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Original article

New cytosine derivatives as inhibitors of DNA methylation

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ABSTRACT

DNA cytosine methylation catalyzed by DNA methyltransferase 1 (DNMT1) is an epigenetic method of gene expression regulation and development. Changes in methylation pattern lead to carcinogenesis. Inhibition of DNMT1 activity could be a good strategy of safe and efficient epigenetic therapy. In this work, we present a novel group of cytosine analogs as inhibitors of DNA methylation. We show new methods of synthesis and their effect on *in vitro* reaction of DNA methylation. Almost all of analyzed compounds inhibit DNA methyltransferase activity in the competitive manner. K_i values for the most potent compound 4-N-furfuryl-5,6-dihydroazacytosines is 0.7 μM. These compounds cause also a decrease of 5-methylcytosine (m^5C) level in DNA of mammalian HeLa and HEK293 cells.

1. Introduction

The expression of the genetic information is regulated through epigenetic mechanisms such as methylation of cytosine in DNA, modifications of histones and noncoding RNAs. Methylation of cytosine residues at C-5 position is catalyzed by DNA methyltransferases [1]. In eukaryotic DNA about 3–8% of all cytosine residues are methylated. This modification occurs predominately at 5′-CpG-3′ dinucleotides that are methylated up to *ca.* 70%. Aberrations in specific DNA methylation pattern are among the main reasons of cancer development [2–5]. The crucial enzyme in this process is DNA methyltransferase 1 (DNMT1) that maintains an altered methylation pattern by copying it from a parent to a daughter DNA strand after replication [3]. Correlation of hypermethylation of promoter regions with transcriptional silencing of tumor suppressor genes (TSG) in carcinogenesis is very well established [6]. Inactivation of DNMT1 may lead to a passive demethylation of promoter and in a consequence might restore an expression of TSG and inhibit tumorogenesis [2,7,8]. Thus, down regulation of DNMT1 seems to be a promising strategy in the anticancer therapy.

Until now several DNMT1 inhibitors have been identified. They can be divided into four groups. The first one includes cytidine analogs like 5-azacytidine, 5-aza-2-deoxycytidine and zebularine [6,9]. After phosphorylation they are incorporated into newly synthesized DNA at cytosine sites [10]. The lack of the proton at position 5 of these cytosine derivatives stabilizes the covalent bond between DNA and enzyme (Fig. 1). Thus, the trapped DNMT1 molecules are excluded from the pool of active enzymes and formation of these covalent protein–DNA complexes is considered as a reason of a high toxicity of those inhibitors [6]. This leads to a decrease in the level of methylation of replicated DNA [9]. It has been suggested that 5-azacytidine affects also other cellular processes via altering expression of genes which promoters were not regulated by DNA methylation prior to the treatment [11]. The another group of DNMT1 inhibitors includes small non-nucleoside natural compounds, like EGCG (epigallocatechin-3-gallate) and curcumin however, it has been suggested that the observed inhibition of DNMT1 might be effected by free radicals formed during degradation of EGCG rather than its direct interaction with enzyme [12–14]. Another compound of that group, phthalimide RG108 functions as a competitive inhibitor and binds directly to DNMT1 active site [15]. A broad application of RG108 is however limited due to its high hydrophobicity [16]. The third group of DNMT1 inhibitors consists of SAM (S-adenosyl-L-methionine) analogs lacking the methyl group [17]. One of them, sinefungin, increases the rate of m^5C deamination to thymine [18]. Finally, the fourth

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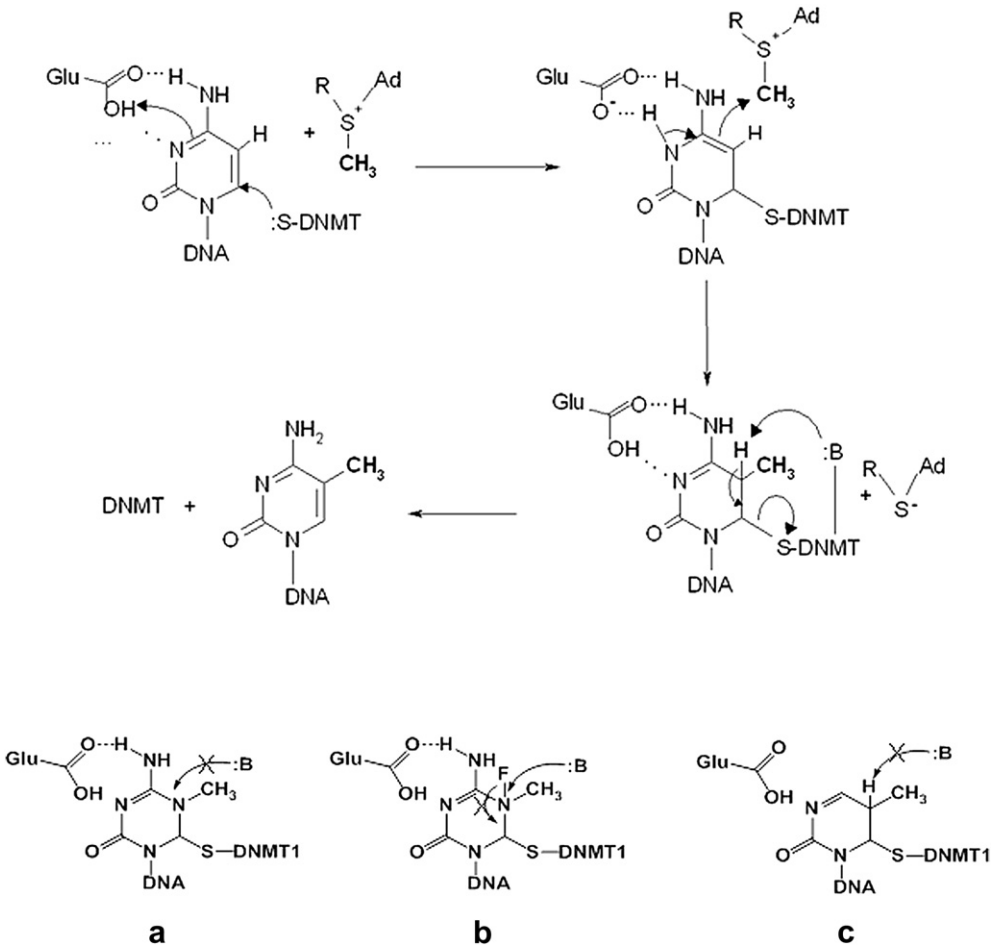


Fig. 1. Mechanism of reaction catalyzed by cytosine C5 DNA methyltransferase. The target cytosine in DNA interacts with active site residues (I) of DNMT1 to facilitate cysteine nucleophilic attack at the C6 position. Nucleophilic attack disrupts the pyrimidine's aromaticity, generating the reactive covalent adduct (II) which readily undergo electrophilic addition of methyl group through methylation (III). Finally deprotonation of position C5 leads to restoration of C–C double bond due to β-elimination (IV). Lack of proton at position 5 of 5-aza-dC (a) as well as the presence of a poor leaving group in 5-F-dC (b) or lack of amino group at C4 in zebularine (c) prevent the restoration of double bond.

group of inhibitors consists of procaine and its derivative procainamide. Through binding to CpG-rich sequences these compounds induce a spatial hindrance preventing DNA methylation [17,19–21].

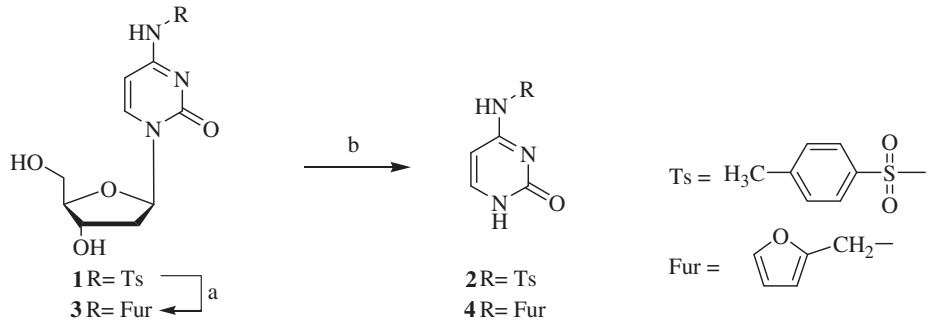
The persistent shortage of effective anticancer drugs makes searching for new inhibitors of DNMT1 a widely recognized challenge. The aim of our study was to find a new, effective and non-toxic inhibitors of the enzyme, which could be used in anti-cancer therapy.

