Design and synthesis of N-benzoyl amino acid derivatives as DNA methylation inhibitors

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Davide Garella,[a,*] Sandra Atlante,[b] Emily Borretto,[a] Mattia Cocco,[a] Marta Giorgis,[a] Annalisa Costale,[a] Livio Stevanato,[a] Gianluca Miglio,[a] Chiara Cencioni,[b] Eli Fernández-de Gortari,[c] José L. Medina-Franco,[c] Francesco Spallotta, [b] Carlo Gaetano,[b,*] Massimo Bertinaria[a]

[a]*Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, via P. Giuria 9, 10125, Torino, Italy.*

[b]*Division of Cardiovascular Epigenetics, Department of Cardiology, Goethe University, Theodor-Stern-Kai 7, 60590, Frankfurt am Main, Germany.*

[c]*Facultad de Química, Departamento de Farmacia, Universidad Nacional Autónoma de México, Avenida Universidad 3000, Mexico City 04510, México*

[*]Corresponding authors: D. Garella, Tel.: +39 0116707649; fax: +39 0116707687. E-mail address: davide.garella@unito.it. C. Gaetano, Tel.: +49 069630187963; fax: +49 069630185277. E-mail address: gaetano@em.uni-frankfurt.de.

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Abstract

The inhibition of human DNA Methyl Transferases (DNMT) is a novel promising approach to address the epigenetic dysregulation of gene expression in different diseases. Inspired by the validated virtual screening hit NSC137546, a series of *N*-benzoyl amino acid analogues was synthesized and obtained compounds were assessed for their ability to inhibit DNMT-dependent DNA methylation in vitro. The biological screening allowed the definition of a set of preliminary structure-activity relationships and the identification of compounds promising for further development. Among the synthesized compounds, *L*-glutamic acid

derivatives **22**, **23**, and **24** showed the highest ability to prevent DNA methylation in a total cell lysate. Compound **22** inhibited DNMT1 and DNMT3A activity in a concentration-dependent manner in the micromolar range. In addition, compound **22** proved to be stable in human serum and it was thus selected as a starting point for further biological studies.

Introduction

Epigenetic modifications play an essential role in the establishment and regulation of cellular differentiation and gene expression.[1,2] DNA methylation is the most stable epigenetic mark in humans.[3] The DNA methylation occurs at the C5 position of the cytosine ring, particularly in a CpG dinucleotide context, through the action of three active DNA methyltransferases (DNMTs): DNMT1, DNMT3A and DNMT3B. These enzymes catalyze the transfer of a methyl group from *S*-adenosyl-*L*-methionine (SAM) to the C5-cytosine.[4] DNMT1 is responsible for DNA methylation maintenance during cell replication by methylation of newly synthesized DNA strands; however, it was hypothesized that this enzyme can also participate in the *de novo* methylation process.[5] DNMT3A and DNMT3B are responsible for *de novo* DNA methylation being able to methylate both unmethylated and hemimethylated DNA strands.[6,7] Another protein, lacking enzymatic activity, namely DNMT3L, is capable of interacting with DNMT3A and DNMT3B with the consequence of stimulating their catalytic activity.[8]

In human genome CpG dinucleotides are typically clustered in regions called CpG islands, which are located in the proximal promoter of more than half of all human genes.[9] When promoter CpG islands are methylated, the corresponding gene is repressed because of poor recognition by transcription factors and by other methyl-binding proteins (MBDs) involved in chromatin remodeling and reorganization.[10]

Aberrant DNA methylation, or the failure to maintain the appropriate DNA methylation status, results in the expression of non-optimal level of gene-associated proteins, which could trigger or exacerbate different pathological responses. For instance, in cancer cells,[11] DNA hypermethylation of CpG islands, joined to a global hypomethylation, give rise to genomic instability and inactivation of cancer-suppressor genes.[12,13] Altered DNA methylation has also been found to regulate synaptic plasticity in post-mitotic neurons[14] and DNMT1, DNMT3A have been recently proposed as new targets for antipsychotic therapy.[15] Moreover, hypermethylation of DNA sequences has been linked to the onset of cardiac fibrosis.[16]

On these bases, the potential therapeutic application of DNMT inhibitors is nowadays actively studied.[17-19] A number of DNMT inhibitors has been developed. They can be classified into two general subsets: nucleoside and non-nucleoside DNMT inhibitors (Figure 1).

The first subset comprises 5-azacytidine (5-AZA) and 5-aza-2′-deoxycytidine (decitabine), which are drugs currently employed in the treatment of myelodysplastic syndromes (MDS), chronic myelomonocytic leukemia (CMML), and acute myeloid leukemia (AML). 5-AZA and decitabine are imported into the cell, phosphorylated, and actively integrated in the DNA structure where they act through covalent inhibition of DNMTs. This complex set of events results in an easy onset of resistance and poor reproducibility of action with more than 1000-fold variability in cancer cell lines as demonstrated with decitabine.[20] A second generation of nucleoside analogues is currently under study, among them: 5-fluoro-2′-deoxycytidine, 5,6-dihydro-5-azacytidine (DHAC) and zebularine. The main drawback of these nucleoside DNMT inhibitors is expected to lie in their mechanism of action, similar to that of first generation drugs.

Non-nucleoside DNMT inhibitors are represented by a heterogeneous subset of compounds which can directly inhibit the enzyme. Different compounds have been identified either from natural sources, from screening campaigns, or thanks to synthetic efforts.[17]

Natural products, such as parthenolide, curcumin, nanaomycin A, are proposed to act by covalent binding to the catalytic cysteine residue in the enzyme pocket.[21-23] A main disadvantage of these compounds could be associated with their lack of selectivity that may reflect in promiscuous binding to other cellular targets.

