoside-specific interactions in mediating the inhibitory effect of our synthetic amino acid–sugar conjugate on colony formation by metastatic cancer cells.

In future, we will evaluate novel structures belonging to the family of the amino acid–sugar conjugates, and some unmodified artificial amino acids, to obtain novel and effective candidates as anti-metastatic drugs.

Conclusion

As a prosecution of our recent work on anti-metastatic artificial Amadori compounds (Roviello et al. 2017), we have described a novel amino acid–sugar conjugate, denominated by us Lac-L-TTA, studied in analogy to Fru-L-TTA. We have found that the non-natural thiophenyl-substituted triazolyl-thione L-alanine undergoes the Amadori rearrangement also with the natural disaccharide, leading to a glycosylated amino acid structurally similar to the natural Amadori compounds but bearing a lactose moiety in place of the fructose ring. In consideration of the anti-metastatic potential of the Amadori products, we evaluated also for our novel compound Lac-L-TTA this biological property. In this respect, we documented a ~70% inhibition of colony formation of PC-3 cell lines at a 2 mM concentration, which is an activity significantly higher (~twofold) than that previously reported not only for Fru-L-TTA, but also for several natural Amadori compounds. Nevertheless, we plan to perform novel structural modifications of the lactosyl-amino acid, for example by substituting the lactose moiety by another sugar or by employing D-TTA instead of L-TTA, in view of an optimization of the anti-metastatic properties of this family of drugs, object of our future efforts in the development of new anticancer strategies.

Acknowledgements We thank Prof. Antonio Roviello for his precious suggestions and Mr. Leopoldo Zona for his invaluable technical assistance. We are grateful to Dr. Virginia Carbone and Dr. Rosa Anna Siciliano from Istituto di Scienze dell'Alimentazione—CNR for their contribution to the MS characterization of Lac-L-TTA. Finally, we thank Regione Campania for the research grant received for the research project "Nuovi sistemi nucleopeptidici per applicazioni diagnostiche" under the research program Legge Regionale n. 5 (annualità 2007).

Compliance with ethical standards

Conflict of interest There are no potential conflicts of interest in this research.

Ethical approval This article does not contain any study with human participants or animals performed by any of the authors.

References

Campo VL, Carvalho I, Allman S, Davis BG, Field RA (2007) Chemical and chemoenzymatic synthesis of glycosyl-amino acids and glycopeptides related to *Trypanosoma cruzi* mucins. Org Biomol Chem 5:2645–2657

Di Fabio G, De Capua A, De Napoli L, Montesarchio D, Piccialli G, Rossi F, Benedetti E (2001) A new strategy for the solid-phase synthesis of glycoconjugate biomolecules. Synlett 3:341–344

Esposito G, Teta R, Miceli R, Ceccarelli LS, Della Sala G, Camerlingo R, Irollo E, Mangoni A, Pirozzi G, Costantino V (2015) Isolation and assessment of the in vitro anti-tumor activity of smenothiazole A and B, chlorinated thiazole containing peptide/polyketides from the Caribbean sponge, *Smenospongia aurea*. Mar Drugs 13:444–459

Estendorfer S, Ledl F, Severin T (1990) Formation of an aminoreductone from glucose. Angew Chem Int Ed Engl 29:536–537

Gallas K, Pototschnig G, Adanitsch F, Stütz AE, Wrodnigg TM (2012) The Amadori rearrangement as glycoconjugation method: synthesis of non-natural C-glycosyl type glycoconjugates. Beilstein J Org Chem 8:1619–1629

Glinsky GV, Price JE, Glinsky VV, Mossine VV, Kiriakova G, Metcalf JB (1996) Inhibition of human breast cancer metastasis in nude mice by synthetic glycoamines. Cancer Res 56:5319–5324

Gruner SAW, Locardi E, Lohof E, Kessler H (2002) Carbohydrate-based mimetics in drug design: sugar amino acids and carbohydrate scaffolds. Chem Rev 102:491–514

Horikawa H, Okada M, Nakamura Y, Sato A, Iwamoto N (2002) Production of hydroxyl radicals and α-dicarbonyl compounds associated with Amadori compound-Cu 2 complex degradation. Free Radic Res 36:1059–1065

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61:69–90

Kumar V, Ramesh NG (2007) A versatile strategy for the synthesis of N-linked glycoamino acids from glycals. Org Biomol Chem 5:3847–3858