We focused our interest on the new analog of cytosine, 4-N-furfurylcytosine (FC), that has been found by us in DNA [manuscript in preparation]. It is a product of cellular oxidation of DNA in which furfural moiety resulted from hydroxylation at the carbon 5′ of deoxyribose units of DNA reacts with N-4 of cytosine residues nearby [22–24]. We considered FC as a leading compound for searching inhibitors of DNA methyltransferase. Here we show that 4-N-furfurylcytosine and its derivatives inhibit reaction of DNA methylation. In addition to that, for all compounds either new methods of synthesis or modifications of earlier published procedures are presented.

2. Chemistry

A series of cytosine derivatives was synthesized using two approaches. In the first one a nucleoside was used as a substrate in which the sugar residue is treated as a "protecting group" of nucleobase (**1**, **3**, **27**, **28**) (Schemes 1 and 5). Thus, one can take an advantage of plethora of synthetic procedures that lead to nucleoside modifications. After an acid-catalyzed hydrolysis of N-nucleosidic bond the following nucleobases: **2**, **4**, **29**, **30** (Schemes 1 and 5) were obtained [25]. Synthesis of cytosine derivatives starting from 4-N-p-toluenesulfonyl-2′-deoxycytidine (**1**) was carried out according to procedure described earlier [26]. The reaction of **1** with a series of primary alkyl amines carried out at higher temperature (70 °C) in pyridine gave expected cytidine derivatives in very high yield (Scheme 1). 4-N-substituted cytosines: **2**, **4** (Scheme 1) were obtained by the cleavage of N-glycosidic bond in **1** or **3** (Scheme 1) with hydrochloric acid in refluxing methanol [25].

The second approach was based on reaction of cytosine with aldehydes leading to formation of Schiff base derivatives followed by their reduction with sodium borohydride in methanol (Scheme 2). This method had been already described [27,28] but we have used other conditions of this reaction. With this approach we received many compounds in short time without protecting of cytosine at N-1 position step. The reaction of cytosine with 'aromatic' aldehydes was found to proceed smoothly and quantitatively in anhydrous methanol in the presence of magnesium methanolate as water 'consumer' (**4**–**9**, **24**, **25**) (Schemes 2 and 4). In the case of alkyl aldehyde as a substrate, the reaction was found to be efficiently catalyzed by acetic acid (**10**–**12**) (Scheme 2). The use of basic conditions (methanolate) for alkyl aldehydes led to condensation products. The Schiff bases were reduced either with



Scheme 1. Synthesis of cytosine derivatives via acid-catalyzed hydrolysis N-glycosidic bond of nucleosides. Reagents and reaction conditions: (a) furfurylamine (5 equiv), pyridine, 70 °C, 12 h; (b) HCl (2.4 equiv) in MeOH aq., reflux, 4 h.

sodium borohydride or borane dimethyl sulfide complex to corresponding products: **4**–**9**, **13**, **14**, **24**, **25** (Schemes 2 and 4).

Modification of 4-N-furfurylcytosine (**4**) and 4-N-benzylcytosine (**7**) at C-5 position with paraformaldehyde done in the presence of triethylamine led to synthesis of 5-hydroxymethyl-4-N-furfurylcytosine (**15**) in high yield and 5-hydroxymethyl-4-N-benzylcytosine (**16**) in lower yield (Scheme 3) [29]. These compounds were used furthermore as substrates in synthesis of a few other new compounds (**17**–**23**).

Acetylation of **15** and **16** with acetic anhydride gave 5-acetyloxymethyl derivatives **17** and **18** respectively (Scheme 5). The reaction of **15** and **16** with sodium metabisulfite in water led to formation of 5-methyl sulphonic acid derivatives **19** and **20** respectively in good yields (Scheme 3).

The 5-hydroxymethyl group of **15** and **16** could be oxidized with manganese dioxide in water giving appropriate 5-formyl derivatives **21** and **22** (Scheme 3) in moderate yields. The derived aldehydes could be then used to obtain Schiff bases in reaction with primary amines. Thus, reaction of **21** with n-butylamine (Scheme 3) followed by reduction with sodium borohydride gave 5-(N-butyloamine)methyl-4-N-furfurylcytosine (**23**).

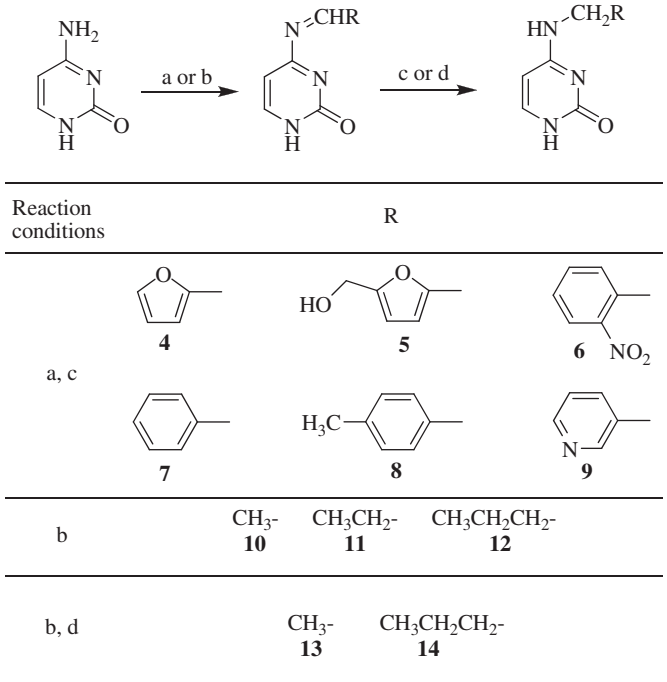
On the other hand, 5-hydroxymethyl-4-N-furfurylcytosine (**15**) was transformed into a methanesulfonyl ester with methanesulfonyl chloride in pyridine and subsequently reacted with amine to obtain **23** in high yield (Scheme 3).

Another approach was applied for synthesis of 4-N-furfuryl-5-methylcytosine (**29**) and 4-N-benzyl-5-methylcytosine (**30**). 5-Methylcytosine and its nucleosides are rather expensive although commercially available. Thus, we have taken advantage of using 3′,5′-di-O-acetylthymidine and transforming it into 5-methyl-4-(1,2,4-triazol-1-yl)-1-(β-D-3,5-di-O-acetyl-2-deoxyribofuranosyl)pyrimidin-2(1H)-one (**26**) [30–32]. Reaction of triazole nucleoside **26** and furfurylamine or benzylamine resulted in 5-methyl-4-N-furfuryl-2′-deoxycytidine (**27**) and appropriate 5-methyl-4-N-benzyl-2′-deoxycytidine (**28**) and these could be easily transformed into desired 5-methyl-4-N-furfurylcytosine (**29**) and 5-methyl-4-N-benzylcytosine (**30**) after cleavage of N-nucleoside bond (Scheme 5) [25].

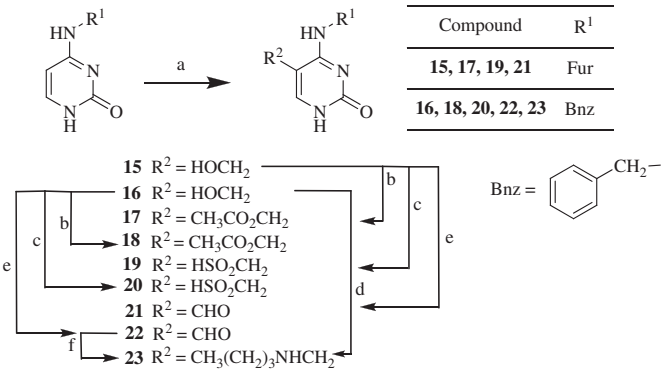
3. Results

3.1. Inhibitory activity of cytosine analogs in in vitro DNA methylation assay

Inhibitory activity of each compound was analyzed in *in vitro* DNA methylation reaction catalyzed by DNA methyltransferase from *Spiroplazma*. Sequence homology between human DNMT1 (Gen Bank Acc no X63692) and M.SssI (X17195.1) is low. However



Scheme 2. Synthesis of cytosine derivatives via acidic/basic route. Reagents and reaction conditions: (a) $Mg(MeO)_2$ (5 equiv), aromatic aldehyde (6 equiv), MeOH, 55 °C, 3 h; (b) AcOH (5 equiv), CH_3COH or CH_3CH_2COH or $CH_3CH_2CH_2COH$ (4 equiv), MeOH, reflux, 3 h; (c) $NaBH_4$ (9 equiv), 0.5 h, MeOH, rt; (d) 1) 2 equiv BH_3-DMS, THF, CH_2Cl_2, 12 h. 2) HCl, MeOH aq.