Hydralazine, procaine, procainamide, isoxazoline derivatives, and the natural compound (-)-epigallocatechin-3-gallate are non-covalent inhibitors. They bind into the enzyme pocket through a complex network of hydrogen bonds in a 2′-deoxycytidine-like binding mode.[24,25] Moreover, thanks to the use of computational techniques and *ad hoc* medicinal chemistry design, an increasing number of small molecules DNMT inhibitors is now emerging.[26]

A few lead structures have been identified and their rational modulation has been pursued. Most studied model compounds comprise RG108, SGI-1027, NSC14778, NSC319745, and their analogues, which act by competing with SAM or with DNA-strand for binding into the respective enzymatic site.[27-32] Furthermore, compounds such as NSC14778 are being used as starting point of computer-assisted drug repurposing of novel hypomethylating agents.[33]

Insert Figure 1

Figure 1. Structure of representative DNMT inhibitors.

So far, the therapeutic development of non-nucleoside DNMT inhibitors was hampered by the relatively poor inhibitory activity, the lack of isoform selectivity, and a significant cytotoxicity. Therefore, new potent and selective DNMT inhibitors are urgently needed.

Recently, virtual screening of the National Cancer Institute (NCI) compounds library allowed the identification of a glutamic acid derivative, namely NSC137546 (Figure 1), as a potential DNMT1 inhibitor. Of note, in the previous study, the stereochemistry of tested NSC137546 was not defined. This virtual screening hit showed a moderate selective inhibition of DNMT1 vs DNMT3B at 100 μM concentration. Since the virtual screening was conducted at the substrate binding site of DNMT1 it was hypothesized that the active compound binds into this pocket, although this was not experimentally checked.[32] Indeed, currently there is not reported a crystallographic structure of NSC137546 bound to DNMT that provide evidence for the actual binding site. Actually, for most of the small molecule DNMT inhibitors described to date, the experimental binding site remains unknown. However, molecular docking has been helpful to propose binding models that require, of course, experimental validation. Inspired by the chemical structure of NSC137546 we decided to explore the chemistry of the *N*-benzoyl amino acidic scaffold with the aim of improving DNMT1 activity and of investigating the selectivity against DNMT3A and DNMT3B. Since the predicted docking pose of the (S)- and (R)-forms are largely similar, in this work we selected the (S) stereochemistry to make use of natural amino acids. Therefore, the structure of 1(*S*)-2-(2,6-dichlorobenzamido) pentanedioic acid (**1**), used as the model template, was modulated according to three different approaches (A-C, Figure 2).

Insert Figure 2

Figure 2. Structure of model compound **1** and chemical modulation strategies applied.

Synthesis of derivatives bearing natural aminoacids as the terminal acidic portion (A moiety in Figure 2) was initially considered, in order to compare a series of sterically homogenous analogues. A first series of 27 compounds was designed (Schemes 1-3). Derivatives **1**-**8** were prepared to explore the structure-activity relationships (SAR) of the A moiety (Figure 2). Compound **9**, the amino analogue of **1**, was synthesized to verify the role of the amide bond (B moiety) in the activity of this class of compounds. Finally, modulation

of the aromatic substructure (C moiety), as in derivatives **10**-**27** (Scheme 3), was performed to gain preliminary SAR information concerning the stereo electronic properties of the aromatic ring.
The synthesis, the ability of the synthesized compounds to inhibit DNA methylation in vitro, and their preliminary SAR are described. The ability of selected compounds to inhibit DNA methylation in cell lysate over expressing DNMT1, DNMT3A and DNMT3B isoforms is reported. The action of selected compounds on isolated DNMT1 and DNMT3A enzymes is also reported. Finally, the characterization of the putative binding mode of derivative **22** in the substrate binding site of DNMT1 and DNMT3A is proposed.

Methods and Materials

Commercially available reagents and solvents were used without further purification, unless otherwise noted. Reaction progress, was monitored by TLC on pre-coated silica plates (Merk 60 F_{254}, 250 μm thickness) and spots were stained by ceric ammonium molibdate, $KMnO_4$ (0.5 g in 100 mL 0.1 N NaOH) and UV light. Reactions under MW irradiation were carried out in the Synthwave™ oven (Milestone). Melting points were measured with a capillary apparatus (Büchi 540). All the compounds were routinely checked by ^1H and ^{13}C NMR (Bruker Avance 300) at 300 and 75 MHz respectively. Chemical shifts (δ) are given in ppm relative to internal standard TMS (0.00 ppm) or residual solvent peaks ($CDCl_3$ = 7.26 ppm, MeOD = 3.31 ppm, DMSO-d_6 = 2.50 ppm and D_2O = 4.79 ppm). ^1H NMR coupling constants (*J*) are reported in Herz (Hz) and multiplicity in indicated as follow: s, singlet; bs, broad singlet; d, doublet; dd, doublet of doublet; t, triplet; m, multiplet; Im, imidazole ring; Ph, phenyl ring. Low resolutions mass spectra were recorded on Finnigan-Mat TSQ-700 in chemical ionization (CI) using isobutane. ESI-MS spectra were recorded on a Micromass Quattro micro™ API (Waters, Milford, MA). The purification was performed by flash-chromatography (CombiFlash Rf® Teledyne ISCO) on appropriate columns (silica gel). Anhydrous sodium sulfate was used as drying agent for the organic phases. Organic solvents were removed under vacuum at 30 °C. Purity of compounds was checked by UHPLC (PerkinElmer) Flexar 15, equipped with UV-Vis diode array detector using an Acquity UPLC CSH Phenyl-Hexyl 1.7 μm 2.1×50 mm column (Waters) and H_2O/CH_3CN containing 0.1 % CF_3COOH and H_2O/CH_3OH containing 0.1 % CF_3COOH solvent systems. Detection was performed at λ = 200, 215, 254 nm. The analytical data confirmed that the purity of the products was ≥ 95%.