Mehlen P, Puisieux A (2006) Metastasis: a question of life or death. Nat Rev Cancer 6:449–458

Mossine VV, Chopra P, Mawhinney TP (2008) Interaction of tomato lycopene and ketosamine against rat prostate tumorigenesis. Can Res 68:4384–4391

Mottram DS (1994) Flavor compounds formed during the Maillard reaction: thermally generated flavors. Maillard Microw Extrus Process 543:104–126

Rakosi K, Szolomajer-Csikos O, Kalmar L, Szurmai Z, Kerekgyarto J, Toth GK (2011) Synthesis of *N*-glycopeptides applying glycoamino acid building blocks with a combined Fmoc/Boc strategy. Protein Pept Lett 18:679–683

Risseeuw MD, Overhand M, Fleet GW, Simone MI (2007) A compendium of sugar amino acids (SAA): scaffolds, peptide- and glyco-mimetics. Tetrahedron Asymmetry 18:2001–2010

Roviello GN, Moccia M, Sapio R, Valente M, Bucci EM, Castiglione M, Pedone C, Perretta G, Benedetti E, Musumeci D (2006) Synthesis, characterization and hybridization studies of new nucleo-γ-peptides based on diaminobutyric acid. J Pept Sci 12:829–835

Roviello GN, Musumeci D, Moccia M, Castiglione M, Sapio R, Valente M, Bucci EM, Perretta G, Pedone C (2007) dabPNA: design, synthesis, and DNA binding studies. Nucleosides Nucleotides Nucleic Acids 26:1307–1310

Roviello GN, Benedetti E, Pedone C, Bucci EM (2010a) Nucleobase-containing peptides: an overview of their characteristic features and applications. Amino Acids 39:45–57

Roviello GN, Musumeci D, De Cristofaro A, Capasso D, Di Gaetano S, Bucci EM, Pedone C (2010b) Alternate dab-aegPNAs: synthesis, nucleic acid binding studies and biological activity. Mol BioSyst 6:199–205

Roviello GN, Gaetano SD, Capasso D, Franco S, Crescenzo C, Bucci EM, Pedone C (2011a) RNA-binding and viral reverse transcriptase inhibitory activity of a novel cationic diamino acid-based peptide. J Med Chem 54:2095–2101

Roviello GN, Musumeci D, Bucci EM, Pedone C (2011b) Evidences for supramolecular organization of nucleopeptides: synthesis, spectroscopic and biological studies of a novel dithymine L-serine tetrapeptide. Mol BioSyst 7:1073–1080

Roviello GN, Vicidomini C, Di Gaetano S, Capasso D, Musumeci D, Roviello V (2016a) Solid phase synthesis and RNA-binding activity of an arginine-containing nucleopeptide. RSC Adv. 6:14140–14148

Roviello GN, Roviello V, Autiero I, Saviano M (2016b) Solid phase synthesis of TyrT, a thymine–tyrosine conjugate with poly(A) RNA-binding ability. RSC Adv. 6:27607–27613

Roviello GN, Iannitti R, Roviello V, Palumbo R, Simonyan H, Vicidomini C (2017) Synthesis and biological evaluation of a novel Amadori compound. Amino Acids. doi:10.1007/s00726-016-2363-4

Russo A, Aiello C, Grieco P, Marasco D (2016) Targeting "Undruggable" proteins: design of synthetic cyclopeptides. Curr Med Chem 23:748–762

Saghyan AS, Simonyan HM, Petrosyan SG, Geolchanyan AV, Roviello GN, Musumeci D, Roviello V (2014) Thiophenyl-substituted triazolyl-thione L-alanine: asymmetric synthesis, aggregation and biological properties. Amino Acids 46:2325–2332

Tai S, Sun Y, Squires JM, Zhang H, Oh WK, Liang C-Z, Huang J (2011) PC3 is a cell line characteristic of prostatic small cell carcinoma. Prostate 71:1668–1679

Teta R, Irollo E, Della Sala G, Pirozzi G, Mangoni A, Costantino V (2013) Smenamides A and B, chlorinated peptide/polyketide hybrids containing a dolapyrrolidinone unit from the Caribbean sponge *Smenospongia aurea*. Evaluation of their role as leads in antitumor drug research. Mar Drugs 11:4451–4463

Wang Y, Nangia-Makker P, Tait L, Balan V, Hogan V, Pienta KJ, Raz A (2009) regulation of prostate cancer progression by Galectin-3. Am J Pathol 174:1515–1523