Scheme 3. Synthesis of 4-N-furfurylcytosine and 4-N-benzylcytosine derivatives with modification at 5-C position. Reagents and reaction conditions: (a) CH_2O (2.3 equiv), Et_3N (10 equiv), H_2O, 65 °C, 12 h; (b) Ac_2O (1.2 equiv), Py, rt, 3 h; (c) sodium metabisulfite (2 equiv), water, 55 °C, 24 h; (d) 1. Py, MsCl (1.5 equiv), rt, 0.5 h; 2. n-butylamine (3 equiv), 55 °C, 1 h; (e) water, MnO_2 (3 equiv), 55 °C, 24 h; (f) 1), n-Butylamine (5 equiv), HCl (2.5 equiv), MeOH, reflux, 8 h; 2) $NaBH_4$ (2 equiv), reflux, 1 h.

superposition of crystal structure of mouse DNMT1 (sequence homology of mouse AF162282 and human X63692 DNMT1 is 82%) and M.HhaI in their DNA-bound complexes shows that five out of six conserved sequence motifs of their catalytic domain adopt very similar conformation [33]. Moreover comparison of a modeled tertiary structure of M.SssI with M.HhaI shows that amino acid residues which take part in cofactor binding, target recognition and catalysis occupy the same position in three dimensional structure despite a sequence heterogeneity of the genes [33–36]. Based on those findings we have used DNA methyltransferase M.SssI as a model system for analysis of DNA methylation inhibitors.

Our cytosine derivatives were divided into three groups according to modification at exocyclic amino group. The first group consists of furfuryl derivatives of cytosine (Table 1). K_i for 4-N-furfurylcytosine (**4**) was 70 μM. This compound was further modified in different ways. Substitution of furfuryl moiety of **4** with hydroxymethyl group (**5**) caused tenfold increase of K_i. Introduction of methyl group at C-5 position (**29**) resulted in 4.6 times increase of inhibitory potency while addition of hydroxymethyl group (**15**) resulted in decreasing of inhibitory potency (30 times) relative to activity of **29**. The presence of 2′-deoxyribose (**3**, **27**) increased K_i values.

Substitution at C-5 with acetoxymethyl group (**17**) slightly (1.5 times) decreases K_i value comparing to leading compound. Exchange of 5-(N-butyloamine)methyl group in **23** into formyl group (**21**) or modification with sulfonyl group (**19**) resulted in decreasing of inhibitory activity. The highest value of K_i of all analyzed compounds which is 0.7 μM for 4-N-furfuryl-5,6-dihydro-5-azacytosine (**24**): is 100 times lower than K_i of a starting compound **4**. The increase of the hydrophobicity of compound **4** caused by benzyl moiety (**7**) (Table 2) enhances its inhibitory potential sevenfold. However **7** is less active than **24**. Similarly by changes of furfuryl (**24**) with benzyl group in **25** did not improve the activity. Substitution of benzyl moiety in **7** with methyl group in *para* position (**8**) resulted in 2.6 fold increase of K_i. Changes of benzyl into 3-picolyl group (**9**) resulted in decrease of the inhibitory activity. Among benzyl derivatives the most active compound is **30**. Comparison of furfuryl and benzyl derivatives (**16**, **18**, **20**, **22**) shows that these substituted with benzyl group are more potent, but still their K_i values are higher than observed for **24**. Within this group the derivative **6** is noncompetitive inhibitor.

The third group consists of derivatives substituted with aliphatic chains at 4-N (Table 3). All of them act as uncompetitive inhibitors as determined from Dixon plot [37]. In these cases K_i' values were determined by Cornish-Bowden method where S/V (concentration of substrate *vs* velocity of reaction) were plotted against I (concentration of inhibitor) concentrations [38]. Because K_i' values were very high these compounds were not investigated further. For comparison 5-aza-cytosine (**31**), RG108 (**32**) and EGCG (**33**) inhibit the activity of SssI in our test with K_i – 5500, 0.23 and 0.028 μM, respectively (Table 1).

3.2. Inhibition of human DNMT1 activity in colorimetric assay

The most potent inhibitor of M.SssI from furfuryl derivative of cytosine is compound **24** ($K_i = 0.7$ μM; $IC_{50} = 2.73$ μM). In order to check its inhibitory potency in reaction catalyzed with human DNMT1 we used non radioactive *in vitro* enzymatic assay. Analysis was carried out in presence of 5 μg of nuclear extract and 10, 100 and 500 μM concentration compound **24**. Under assay conditions **24** shows dose-dependent activity and inhibit of human DNMT1 with $IC_{50} = 22$ μM. In concentration of 10 μM **24** inhibits 55% of human DNMT1 activity. Because one of the known best inhibitors, RG108, was shown to inhibit only 11% of DNMT1 at concentration of 100 μM [39], to compare our results we used EGCG. In our experiment EGCG inhibit 42% of human DNMT1 at the same concentration as **24** (10 μM). In DNMT1 assay both compounds are less potent than in M.SssI inhibition assay (Fig. 2).

3.3. Effect of test compounds on DNA methylation of different cell lines

HeLa and HEK293 cells were treated with compounds **7**, **24**, **29**, to study their influence on the global level of DNA methylation. Genomic DNA was isolated, digested into nucleotides and labeled with [γ-^{32}P]ATP. The mixture of nucleotides was analyzed by two-dimensional thin layer chromatography on cellulose coated plates (Fig. 3a). Amount of m^5C was calculated according to equation: $R = m^5C/(m^5C + dC + dT) \times 100$ [40]. The compounds **7**, **24** and **29** reduce m^5C level in DNA of HeLa and HEK293 cells (Fig. 3b). IC_{50} values (concentration of inhibitor which causes 50% decrease in methylation level) for these compounds determined for HeLa cells were 31, 26 and 88 μM, respectively. For HEK293 IC_{50} for compounds **7**, **24** and **29** were above 100 μM, whereas for 4-N-furfuryl-5,6-dihydro-5-azacytosine this value is lower (75 μM).

Detailed analysis of the products of hydrolysis of DNA isolated from cell growing in the presence of inhibitors did not show detectable amount of analyzed compounds (Fig. 3a).

4. Discussion

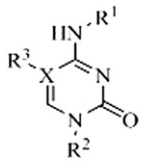
Until now the best DNMT1 inhibitor which is being used in clinical practice is 5-azacytidine, which is a simple derivative of the substrate. However, that compound is too toxic to meet the demands of modern medicine [41]. Therefore we have been looking for a new analogs of the substrate of DNA methylation. We chose N-4-furfurylcytosine as a starting compound.

Analysis of the crystal structure of DNA methyltransferase HhaI showed that the cytosine binding site is located in largely hydrophobic pocket composed of polar amino acids residues [42]. Thus, to fulfill the prerequisite that DNMT1 inhibitors should be a hydrophobic derivative of a substrate, we used 4-N-furfurylcytosine as a starting compound. Recently we have found that it occurs naturally in DNA from calf thymus [manuscript in preparation]. Plausible mechanism of its formation by the intramolecular rearrangement of oxidatively modified deoxyribose at C-5′, has been already proposed for kinetin, another furfural derivative [22–24].

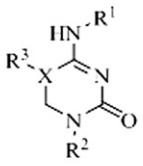
The inhibitors which directly interact with DNMT1 enzyme are expected to be less toxic comparing to those which are incorporated into the DNA like 5-azacytidine, 5-aza-2′-deoxycytidine and zebularine and form DNA–protein adduct through the formation of covalent bond with the enzyme [1]. Dixon plot analysis showed that almost all compounds act as competitive inhibitors, with exception of compounds **6** and **10**–**14** which are noncompetitive and uncompetitive ones, respectively. In our *in vitro* assay, analyzed compounds show significant inhibition of the enzyme activity without being incorporated into DNA chain, in contrast to 5-azacytidine which is active only when the inhibitor is within the DNA chain. In our test 5-aza-cytosine inhibit SssI activity with K_i 5500 μM, while K_i for RG108 is 0.23 μM. It means that cytosine derivative might effectively inhibit methyltransferase activity through direct interaction with the enzyme.