Detailed synthetic procedures are fully described in Supporting Information. Characterization data of **10-14, 16, 22, 24**,[35] **15**,[36] **18**,[37] **19**,[38] **21**,[39] are in keeping with those reported.

Chemistry

General procedure for the synthesis of compounds 1-6, **10-27**

Carboxylic acid (2.6 mmol, 1 eq) was solubilized in $SOCl_2$ (22 mmol, 8.5 eq) and irradiated under pressure in a microwave oven (5 bar, N_2) at 120 °C for 1.5 h. The solvent was removed under reduced pressure, and the residual oil was solubilized in 1,4-dioxane (5 mL) and added dropwise to a stirring solution of appropriate aminoacid (2.6 mmol) and Na_2CO_3 (6.5 mmol) in water (5 mL). The mixture was stirred overnight at room temperature, poured in 1N HCl (40 mL) and extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with brine, dried with anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was triturated with CH_2Cl_2 (about 30 mL) and the obtained precipitate was filtered. The product was purified (when required) by flash chromatography (CombiFlash, gradient CH_2Cl_2/MeOH 0.1%CF_3COOH).

General procedure for the synthesis of compounds 7 and 8

To the compound **3** (500 mg, 1.6 mmol) solubilized in THF (5 mL) was added DCC (453 mg, 2.2 mmol). The mixture was stirred overnight at room temperature. The mixture was filtered, and the filtrate was partially concentrated under reduced pressure. The appropriate amine (isopropyl amine or benzylamine, 1.6 mmol) was added to the residue and the mixture was stirred neat (overnight) at room temperature. The solution was poured in water and extracted with Et_2O (1 x 20 mL). The pH was adjusted to 1-2 with 1N HCl, and the aqueous phase was extracted with EtOAc (3 x 20 mL). The organic phases were washed with brine, dried, filtered and concentrated under reduced pressure to obtain the desired compound without any further purification.

General procedure for the synthesis of (*S*)-Sodium-2-((2,6-dichlorobenzyl)amino)pentanedioate (9)

L-glutamic acid (600 mg, 4.0 mmol) and NaOH (320 mg, 8.0 mmol) were solubilized in H_2O/MeOH 1:1 (20 mL). 2,6-Dichlorobenzaldehyde (700 mg, 4.0 mmol) was added and the mixture was stirred at room temperature for 2 h. $NaBH_4$ (227 mg, 6.0 mmol) was added portionwise and the mixture was stirred

overnight at room temperature. The pH of the mixture was adjusted to 5-6 adding acetic acid. After stirring for further 45 min, the solvent was completely removed and the sticky mass was treated with EtOH (30 mL) to obtain the precipitation of a white solid which was filtered and dried to afford the final compound **9** as a white solid.

Biology

Total DNMT activity assay

All compounds were screened for total DNMT activity using a DNA methyltransferase (DNMT) activity/inhibitor assay kit (Epigentek) according to manufacturer instruction. To measure the effects of the compounds on DNMT activity, 35 µg of total HaCaT cellular extract freshly prepared in RIPA buffer (Tris HCl pH 7.4 10 mM, NaCl 150 mM, NP-40 1%, sodium deoxycholate (DOC) 1%, SDS 0.1%, glycerol 0,1%, Protease Inhibitors Cocktail) were incubated with 50 µM and 100 µM of the different compounds in DMSO (1% final conc.) or vehicle alone (1% DMSO) at 37 °C for 2h. As negative control the lysate was denatured at 100 °C for 30 min. Total lysate was used in order to avoid the loss of possible coenzyme and complex formation. The amount of methylated DNA, which is proportional to enzyme activity, was colorimetrically detected by a plate reader at 450 nm (EnSpire® Multimode Plate Reader – Perkin Elmer).

Inhibition of DNA methylation in DNMT1, DNMT3A and DNMT3B overexpressing cell lysates.

Compounds **22**, **24** were tested to evaluate their selectivity on the different DNMT isoforms. In order to work selectively on DNMT1, DNMT3A and DNMT3B, HEK293T cells were transfected with the plasmids containing the three different DNMTs' sequences and mock control. Cells were transfected with 2.5 µg of expression plasmid using Lipofectamine 3000 reagent (Invitrogen) according the manufactures instruction. Plasmids pcDNA3/Myc-DNMT1 (Addgene plasmid # 36939), pcDNA3/Myc-DNMT3A (Addgene plasmid # 35521) and pcDNA3/Myc-DNMT3B1 (Addgene plasmid # 35522) were a gift from Arthur Riggs.[40,41] The presence of exogenous DNMTs was checked by western blot (not shown) and afterwards the transfected cells were freshly lysed in RIPA buffer as above. 35 µg of cellular extract were incubated with selected compounds at different concentration in the 1 µM - 150 µM range (1% DMSO final conc.) or with vehicle alone (1% DMSO) at 37 °C for 2 h. RG108 (Cayman) was used as positive controls, while as negative

control, lysates were denatured at 100 °C for 30 min. DNMT activity was detected by DNMT activity/inhibitor assay kit (Epigentek). Data are presented as means ± SD; each compound was tested at least three times.

Inhibition of DNMT1 and DNMT3A activity on isolated enzyme.

To assess the specific interaction of DNMT inhibitors with DNMT1 or DNMT3A, the activity assay was performed on the immunoprecipitated enzymes after their overexpression. After DNMT transfection, HEK293T cells were freshly lysed in RIPA buffer as described above (supplemented with Protease Inhibitors Cocktail), and DNMT1 and DNMT3A were immunoprecipitated using Paramagnetic beads (Ademtech's Bioadembeads) as previously described.[42] Briefly, 600 µg of transfected cell extract were incubated at 4 °C for 2 h with DNMT antibodies: anti-DNMT1 (6 µg, mouse, monoclonal; Abcam), anti-DNMT3A (6 µg, mouse, monoclonal; Abcam). Normal mouse IgG (Santa Cruz Biotechnology, Inc.) was used as immunoprecipitation control. Then, the immunocomplexes were incubated at 4 °C for 2 h with 60 µl of paramagnetic beads. All the immunoprecipitation steps have been performed on ice in order to preserve the enzymatic activity. After elution, the enriched enzymes were incubated with compounds **1** or **22** at 100 µM (1% DMSO final conc.) or vehicle alone (1% DMSO) at 37 °C for 2 h. RG108 (Cayman) was used as reference, while as negative control, lysates were denatured at 100 °C for 30 min. DNMT activity was detected by DNMT activity/inhibitor assay kit (Epigentek). Data are presented as means ± SD; each compound was tested at least three times. Western blotting analysis was performed, according to standard procedure, to check the immunoprecipitation.

Stability of compound 22 in pH 7.4 phosphate-buffered solution

A stirred solution of compound **22** in pH 7.4 phosphate-buffered solution (final concentration 2 mg/mL) was maintained at 37 ± 0.5 °C for 48 h. At different time intervals 100 µL of this solution were withdrawn, diluted to 1 mL with methanol containing 1% CF_3COOH and 5 µL of the resulting solution were analyzed by RP-UHPLC using a Flexar UHPLC (Perkin Elmer) equipped with a Flexar Solvent Manager 3-CH-Degasser, a Flexar-FX UHPLC autosampler, a Flexar-FX PDA UHPLC Detector, a Flexar-LC Column Oven, and a

Flexar-FX-15 UHPLC Pump. The analytical column was an Acquity CSH™ (2.1 x 100 mm, 1.7 μm particle size) (Waters) column. The samples were analyzed using an isocratic method employing a mobile phase consisting of methanol/water (90/10) containing 0.1% trifluoroacetic acid (flow rate 0.6 mL/min). The column effluent was monitored at λ = 220 nm referenced against a λ = 360 nm wavelength. Quantitation was done using calibration curves of compound **22** chromatographed under the same conditions. The linearity of the calibration curves was determined in a concentration range of 1-3 mg/mL (r^2> 0.98). Data analysis was performed using Chromera Manager (Perkin Elmer). All experiments were run in triplicate.

Stability of compound 22 in human serum

A solution of compound **22** (20 mg/mL) in methanol was added to human serum (sterile-filtered from human male AB plasma, Sigma-Aldrich) preheated at 37 °C to obtain a final concentration of 2 mg/mL. The resulting solution was incubated at 37 ± 0.5 °C; at appropriate time intervals, 100 μL of the reaction mixture were withdrawn and added to 900 μL of methanol containing 0.1% trifluoroacetic acid in order to deproteinize the serum. The sample was vortexed, and then centrifuged for 5 min at 1500 g. The clear supernatant was filtered by Captiva PES 0.2 μm filters (Agilent) and analyzed by RP-UHPLC.
HPLC analyses was performed with a Flexar UHPLC (Perkin Elmer) equipped with a Flexar Solvent Manager 3-CH-Degasser, a Flexar-FX UHPLC autosampler, a Flexar-FX PDA UHPLC Detector, a Flexar-LC Column Oven, and a Flexar-FX-15 UHPLC Pump. Data analysis was done using a Chromera Manager (Perkin Elmer). The analytical column was an Acquity CSH™ (2.1 x 100 mm, 1.7 μm particle size) (Waters) column. The samples (1 μL, injection volume) were analyzed using an isocratic method employing a mobile phase consisting of methanol/water (80/20) containing 0.1% trifluoroacetic acid at a flow rate of 0.2 mL/min. The column effluent was monitored at a λ = 220 nm referenced against a λ = 360 nm wavelength. Quantitation was done as indicated above. All experiments were run in triplicate.

Molecular docking

All flexible ligand docking and scoring calculations were performed with ICM-Pro, version 3.8-4. ICM is based on Monte Carlo optimization of the ligand internal coordinates in the space of pocket grid potential maps. The crystallographic structures of DNMT1 (PDB ID: 3PTA) and DNMT3A (PDB ID: 2QRV) were

employed. Before docking, the structures of the proteins were prepared using ICM using default settings. To ensure the convergence of the Monte Carlo algorithms three different cycles was perform for each docked ligand. For the best ten poses a manually clustering was performed and the docked poses were checked visually. Visualizations and analysis of the protein-ligand complexes were conducted with ICM. The 2D interaction diagram was generated with the Molecular Operating Environment (MOE) software, version 2014.09.[43]

Statistics

Data were collected and analysed blind by an observer. Results were expressed as mean ± SD of at least three experiments done in triplicate. Statistical significance was evaluated by ANOVA and Bonferroni post-hoc test (Prism 5, GraphPad Software, La Jolla, CA, USA). Differences were judged statistically significant when $P < 0.1$.