Inhibitory activity of N-4-furfuryl-5,6-dihydro-5-azacytosine (**24**) is 3 times weaker than – RG108. However **24** an analog of 5,6-dihydro-5-azacytosine with saturated pyrimidine double bond between N-5 and C-6 forms reversible complex with DNMT1 and reduces its toxicity [43]. To check its activity against human DNMT1, additional enzymatic assay was performed. In this assay we used nuclear extract from HeLa cells and hemimethylated DNA

Table 1
Inhibition of SssI enzyme in DNA methylation *in vitro* assay by furfuryl and benzyl derivatives of cytosine.

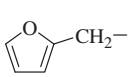
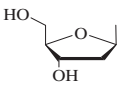
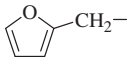
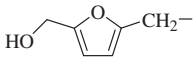
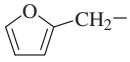
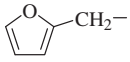
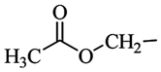
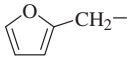
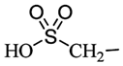
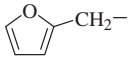
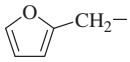
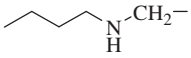
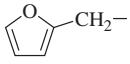
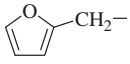
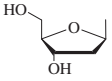
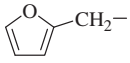


3-23, 26-30



24, 25

.

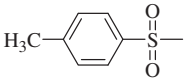
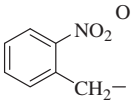
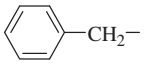
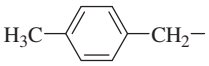
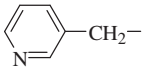
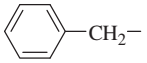
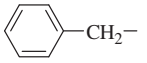
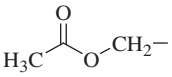
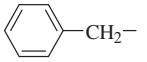
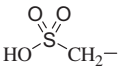
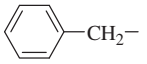
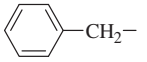
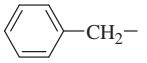
Compound	R^1	R^2	R^3	X	K_i (μM)[a]	M.W. (Da)	H-bond acceptor/donor atom[d]	*c* Log *P*[b]	tPSA (Å)[c]
3			H	C	170	307	8/3	−0.22	103.6
4		H	H	C	70	191	5/2	0.3	62.7
5		H	H	C	700	221	6/3	−0.22	82.9
15		H	$HOCH_2$–	C	440	221	6/3	−0.41	82.9
17		H		C	50	263	7/2	−0.18	89
19		H		C	112	285	8/3	−0.96	117
21		H	HCO–	C	83	219	6/2	−0.55	79.7
23		H		C	960	276	6/3	0.97	74.7
24		H	H	N	0.7	194	6/3	0.53	74.7
27			CH_3–	C	250	321	8/3	0.13	103.6
29		H	CH_3–	C	15	205	5/2	0.65	62.7
31	H	H	–	N	5500	112	5/3	−1.24	84.6
32		RG108			0.23	334	6/2	2.7	92.1
33		EGCG			0.028	458	11/8	2.2	197.3

[a] All derivatives are competitive inhibitors with K_i measured according to Dixon plot.
[b] *c* Log *P* is calculated with ChemDraw Ultra 11.0 programme.
[c] tPSA (topological polar surface area) is calculated with ChemDraw Ultra 11.0 programme.
[d] The numbers of Lipinski's H-bond acceptors/donors were calculated with Discovery Studio 2.5.

as a substrate. N-4-furfuryl-5,6-dihydro-5-azacytosine (**24**) inhibits human DNMT1 with $IC_{50} = 22$ μM, this result is higher than the one observed in the case of SssI ($IC_{50} = 2.72$ μM) but in the experiment with human DNMT1 we could not precisely determine neither the concentration of DNMT1 (only total amount of protein was measured) nor DNA substrate, which makes impossible to determinate the K_i value.

Analysis of the effect of these three potent compounds on DNA methylation in cell lines (**7**, **24**, **29**) (Schemes 2, 4 and 5) showed that they reduce the methylation level in HEK293 and much more

Table 2
Inhibition of SssI enzyme in DNA methylation *in vitro* assay by benzyl derivatives of cytosine.

Compound	R^1	R^2	R^3	X	K_i (μM)[a]	M.W. (Da)	H-bond acceptor/donor atoms[d]	*c* Log *P*[b]	tPSA (Å)[c]
2		H	H	C	550	265	6/2	1.45	87.6
6		H	H	C	6400	246	7/2	0.74	105.3
7		H	H	C	10	201	4/2	1.68	53.4
8		H	H	C	26	215	4/2	2.17	53.4
9		H	H	C	400	202	5/2	0.35	65.8
16		H	$HOCH_2$–	H	135	231	5/3	0.97	73.7
18		H		C	23	273	6/2	1.2	79.7
20		H		C	33	295	7/3	0.43	107.8
22		H	HCO–	C	35	229	5/2	0.84	70.5
25		H	H	N	20	204	5/3	1.91	65.5
30		H	CH_3–	C	3.6	215	4/2	2	53.4

[a] Almost all derivatives are competitive inhibitors with K_i measured according to Dixon plot. Compound **6** is noncompetitive inhibitor.
[b] *c* Log *P* is measured as indicated above.
[c] tPSA is measured as indicated above.
[d] Numbers of Lipinski's H-bond acceptors/donors are measured as indicated above.

in HeLa cells. Having in mind that copying of the methylation pattern follows replication process, one can expect that increased rate of cancer cells divisions will result in greater reduction of DNA methylation in comparison to normal cells after treatment with these inhibitors.

All presented compounds were checked to fulfill Lipinski's rules [44]. According to them the active drug should have not more than 5 hydrogen bond donors and not more than 10 hydrogen bond acceptors, M_w lower than 500 Da and log *P* (octanol–water partition coefficient) less than 5 [44]. Therefore a perfect inhibitor of DNMT1 should be a small molecule, able to bind specifically to catalytic domain of enzyme and inhibit its activity without any side effects [20]. All analyzed compounds clearly fulfill the Lipinski's rules and have less than 5 and 10 hydrogen bond donors and acceptors, respectively. Their molecular weight and *c* log *P* (calculated log *P*) are less than 500 Da and 5, respectively (Table 1).

Table 3
Inhibition of SssI enzyme in DNA methylation *in vitro* assay by aliphatic derivatives of cytosine.

Compound	R^1	R^2	R^3	X	K_i (μM)[a]	M.W. (Da)	H-bond acceptor/donor atoms[d]	*c* Log *P*[b]	tPSA (Å)[c]
10	CH_3CH–	H	H	C	5600	137	4/1	−0.17	53.8
11	CH_3CH_2CH–	H	H	C	3400	151	4/1	0.48	53.8
12	$CH_3CH_2CH_2CH$–	H	H	C	800	165	4/1	0.9	63.8
13	CH_3CH_2–	H	H	C	6000	139	4/2	0.15	53.4
14	$CH_3CH_2CH_2$–	H	H	C	1000	167	4/2	1.05	53.4

[a] All derivatives are uncompetitive inhibitors with K_i measured according to Cornish-Bowden's plot.
[b] *c* Log *P* is measured as indicated above.
[c] tPSA is measured as indicated above.
[d] Numbers of Lipinski's H-bond acceptors/donors are measured as indicated above.

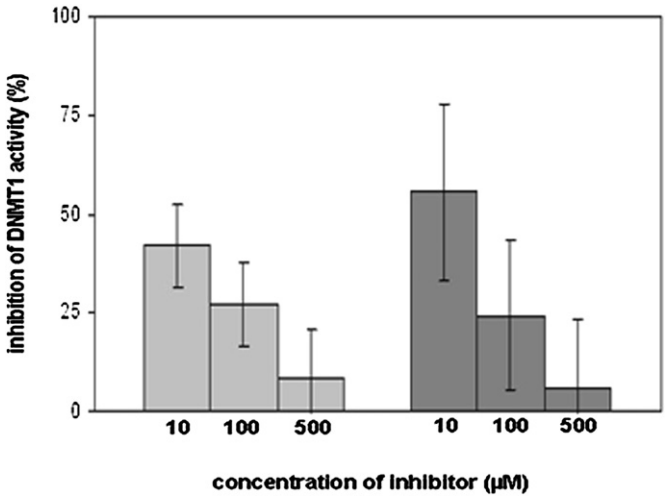


Fig. 2. Plot of DNMT1 activity in the presence of different concentration of inhibitors: N-4-furfuryl-5,6-dihydro-5-azacytosine (**24**) and EGCG (**33**). Percentage of change in enzymatic activity was determined in relation to DNMT1 without inhibitor. Light gray bars – EGCG, dark gray bars – compound **24**.