Results and Discussion

Chemistry

Compound **1**, and the analogues **2-6**, modified in the acidic moiety, were synthesized according to the procedure reported in Scheme 1. 2,6-Dichlorobenzoic acid was converted into the corresponding acyl chloride irradiating under pressure (5 bar, N_2) in a microwave oven at 120 °C using $SOCl_2$ for 1.5 h. This procedure allowed the use of a parallel chemistry approach generating five acyl chloride derivatives in a single run in almost quantitative yields. After $SOCl_2$ evaporation, the crude product was added dropwise to a stirring solution of the appropriate (S)-aminoacid in water using Na_2CO_3 as the base. The mixture was stirred at room temperature to afford compounds **1-6** in 40-72% non-optimized yields (Scheme 1). The use of water, as the preferred solvent allowed for amino acids dissolution, but surely affected the reaction yield. Nevertheless, it avoided the use of polar aprotic solvents (e.g. DMF) which could complicate isolation and purification of the final compounds. Compounds **7** and **8** were synthesized by coupling of **1** with 2-propylamine or benzylamine using dicyclohexyl carbodiimide (DCC) in THF in 50% and 55% yield respectively (Scheme 1). The structural identity of compounds **7** and **8** was supported by 2D-NMR spectra. For both compounds, the 2D-COSY experiments allowed to discriminate the proton in position 2 from the chiral proton in position 4. The 2D-HMBC experiments showed the correlation of the chiral proton with both

the amide carbon atoms, confirming that the new amide bond was formed at the alpha acid (see Supporting Information).

Scheme 1. Synthesis of 2,6-dichlorobenzoyl aminoacid derivatives **1**-**8**.

Insert scheme 1

Reagents and conditions. a) (i) $SOCl_2$, MW irradiation 120 °C, N_2 (5 bar), 1.5 h; (ii) *L*-amino acid, Na_2CO_3, H_2O, RT, 12 h. b) (i) DCC, THF, RT, 12 h; (ii) R-NH_2, RT, 12 h.

The amino derivative **9** was obtained in 70% yield via reductive amination of 2,6-dichlorobenzaldehyde with *L*-glutamic acid and $NaBH_4$ (Scheme 2).

Scheme 2. Synthesis of compound **9**.

Insert Scheme 2

Reagents and conditions. a) (i) *L*-glutamic acid, NaOH, H_2O/MeOH 1/1, RT, 2 h; (ii) $NaBH_4$, RT, 12 h.

Derivatives **10**-**27** (Scheme 3) were synthesized by reaction of *L*-glutamic acid or 4-aminobutanoic acid with the appropriately substituted benzoyl chloride using the expeditious protocol previously developed for the synthesis of compounds **1**-**6**.

Scheme 3. Synthesis of compounds **10**-**27.**

Insert scheme 3

Reagents and conditions. a) (i) $SOCl_2$, MW irradiation 120 °C, N_2 (5 bar), 1.5 h; (ii) *L*-glutamic acid, Na_2CO_3, H_2O, RT, 12 h. b) (i) $SOCl_2$, MW irradiation 120 °C, N_2 (5 bar), 1.5 h; (ii) 4-aminobutanoic acid, Na_2CO_3, H_2O, RT, 12 h.

Biological activity

Inhibition of total DNA methylation

The ability of the newly synthesized compounds to inhibit DNA methylation was evaluated on HaCaT total cell lysate in order to avoid the loss of possible coenzyme(s) and complex formation. Compounds were incubated at either 100 µM or 50 µM for 2 h at 37 °C in a freshly obtained cell lysate. The use of two

different concentrations of test compounds was chosen to overcome cellular variability and to afford reliable results, thus avoiding the identification of false positive hits.

The amount of methylated DNA was quantified using the EpiQuik DNA methyl transferases activity/inhibition assay in comparison with vehicle treated (DMSO 1%) cell lysate. Results, expressed as the percentage of the residual DNA methylation, are reported in Table 1. Vehicle alone had no effect on DNA methylation in our test system. We would like to point out that many studies highlighted that, depending on the experimental conditions of the enzymatic assay, the inhibitory potency can vary greatly for the same compound; critical factors are the DNA methyltransferase employed, the method of detection, the concentration of the cofactor and the nature of the DNA duplex used as the substrate.[34]

Table 1. Ability of compounds **1**-**27** to inhibit total DNA methylation expressed as residual relative DNA methylation.

Compound	**Residual DNA methylation (%)**[a] (mean ± SD[b])		**Compound**	**Residual DNA methylation (%)**[a] (mean ± SD[b])	
	100 µM	**50 µM**		**100 µM**	**50 µM**
1	61 ±12[c]	96 ±6	**15**	72 ±7	82 ±6
2	60 ±2[c]	87 ±15	**16**	75 ± 2	89 ±5
3	99 ±11	122 ±26	**17**	69 ±3	97 ±2
4	126 ± 29[c]	96 ±2	**18**	58 ±3[c]	92 ±4
5	90 ± 1	98 ±22	**19**	64 ±11	93 ±6
6	58 ±7[c]	104 ± 15	**20**	81 ± 2	85 ±5
7	82 ± 16	118 ± 1[c]	**21**	60 ±5[d]	86 ± 18
8	94 ±39	90 ± 17	**22**	45 ± 1[c]	63 ± 16[d]
9	63 ± 1[d]	111 ± 2	**23**	51 ±9[c]	68 ±3[d]
10	116 ±9	108 ±36	**24**	68 ±21[d]	65 ± 1[d]
11	85 ±20	122 ± 16	**25**	74 ±4	95 ±10
12	72 ±23	100 ± 1	**26**	107 ±10	103 ±11
13	57 ±4[c]	106 ±13	**27**	99 ±14	93 ±5
14	70±6[c]	100 ± 16			