Similarly, calculated tPSA (topological polar surface area) for all compounds is less than 140 Å^2 what is necessary for efficient transport through the membranes. Moreover, tPSA values lower than 60 Å^2 as calculated i.e. for **7**, **8**, **30** (Schemes 2 and 5) and indicates the possibility of crossing the blood–brain barrier. The analyzed derivatives fulfill also a criterion of aromaticity which is extremely important for bioavailability and drug–target interaction via van der Waals forces or π-stacking. A number of aromatic rings larger than two, reduce hydrophilicity and lead to a poor drug distribution within the body [45]. Moreover, our cytosine derivatives take advantage of being small molecules which can be used in a rapid, dose-dependent and reversible manner [45].

In conclusion, we showed a new family of cytosine derivatives having a big potential to inhibit DNA methyltransferase activity and methods of their synthesis.

5. Experimental section

5.1. Chemistry

All reagents were commercially available or their synthesis has been described [26]. Solvents except of methanol were used without further purification. Reaction progresses were usually monitored with TLC using Merck silica gel 60 F254 plates and UV light at 254 nm. All chromatographic purifications were carried out on the silica gel 60 0.0063–0.200 mm (Merck) and on the silica gel 60 0.040–0.063 mm or on the silanized silica gel 60 0.063–0.200 mm (Merck). ^{1}H NMR (300 MHz) spectra were recorded on a Varian 300 MHz spectrometer using DMSO-d_6 as a solvent. ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer or a Varian 300 MHz spectrometer using DMSO-d_6, D_2O-d_2 or MeOH-d_4 as solvents. Tetramethylsilane (TMS) was used as an internal standard. Mass spectra (EMS) were measured with ESI Micro Q-TOF (Bruker).

5.1.1. 4-N-p-Toluenesulfonylcytosine (**2**)

To the flask containing 4-N-toluenesulfonyl-2′-deoxycytidine (**1**) (495 mg, 1.3 mmol) acetonitrile (10 mL), methanol (2 mL) and hydrochloric acid (0.26 mL, 3.12 mmol, 2.4 equiv) were added. The reaction was refluxed for 3 h. After that the pH was adjusted with KOH (175 mg, 3.12 mmol, 2.4 equiv) in methanol (10 mL), and the solvents were evaporated. The dry residue was dissolved with water. Product was extracted with methylene chloride and cleaned with active carbon. Evaporation of organic solvents gave 4-N-p-toluenesulfonylcytosine (**2**) as a white powder.

Yield 78%, 269 mg. MS (ESI, pos.) *m/z*: 288 $[M + Na]^+$, 553 $[2M + Na]^+$. ^{1}H NMR: δ 2.4 (s, 3H, CH_3); 6.4 (d, $J = 7.5$ Hz, 1H, H-5); 7.4 (d, $J = 8.1$ Hz, 1H, H-6); 7.6 (d, $J = 7.5$ Hz, 2H, H-8, H-10); 7.7 (d, $J = 8.1$ Hz, 2H, H-9, H-11); 7.6 (s, 1H, NH-7). ^{13}C NMR (75 MHz, CD_3CN-d_3): δ 22.13; 98.28; 127.85; 131.16; 141.10; 145.08; 145.48; 153.70; 161.90.

5.1.2. 4-N-Furfuryl-2′-deoxycytidine (**3**)

4-N-p-Toluenesulfonyl-2′-deoxycytidine (**1**) [26] (1.1 g, 2.88 mmol, 1 equiv) and furfurylamine (1.4 g, 14.39 mmol, 5 equiv) were added to anhydrous pyridine (25 mL). The reaction flask was closed and kept in an oven at 80 °C for 12 h. Next, dichloromethane (25 mL) was added and the mixture was extracted with water (3 × 25 mL). The aqueous layer was evaporated under reduced pressure to give a product **3** as a white foam.

Yield 85%, 752 mg. MS (ESI, pos.) *m/z*: 308 $[M + H]^+$, 330 $[M + Na]^+$, 615 $[2M + H]^+$, 637 $[2M + Na]^+$. ^{1}H NMR: δ 1.9 (m, 1H, H-2′); 2.0 (m, 1H, H-2″); 3.5 (d, $J = 2.4$ Hz, 2H, H-5′); 3.7 (q, $J = 3.9$ Hz, $J = 6.8$ Hz, 1H, H-4′); 4.2 (q, $J = 3.2$ Hz, $J = 5.5$ Hz, 1H, H-3′); 4.5 (d, $J = 5.5$ Hz, 2H, H-8); 4.9 (m, 1H, 5′-OH); 5.2 (m, 1H, 3′-OH); 5.8 (d, $J = 7.4$ Hz, 1H, H-5); 6.1 (t, $J = 6.1$ Hz, 1H, H-1′); 6.3 (q, $J = 0.7$ Hz, $J = 3.2$ Hz, 1H, H-9); 6.4 (q, $J = 1.8$ Hz, $J = 3.2$ Hz, 1H, H-10); 7.3 (d, $J = 7.9$ Hz, 1H, H-6); 7.6 (q, $J = 0.7$ Hz, $J = 1.8$ Hz, 1H, H-11); 8.0 (t, $J = 5.3$ Hz, 1H, NH-7). ^{13}C NMR (75 MHz, DMSO-d_6): δ 36.42; 38.67; 61.34; 70.37; 84.89; 87.19; 94.41; 107.24; 110.48; 140.24; 142.23; 151.75; 154.92; 163.11.

5.1.3. 4-N-Furfurylcytosine (**4**)

4-N-Furfuryl-2′-deoxycytidine (**3**) (731 mg, 2.38 mmol, 1 equiv) was dissolved in water (18 mL) and concentrated hydrochloric acid (1.8 mL, 8.4 equiv) was added. The mixture was refluxed for *ca.* 1 h while the reaction mixture turned yellowish. After cooling to room temperature the pH of the mixture was adjusted to *ca.* 7 with 1 M aqueous solution of potassium hydroxide. Then the solvents were removed by evaporation under the reduced pressure. The 4-N-furfurylcytosine (**4**) was purified by silanized silica gel column chromatography and was eluted with up to 10% of the methanol in warm water (40–45 °C). Product was obtained as a white powder in yield 90%, 409 mg.

5.1.4. Derivatives of cytosine with aryl substituent at 4-N position

Cytosine (300 mg, 2.70 mmol, 1 equiv) and appropriate aromatic aldehyde (16.20 mmol, 6 equiv) were added to methanol (*ca.* 25 mL) containing magnesium methylate (1.2 g, 13.50 mmol, 5 equiv). The reaction was carried for 3 h at 55 °C. Then sodium borohydride (923 mg, 24.30 mmol, 9 equiv) was slowly added to the mixture and the reduction reaction was carried for 30 min at room temperature. After that, the pH of the mixture was adjusted to 7 with conc. HCl and then the solvents were removed by evaporation under the reduced pressure. Water (25 mL) was added to the residue and the mixture was extracted with ethyl acetate (25 mL). The organic layer was extracted with aqueous solution of hydrochloric acid (0.05 M, 25 mL). The combined aqueous extracts were neutralized with aq. potassium hydroxide (1 M, 1.3 mL) and extracted with ethyl acetate (25 mL). The organic layer was dried with magnesium sulfate and then evaporated under the reduced pressure to give a product as a white solid. The yields range: from 55 to 86%.