[a] Determined in fresh HaCaT cell lysate using Epiquik DNA methyltransferase activity/inhibition assay. [b] Data are expressed as percentage of residual methylated DNA relative to vehicle (DMSO 1%) treated cell lysate. Results are the mean of at least three independent experiments run in triplicate. [c] $P< 0.05$ vs vehicle; [d] $P< 0.1$ vs vehicle; ANOVA and Bonferroni post-hoc test.

The ability of synthesized compounds to inhibit total DNA methylation, although initially studied only at two fixed concentrations (100 and 50 µM), was helpful to draw preliminary SAR indications and to address future chemical modulation of this class of compounds.

Compound **1** (100 µM) significantly decreased DNA-methylation by 39 ± 12% ($P<0.05$). Removal of the α-carboxylic group (**2**) afforded an inhibition of DNA-methylation (40 ± 2%; $P< 0.05$) comparable to that

showed by **1**. Both **1** and **2** were un-effective when tested at 50 μM (Table 1). Conversion of α-COOH into a substituted amide gave compounds **7** and **8**, showing no significant ability to prevent DNA methylation under our assay conditions. The obtained results indicated that substitution of α-carboxylic group in compound **1** with uncharged and bulky groups (compounds **7** and **8**) is not a promising strategy to improve DNMT inhibition in this series of *L*-glutamic acid derivatives, while the role of free α-COOH needs further studies to be fully elucidated.

The modulation of the γ-carboxylic functionality was then considered. The replacement of this carboxylic group with a carboxyamido group (**3**), or its removal (**4**), resulted in a complete loss of activity (Table 1). The replacement of the γ-COOH with basic groups furnished compounds **5** and **6** bearing a guanidine and imidazole ring in γ-position, respectively. Results showed that the imidazole derivative **6** could conserve the inhibitory activity (42 ± 7% inhibition; $P< 0.05$) while the strongly basic **5** reflected in a complete loss of activity. This result was not unexpected taking into account that γ-COOH in **1** is negatively charged at pH 7.4, while **5** is positively charged. The imidazole ring in **6** (calculated pK_a 7.08) is also present in a positively charged form for about 32% at pH 7.4. Consequently, its residual ability to decrease DNA methylation could be attributed to other properties of this moiety (e.g., metal complexing ability or 1,3-prototropic tautomerism). Unfortunately, similarly to derivatives **1** and **2**, compound **6** did not exert any detectable inhibitory effect at lower concentration (50 μM; Table 1). These preliminary SARs suggest that a putative electrostatic interaction, rather than just hydrogen bonding, could be involved in the binding of the γ-COOH group of **1** with its target (*vide infra*).

Interestingly, when the amide bond of **1** was reduced to afford the amino derivative **9** (B moiety in Figure 2) a behavior similar to that of the parent compound was found, with inhibition of methylation at 100 μM (37 ± 1%; $P<0.1$) and no significant inhibition at 50 μM.

Based on the preliminary SAR data discussed above with the modification of the acidic and amide moieties (A and B, respectively in Figure 2), we decided to explore the influence of benzene ring substitution (C moiety) keeping the *L*-glutamic acid side chain present in **1** as the preferred moiety A and the amide group. Moving one chlorine atom from *ortho* to *para* position of benzene ring, to obtain the 2,4-dichloro-substituted compound **10**, afforded an inactive compound. Derivatives **24**, **16**, and **14**, bearing one chlorine atom in *ortho*, *meta* and *para* position, proved able to slightly reduce DNA methylation at 100 μM showing a residual methylation of 68 ± 21 %, 75 ± 2% , and 70 ± 6% respectively. Interestingly, the *o*-chloro

substituted compound **24** maintained an inhibitory activity (35 ± 1% inhibition) at the lower concentration, demonstrating an improvement with respect to the reference **1**.

These preliminary SAR data, at two compound concentrations, suggest that substitution of benzene ring with an halogen atom in *ortho* position could afford derivatives with improved efficacy as compared to double halogen-substitution.

Use of an electron-donating substituent (i.e. a methoxy group) in either position of benzene ring (compounds **15**, **17**, **20**) did not show an increase in the activity when compared to *o*-Cl substituted **24**, when compounds where tested at 50 μM concentration.

In order to obtain more hints about the benzene ring substitution preference, and to verify whether we could further improve the activity of **24**, we synthesized and evaluated a series of compounds (**11**-**13**, **18**, **19**, **21**-**23**, and **26**) bearing substituents endowed with different steric and electronic properties. Among the synthesized compounds **13** (*p*-F substituted), **22** (*o*-Br substituted), and **23** (*o*-F-substituted) showed the most attractive activity, being able to inhibit DNA methylation by 43 ± 4% ($P<0.05$), 55 ± 1% ($P<0.05$), and 49 ± 9%, ($P< 0.05$), respectively at 100 μM. Compounds **22** and **23** were also able to prevent DNA methylation up to a significant extent when tested at 50 μM (37 ± 16% and 32 ± 3% inhibition). Unsubstituted compound **25** as well as *p*-phenyl-substituted derivative **19** retained a modest activity only at 100 μM.