*5.1.4.1. 4-N-Furfurylcytosine (**4**).* Yield 56%, 289 mg. MS (ESI, pos.) *m/z*: 192 $[M + H]^+$, 214 $[M + Na]^+$, 230 $[M + K]^+$. ^{1}H NMR: δ 4.5 (d, $J = 5.4$ Hz, 2H, H-8); 5.6 (d, $J = 7.1$ Hz, 1H, H-5); 6.3 (q, $J = 0.7$ Hz, $J = 3.2$ Hz, 1H, H-9); 6.4 (q, $J = 1.9$ Hz, $J = 3.2$ Hz, 1H, H-10); 7.2 (d,

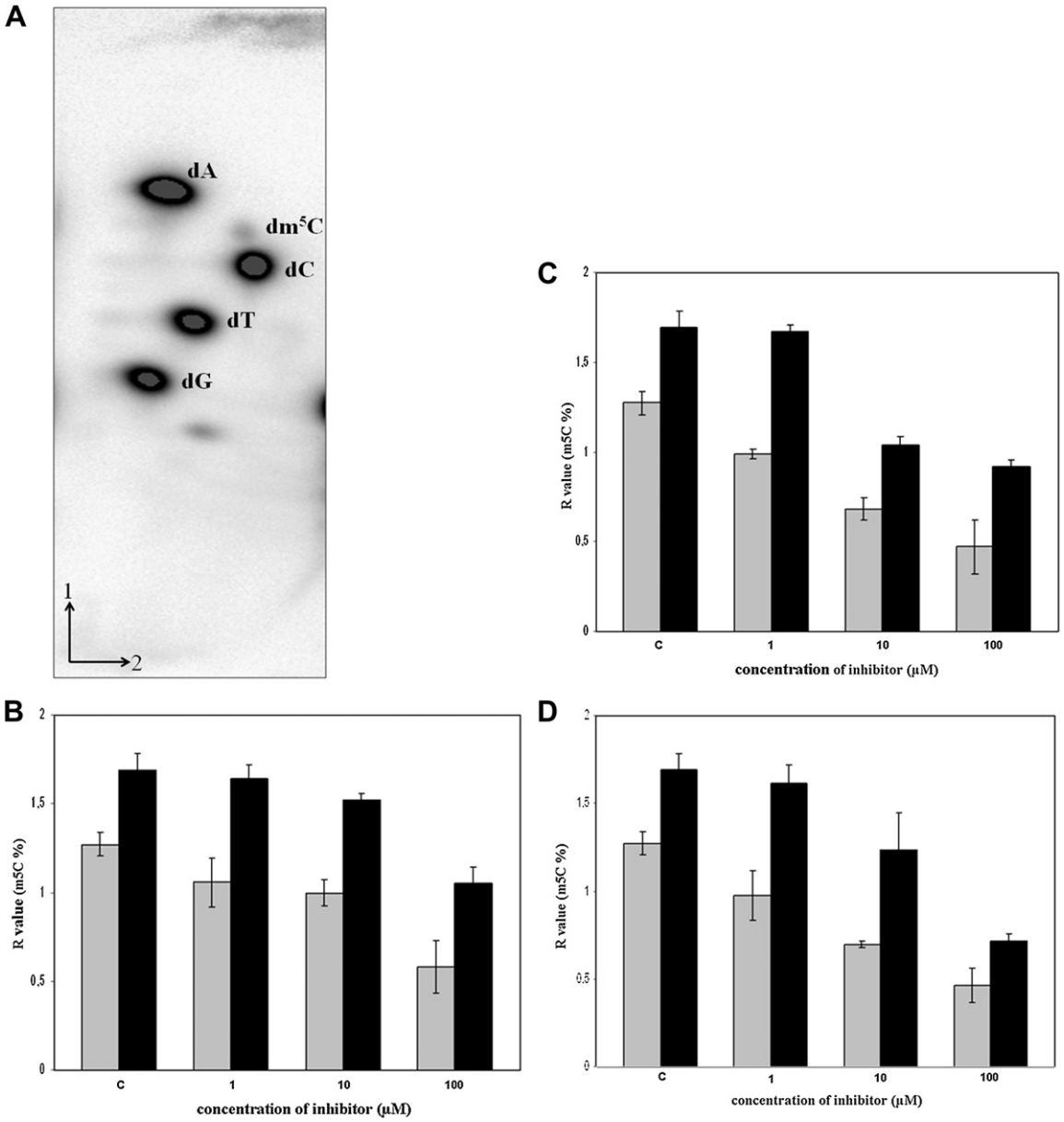
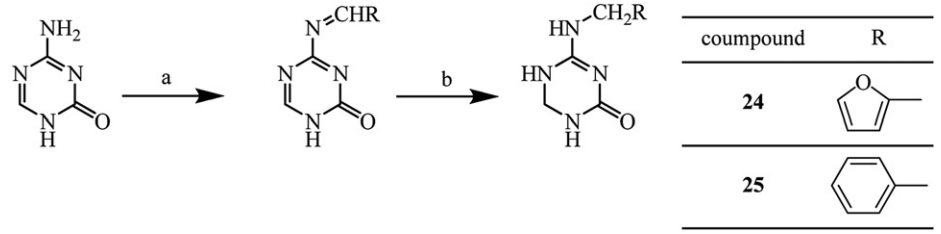


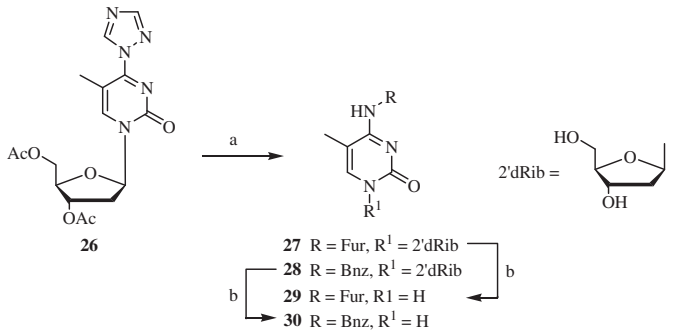
Fig. 3. Panel A, two-dimensional thin layer chromatography (TLC) analysis of [5′-^{32}P] labeled deoxynucleotides obtained by enzymatic hydrolysis from HeLa cell line treated with DNA methyltransferases activity inhibitor, dA – 2′-deoxyadenosine phosphate, dmC – 5-methyl-2′-deoxycytosine phosphate, dC – 2′-deoxycytosine phosphate, dT – 2′-deoxytymidine phosphate, dG – 2′-deoxyguanosine phosphate. A comparison of m^{5}C amount in DNA from HeLa (gray bars) and HEK293 cells (black bars) treated with various concentration (1, 10, 100 μM) of 4-furfuryl-5-methylcytosine (**28**) panel B, 4-N-benzylcytosine (**7**) panel C and 4-furfuryl-5,6-dihydroazacytosine (**24**) panel D.

J = 7.1, 1H, H-6); 7.6 (q, J = 0.7 Hz, J = 1.9 Hz, 1H, H-11); 7.9 (t, J = 5.6 Hz, 1H, NH-7); 10.3 (s, 1H, NH-1). ^{13}C NMR (75 MHz, DMSO-d_6): δ 40.33; 90.10; 107.16; 110.47; 141.82; 142.19; 151.96; 156.55; 164.29.

*5.1.4.2. 4-N-(5-Hydroxymethylfurfuryl)cytosine (**5**).* Yield 62%, 370 mg. MS (ESI, pos.) m/z: 222 $[M + H]^+$, 244 $[M + Na]^+$, 443 $[2M + H]^+$, 465 $[2M + Na]^+$. ^{1}H NMR: δ 4.3 (d, J = 7.2 Hz, 2H, H-11); 4.4 (d, J = 5.7 Hz, 2H, H-8); 5.2 (t, J = 7.6 Hz, 1H, OH); 5.6 (d,



Scheme 4. Synthesis of 5-azacytosine derivatives via basic route. Reagents and conditions: (a) $Mg(MeO)_2$ (5 equiv), aromatic aldehyde (6 equiv), MeOH, 55 °C, 3 h; (b) $NaBH_4$ (9 equiv), 0.5 h, MeOH, rt.



Scheme 5. Synthesis of 5-methylcytosine derivatives via route of acid-catalyzed hydrolysis of N-glycosidic bond. Reagents and reaction conditions: (a) 1) furfurylamine or benzylamine (1.5 equiv), CH_3CN, 50 °C; 2) MeOH, 35% aq. ammonia, 1 h; (b) conc. HCl (3 equiv), MeOH aq., reflux, 4 h.

J = 7.5 Hz, 1H, H-5); 6.1 (m, 2H, H-9, H-10); 8.1 (t, J = 5.5 Hz, 1H, NH-7); 10.3 (s, 1H, NH-9). ^{13}C NMR (100 MHz, DMSO-d_6): δ 40.33; 55.62; 93.24; 107.60; 107.75; 141.82; 151.17; 154.75; 156.67; 164.29.

*5.1.4.3. 4-N-o-Nitrobenzylocytosine (**6**).* Yield 54%, 359 mg. MS (ESI, pos.) m/z: 247 $[M + H]^+$, 269 $[M + Na]^+$, 285 $[M + K]^+$.

^{1}H NMR: δ 4.7 (d, J = 5.8 Hz, 2H, H-8); 5.8 (d, J = 7.3 Hz, 1H, H-5); 7.3 (d, J = 5.8 Hz, 1H, H-6); 7.6 (m, 2H, H-11, H-12); 7.8 (m, 2H, H-9, H-10); 8.1 (m, 1H, NH-7); 10.3 (s, 1H, H-1). ^{13}C NMR (100 MHz, DMSO-d_6): δ 42.58; 96.11; 125.94; 129.43; 131.33; 134.72; 135.12; 142.79; 149.74; 160.49; 166.97.

*5.1.4.4. 4-N-Benzylcytosine (**7**).* Yield 61%, 331 mg. MS (ESI, pos.) m/z: 202 $[M + H]^+$, 224 $[M + Na]^+$, 240 $[M + K]^+$, 403 $[2M + H]^+$, 425 $[2M + Na]^+$, 441 $[2M + K]^+$. ^{1}H NMR: δ 4.5 (d, J = 5.7 Hz, 2H, H-8); 5.7 (d, J = 7.1 Hz, 1H, H-5); 7.2–7.3 (m, 6H, H-6 and H of Ar); 8.0 (t, J = 5.9 Hz, 1H, NH-7); 10.3 (s, 1H, H-1). ^{13}C NMR (100 MHz, MeOD-d_4): δ 45.57; 96.75; 128.78; 129.36; 130.03; 130.44; 130.71; 140.24; 142.83; 161.19; 167.13.

*5.1.4.5. 4-N-p-Methylbenzylcytosine (**8**).* Yield 86%, 499 mg. MS (ESI, pos.) m/z: 216 $[M + H]^+$, 238 $[M + Na]^+$, 431 $[2M + H]^+$. ^{1}H NMR: δ 2.2 (s, 3H, CH_3); 4.5 (d, J = 5.6 Hz, 2H, H-8); 5.6 (d, J = 7.1 Hz, 1H, H-5); 7.2 (d, J = 6.8 Hz, 1H, H-6); 7.5 (m, 2H, H-9, H-10); 7.7 (m, 2H, H-11, H-12); 8.1 (t, J = 5.9 Hz, 1H, NH-7); 10.2 (s, 1-H, NH-1). ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.64; 42.62; 93.20; 127.39; 128.82; 135.87; 136.12; 141.62; 156.72; 164.45.

*5.1.4.6. 4-N-Picolylcytosine (**9**).* Yield 86%, 469 mg. MS (ESI, pos.) m/z: 202 [M]; 225 $[M + Na]^+$, 240 $[M + K]^+$.^{1}H NMR: δ 4.8 (d, J = 5.9 Hz, 2H, H-8); 5.8 (d, J = 7.3 Hz, 1H, H-5); 7.3 (d, J = 7.8 Hz, 1H, H-6); 7.4 (m, 1H, H-10); 7.7 (d, J = 1.5 Hz, 1H, H-9); 8.1 (t, J = 5.9 Hz, 1H, NH-7); 8.4 (m, 1H, H-11); 8.5 (d, J = 1.3 Hz, 1H, H-12); 10.3 (s, 1H, NH-1). ^{13}C NMR (100 MHz, DMSO-d_6): δ 40.57; 93.15; 123.44; 134.72; 135.21; 141.92; 148.10; 148.87; 156.56; 164.50.

5.1.5. Derivatives of cytosine with alkyl substituent at external amino group

Cytosine (400 mg, 3.60 mmol, 1 equiv) and alkyl aldehyde (14.40 mmol, 4 equiv) were added to methanol (*ca.* 35 mL) containing acetic acid (1 mL, 18.00 mmol, 5 equiv). The mixture was refluxed for 3 h. Then, the solvents were removed by evaporation under the reduced pressure. Then, products were isolated using reverse chromatography with mixture of water and acetone. The Schiff bases (2 mmol) were dissolved in methylene chloride (5 mL) and then were reduced with 2 M borane dimethyl sulfide complex in THF (2 mL, 4 mmol, 2 equiv) for 12 h at ambient temperature. After that to the reaction flask water (3 mL) and acetone (10 mL) were added. Then all solvents were evaporated and then conc. HCl (1 mL) in methanol (10 mL) and water (10 mL) were added to reaction mixture. The mixture was left for 12 h. Then the solvents were evaporated and product was separated by silanized silica gel column chromatography with mixture of water and acetone.

*5.1.5.1. 4-N-Ethylidenecytosine acetate (**10**).* Yield 92%, 652 mg. MS (ESI, pos.) m/z: 138 $[M + H]^+$; 170 $[M + MeOH + H]^+$; 192 $[M + MeOH + Na]^+$. ^{1}H NMR: δ 1.2 (d, J = 5.6 Hz, 3H, H-9); 3.1 (s, 3H, CH_3 of anion); 5.2 (q, J_A = 14.3 Hz, J_B = 6.2 Hz, 1H, H-8); 5.5 (d, J = 7.2 Hz, 1H, H-5); 7.2 (d, J = 7.2 Hz, 1H, H-6); 7.8 (d, J = 8.8 Hz, 1H, NH-7); 10.5 (s, 1H, NH-1). ^{13}C NMR (100 MHz, DMSO-d_6): δ 18.96; 34.82; 61.47; 87.73; 120.23; 148.98; 154.96; 180.14.

*5.1.5.2. 4-N-Propylidenecytosine acetate (**11**).* Yield 76–80%, 578–608 mg. MS (ESI, pos.) m/z: 152 $[M + H]^+$; 184 $[M + MeOH + H]^+$; 206 $[M + MeOH + Na]^+$. ^{1}H NMR: δ 0.8 (t, J = 7.6 Hz, 3H, H-10); 1.5 (m, 2H, H-9); 3.1 (s, 3H, CH_3 of anion); 5.2 (q, J_A = 14.8 Hz, J_B = 6.0 Hz, 1H, H-8); 5.5 (d, J = 6.8 Hz, 1H, H-5); 7.2 (d, J = 7.2 Hz, 1H, H-6); 7.7 (d, J = 9.2 Hz, 1H, NH-7); 10.5 (s, 1H, NH-1). ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.86; 22.02; 34.47; 54.60; 80.51; 92.91; 142.55; 156.58; 165.24; 174.66.

*5.1.5.3. 4-N-Butylidenecytosine acetate (**12**).* Yield 48–65%, 389–526 mg. MS (ESI, pos.) m/z: 166 $[M + H]^+$; 198 $[M + MeOH + H]^+$; 220 $[M + MeOH + Na]^+$. ^{1}H NMR: δ 0.8 (t, J = 7.3 Hz, 3H, H-11); 1.2 (m, 2H, H-10); 1.4 (m, 2H, H-9); 3.1 (s, 3H, CH_3 of anion); 5.4 (q, J_A = 15.1 Hz, J_B = 6.3 Hz, 1H, H-8); 5.5 (d, J = 6.8 Hz, 1H, H-5); 7.2 (d, J = 6.8 Hz, 1H, H-6); 7.8 (d, J = 8.7 Hz, 1H, NH-7); 10.5 (s, 1H, NH-1). ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.65; 17.79; 18.81; 34.66; 54.58; 80.23; 92.91; 142.52; 156.54; 165.54; 174.67.

*5.1.5.4. 4-N-Ethylcytosine (**13**).* Yield 57%, 158 mg. MS (ESI, pos.) m/z: 140 $[M + H]^+$; 162 $[M + Na]^+$; 301 $[2M + Na]^+$. ^{1}H NMR: δ 1.1 (t, J = 7.3 Hz, 3H, CH_3); 3.2 (m, 2H, H-8); 5.6 (d, J = 6.8 Hz, 1H, H-5); 7.4 (d, J = 6.8 Hz, 1H, H-6); 7.9 (s, 1H, NH-7); 10.5 (s, 1H, NH-1). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.57; 34.31; 93.25; 141.24; 156.85; 156.91; 164.30; 166.65.

*5.1.5.5. 4-N-Butylcytosine (**14**).* Yield 88%, 294 mg. MS (ESI, pos.) m/z: 168 $[M + H]^+$; 190 $[M + Na]^+$; 335 $[2M + H]^+$; 357 $[2M + Na]^+$. ^{1}H NMR: δ 0.9 (t, J = 7.2 Hz, 3H, CH_3); 1.3 (m, 2H, H-10, H-10′); 1.4 (m, 2H, H-9, H-9′); 3.2 (q, J_A = 12.4 Hz, J_B = 6.4 Hz, 2H, H-8); 5.6 (d, J = 7.2 Hz, 1H, H-5); 7.2 (d, J = 6.8 Hz, 1H, H-6); 7.5 (t, J = 4.8 Hz, 1H, NH-7); 10.2 (s, 1H, NH-1). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.12; 21.11; 32.15; 41.25; 96.31; 141.87; 166.71; 180.37.