Finally, to explore the role of α-COOH group, we combined *o*-chloro substitution (as in **24**) with α-COOH group removal (as in **2**). The obtained derivative **27** proved inactive in our assay, indicating that α-COOH group can play a relevant role in the activity of the *o*-chloro substituted compound **24**.

Inhibition of DNA methylation in cell lysates selectively over expressing DNMT1, DNMT3A and DNMT3B

Based on the results of the inhibition of DNA methylation discussed in the previous section, we selected two of the newly designed molecules, **22** and **24**, to further investigate their DNMT inhibition properties. The choice of o-Cl-substituted compound **24**, which is slightly less active than o-F-substituted **23** (at 100 μM) was done to directly compare **24** *vs* **1** over a larger range of concentrations, in order to test the hypothesis that mono-halo-substituted benzoyl amino acids could be better DNMT inhibitors than disubstituted analogues. The new compounds, along with model compound **1**, and RG108 were studied at seven different concentrations (range 1 - 150 μM) for their ability to prevent DNA methylation in a HEK293T cell lysate selectively over expressing DNMT1, DNMT3A, and DNMT3B. All the tested compounds were able to

inhibit DNMT1- and DNMT3A-dependent DNA methylation in a concentration dependent manner with a residual methylation in the 28-49 % range in cell lysate over expressing DNMT1 (Figure 3), and 39-41% in cell lysate over expressing DNMT3A (Figure 4) at the maximal concentration tested. Interestingly, in this test model, compound **22** proved as active as the reference DNMT inhibitor RG108 maintaining a significant and encouraging inhibition of DNA methylation up to 25 μM (**22** = 41 ± 18 % vs RG108 =47 ± 10 % inhibition in DNMT1 expressing lysate; **22** = 38 ± 6 % vs RG108 = 32 ± 17 % inhibition in DNMT3A expressing lysate). Compounds **1** and **24** proved somewhat less active on both DNMT1 and DNMT3A. All the compounds showed poor or non-significant inhibitory activity in DNMT3B over expressing cell lysate up to 100 μM (Table S1 in the Supporting Information).

The obtained data suggest that these compounds are non-selective DNMT1/DNMT3A inhibitors.

Insert Figure 3

Figure 3. Concentration-response experiments for compounds **1**, **22**, **24** and reference RG108 showing inhibition of DNA methylation in cell lysate selectively overexpressing DNMT1.
HEK293T cells were transfected with plasmids containing DNMT1 sequence and mock control. Fresh cellular extracts were incubated either in the presence of test compounds or with vehicle (1 % DMSO) alone for 2 h. The amount of methylated DNA was determined using Epiquik DNA methyltransferase activity/inhibition assay. Data are expressed as percentage of residual methylated DNA ± SD relative to vehicle (DMSO 1%) treated cell lysate. Results are the mean of at least three independent experiments run in triplicate. * $P < 0.05$, $^{\nabla}$ $P < 0.01$, and $^{\#}$ $P < 0.001$ vs. vehicle treated lysates; ANOVA and Bonferroni post-hoc test.

Insert Figure 4

Figure 4. Concentration-response experiments for compounds **1**, **22**, **24** and reference RG108 showing inhibition of DNA methylation in cell lysate selectively over expressing DNMT3A. HEK293T cells were transfected with plasmids containing DNMT3A sequence and mock control. Fresh cellular extracts were incubated either in the presence of test compounds or with vehicle (1 % DMSO) alone for 2 h. The amount of methylated DNA was determined using Epiquik DNA methyltransferase activity/inhibition assay. Data are expressed as percentage of residual methylated DNA ± SD relative to vehicle (DMSO 1%) treated cell lysate. Results are the mean of at least three independent experiments run in triplicate. * $P < 0.05$, $^{\nabla}$ $P < 0.01$, and $^{\#}$ $P < 0.001$ vs. vehicle treated lysates; ANOVA and Bonferroni post-hoc test.

Inhibition of DNMT1 and DNMT3A in enzymatic-based assays

To further evaluate the ability of compounds **1**, **22** and RG108 to act directly on DNMT1 and DNMT3A, the enzymes were overexpressed in HEK293T cells and enriched by immunoprecipitation using the specific antibodies. Enriched enzymes were treated with 100 μM of compounds **1**, **22** and RG108; DNA methylation inhibition was measured as above. Results are reported in Table 2. All tested compounds are able to inhibit

DNMT1- and DNMT3A-mediated DNA methylation. Compound **22** inhibited both DNMT1 and DNMT3A activity by 42% and 49%, respectively. In this assay, the activity of **1** was very close to that previously reported for NSC137546 (stereochemistry not defined) on recombinant DNMT1.[32] Collectively, these observations suggest that the chemistry of *N*-benzoyl-*L*-glutamic acid derivatives could be further explored to generate mixed DNMT1 / DNMT3A inhibitors.

Table 2. Ability of compounds **1**, **22**, and reference compound RG108 to inhibit DNA methylation mediated by isolated DNMT1 and DNMT3A enzymes.

Compound	**Residual relative enzymatic activity (%)**[a]	
	DNMT1 (mean ± SD[b])	**DNMT3A** (mean ± SD[b])
1	66± 15 [c]	61± 17 [c]
22	58±11 [d]	51±13 [c]
RG108	65±8 [c]	79±3

[a] HEK293T cells were transfected with plasmids containing the different DNMTs' sequences and mock control. Cells were freshly lysed in RIPA buffer, DNMT1 and DNMT3A were immunoprecipitated using Paramagnetic beads and anti-DNMT1, anti-DNMT3A as antibodies. Normal mouse IgG was used as immunoprecipitation control. All the immunoprecipitation steps have been performed on ice in order to preserve the enzymatic activity. The immunoprecipitated enzymes were incubated with compounds **1** and **22** at 100 µM (1% DMSO final conc.) or vehicle alone (1% DMSO) at 37 °C for 2 h. RG108 was used as reference at 100 µM. DNMT activity was detected by DNMT activity/inhibitor assay kit. [b] Data are expressed as percentage of residual enzymatic activity relative to vehicle (DMSO 1%) treated cell lysate ± SD. Results are represented as mean of, at least, three independent experiments run in triplicate. [c] $P < 0.1$, and [d] $P < 0.05$ vs vehicle; ANOVA and Bonferroni post-hoc test.

*Docking studies of **22** with DNMT1 and DNMT3A*

Molecular docking and other computational techniques have shown to be useful to elucidate the binding mode of experimentally known DNMT1 and DNMT3A inhibitors.[26] In order to explore the putative protein-ligand interactions of compound **22**, we conducted flexible docking of its structure within the substrate binding site of the catalytic domain of human DNMT1 and DNMT3A, respectively. Crystallographic structures of DNMT1 (Protein Data Bank, PDB ID: 3PTA)[44] and DNMT3A (PDB ID: 2QRV) were employed.[45] Docking was conducted with the program Internal Coordinates Mechanics (ICM) software.[46] The docking protocol is presented in the Experimental part section. Figure 5 shows a three- and bi-dimensional (3D and 2D) binding model of **22** in complex with DNMT1. According to this model, the γ-carboxylate group of **22** makes two important hydrogen bonds with the side chains of Arg1312 and Arg 1310. An additional hydrogen-arene bond is predicted between the phenyl moiety of **22** with Cys1226. The

overall position of the compound in the binding pocket and, in particular, the interactions with the catalytic Cys1226, suggests that **22** could inhibit DNMT1 by blocking the substrate binding site.

Insert Figure 5

Figure 5. Docking model of compound **22** with the catalytic domain of human DNMT1. The 3D and 2D-interaction map show selected amino acid residues of the binding site. Non-polar hydrogen atoms are omitted for clarity. In the 2D-interaction map green and blue arrows indicate hydrogen bonding to side chain and backbone atoms, respectively. Blue 'clouds' on ligand atoms indicate the solvent exposed surface area of ligand atoms. Light-blue 'halos' around residues indicate the degree of interaction with ligand atoms. The dotted contour reflects steric room for methyl substitution.

Figure 6 depicts the docking model of **22** with DNMT3A. Similar to Figure 5, a 3D model is shown along with a 2D-interaction diagram. In this docking model, the α-carboxylate group makes a hydrogen bond with the side chains of Arg887 but it does not make interactions with the catalytic Cys662. Similar to the binding model with DNMT1, the docking results suggest that **22** could inhibit DNMT3A by blocking the substrate binding site. In both models, with DNMT1 and DNMT3A, the two carboxylate groups are involved in the formation of hydrogen bonds with residues in the binding site. This result is in overall agreement the SAR discussed above.

Insert Figure 6

Figure 6. Docking model of compound **22** with the catalytic domain of human DNMT3A. The 3D and 2D-interaction map show selected amino acid residues of the binding site. Non-polar hydrogen atoms are omitted for clarity. In the 2D-interaction map the color coding and symbols are as in Figure 5.

*Stability of compound **22** under physiological conditions and in human serum*

In view of future in vivo studies, the chemical stability of **22** under physiological conditions and in human serum was checked. In separate experiments, compound **22** was incubated at 37 °C for 48 h in pH 7.4 phosphate-buffered solution and in human serum (Figure S2 in the Supporting Information) at the concentration of 2 mg/mL. The stability was monitored for 48 h by measuring the compound concentration at different time intervals via RP-UHPLC. Compound **22** was completely stable in both the conditions tested.

3. Conclusions

Inspired by the structure of the validated hit compound NSC137546, in this work we synthesized a series of *N*-benzoyl amino acids and explored their ability to inhibit DNMT-dependent total DNA methylation. The SAR derived from this study indicated that the *N*-benzoyl substituted *L*-glutamic acid scaffold is the minimal requirement for the activity of this class of benzamide derivatives. Ortho halogeno-substituted compounds **22**, **23**, and **24** emerged as the most interesting hits. In particular, compound **22** inhibited both DNMT1- and DNMT3A-mediated DNA-methylation in a concentration-dependent manner and proved active on both DNMT1 and DNMT3A isolated by immunoprecipitation. Docking studies suggest putative binding to the substrate site of both DNMT isoforms studied. Compound **22** proved to be stable under physiological conditions in human serum and it is currently being tested in different models of cardiac fibrosis. Results of these studies will be reported in due course.

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Supplementary data

Additional Supporting Information may be found in the online version of this article:

Full characterization of compounds (^{1}H and ^{13}C-NMR spectra of compounds **1-9**, **17**, **20**, **23**, **25-27**. COSY and HMBC-NMR spectra of compounds **7** and **8**), along with detailed experimental procedures, is given in supplementary file.

Table S1: Ability of compounds **1**, **22**, **24**, and reference compound RG108 to inhibit DNA methylation in cell lysate selectively overexpressing DNMT3B.

Figure S2: Stability of compound **22** in human serum.

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