5.1.6. Derivatives of 4-N-arylmethylcytosine with hydroxymethyl substituent at C-5

4-N-Arylmethylcytosine (**4**, **7**) (2.62 mmol, 1 equiv) and paraformaldehyde (180 mg, 6.02 mmol, 2.3 equiv) were added to water (*ca.* 25 mL) containing triethylamine (2.6 g, 26.00 mmol, 10 equiv). The mixture was refluxed for 8 h and then the flask was closed and kept in oven at 60 °C for 12 h. After that the solvents were removed by evaporation under the reduced pressure and ethanol (25 mL) was added to a residue causing to precipitate a white powder. The powder was filtered and dissolved in methanol (0.2–0.5 mL) and again precipitated with ethanol (25 mL). This procedure was repeated two more times.

*5.1.6.1. 4-N-Furfuryl-5-hydroxymethylcytosine (**15**).* Yield 85–95%, 492–550 mg. MS (ESI, pos.) m/z: 222 $[M + H]^+$; 244 $[M + Na]^+$; 465 $[2M + Na]^+$. ^{1}H NMR: δ 4.1 (d, J = 4.9 Hz, 2H, H-13); 4.5 (d, J = 5.3 Hz, 2H, H-8); 5.0 (t, J = 5.2 Hz, .1H, OH); 6.2 (m, 1H, H-12); 6.3

(m, 1H, H-11); 7.2 (t, $J = 5.2$ Hz, 1H, NH-7); 7.3 (s, 1H, H-6); 7.5 (m, 1H, H-10); 10.3 (s, 1H, NH-1). ^{13}C NMR (100 MHz, DMSO-d_6): δ 40.33; 57.13; 104.87; 106.88; 110.44; 140.02; 141.94; 152.27; 156.39; 163.08.

*5.1.6.2. 4-N-Benzyl-5-hydroxymethylcytosine (**16**).* Yield 80–90%, 484–545 mg. MS (ESI, pos.) *m/z*: 232 $[M + H]^+$;254 $[M + Na]^+$; 270 $[M + K]^+$; 485 $[2M + Na]^+$. ^{1}H NMR: δ 4.2 (d, $J = 4.8$ Hz, 2H, H-15); 4.5 (d, $J = 5.7$ Hz, 2H, H-8); 7.2–7.3 (m, 5H, Ar); 7.6 (s, 1H, H-6); 10.5 (s, 1H, NH-1). ^{13}C NMR (100 MHz, DMSO-d_6): δ 45.48; 57.15; 104.96; 126.82; 127.08; 127.39; 128.18; 128.31; 139.53; 141.70; 156.56; 163.22.

5.1.7. Acetylation of 4-N-arylmethyl-5-hydroxymethylcytosine derivatives

The 4-N-aryl-5-hydroxymethylcytosine (**15**, **16**) (0.45 mmol) and acetic anhydride (0.05 mL, 0.54 mmol, 1.2 equiv) were added to pyridine (0.2 mL, 2.71 mmol, 5 equiv). The reaction flask was kept at ambient temperature for 3 h. After that the water (0.5 mL) was added, and all solvents were evaporated. The dry residue was dissolved with water (15 mL). Product was extracted by ethyl acetate (3 × 15 mL). The organic part was dried by sodium sulfate, and then solvent was evaporated receiving clean, white powder product.

*5.1.7.1. 4-N-Furfuryl-5-acetyloxymethylcytosine (**17**).* Yield 90–95%, 107–113 mg. MS (ESI, pos.) *m/z*: 264 $[M + H]^+$; 286 $[M + Na]^+$; 549 $[2M + Na]^+$. ^{1}H NMR: δ 1.9 (s, 3H, CH_3); 4.5 (d, $J = 5.6$ Hz, 2H, H-8); 4.7 (s, 2H, H-13); 6.2 (d, $J = 3.2$ Hz, 1H, H-12); 6.3 (m, 1H, H-11); 7.5 (s, 1H, H-6); 7.5 (m, 1H, H-10); 7.6 (t, $J = 5.21$ Hz, 1H, NH-7); 10.5 (s, 1H, N-H-1). ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.86; 36.57; 57.12; 104.87; 106.87; 110.44; 140.02; 141.94; 152.26; 156.39; 163.08.

*5.1.7.2. 4-N-Benzyl-5-acetyloxymethylcytosine (**18**).* Yield 95%, 117 mg. MS (ESI, pos.) *m/z*: 274 $[M + H]^+$; 296 $[M + Na]^+$; 569 $[2M + Na]^+$. ^{1}H NMR: δ 2.0 (s, 3H, CH_3); 4.5 (d, $J = 5.2$ Hz, 2H, H-8); 4.7 (s, 2H, H-14); 7.2–7.3 (m, 5H, Bz); 7.5 (s, 1H, H-6); 7.8 (t, $J = 6.0$ Hz, 1H, NH-7); 10.5 (s, 1H, H-1). ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.89; 45.21; 60.31; 99.36; 126.54; 126.89; 127.08; 127.37; 128.14; 139.53; 143.90; 156.16; 162.80; 170.52.

5.1.8. Sulfonylation of 4-N-arylmethyl-5-hydroxymethylcytosine

The 4-N-arylmethyl-5-hydroxymethylcytosine (**15**, **16**) (4.83 mmol) and sodium metabisulfite (1.8 g, 9.66 mmol, 2 equiv) were dissolved in water (15 mL) or mixture of water and acetonitrile (2:1, by vol., 15 mL). The reaction was kept in oven at 55 °C for 24 h. After that the solvents were evaporated, the product was isolated by column chromatography using silanized silica gel chromatography with mixture of water and acetone. Then the product was dissolved with methanol (15 mL) and cleaned with active carbon.

*5.1.8.1. 4-N-Furfurylcytosine 5-methyl sulfonic acid sodium salt (**19**).* Yield 85%, 1171 mg. MS (ESI, pos.) *m/z*: 330 $[M + 2Na–H]^+$. ^{1}H NMR: δ 3.5 (s, 2H, H-12); 4.4 (d, $J = 5.1$ Hz, 2H, H-8); 6.3 (d, $J = 2.4$ Hz, 1H, H-11); 6.4 (d, $J = 2.9$ Hz, 1H, H-9); 7.3 (s, 1H, H-6); 7.5 (m, 1H, H-10); 8.1 (t, $J = 5.3$ Hz, 1H, NH-7). ^{13}C NMR (100 MHz, DMSO-d_6): δ 37.12; 50.43; 99.92; 106.77; 110.42; 141.94; 142.62; 152.21; 156.01; 163.93.

*5.1.8.2. 4-N-Benzylcytosine 5-methylsulfonic acid sodium salt (**20**).* Yield 75%, 1069 mg. MS (ESI, pos.) *m/z*: 296 $[M + H]^+$; 318 $[M + Na]^+$; 340 $[2M + Na–H]^+$. ^{1}H NMR: δ 4.2 (s, 2H, H-15); 4.5 (d, $J = 5.6$ Hz, 2H, H-8); 7.2–7.4 (m, 5H, Ar + H-6); 8.1 (t, $J = 5.6$ Hz, 1H, NH-7). ^{1}H NMR (MeOD) δ 3.88 (s, 2H, H-15); 4.65 (s, 2H, H-8); 7.20 (t, $J = 7.6$, 1H, H-14); 7.28 (t, $J = 7.6$ Hz, 2H, H-10, H-12); 7.38 (d, $J = 7.6$ Hz, 2H, H-11, H-13); 7.42 (s, 1H, H-6). ^{13}C NMR (100 MHz, DMSO-d_6): δ 45.48; 68.79; 99.89; 126.51; 127.12; 127.64; 128.12; 129.41; 142.40; 156.26; 163.94.

5.1.9. Oxidation of 4-N-arylmethyl-5-hydroxymetylcytosine

The 4-N-arylmethyl-5-hydroxymethylcytosine (**15**, **16**) (0.91 mmol) and manganese oxide (IV) (MnO_2) (237 mg, 2.73 mmol, 3 equiv) were added to flask followed by water (15 mL) and ethylene dichloride (15 mL). The reaction mixture was intensively stirred at ambient temperature for 24 h. After that, the organic layer was separated and dried with anhydrous magnesium sulfate, and then evaporated to give a product as a white powder.

*5.1.9.1. 4-N-Furfuryl-5-formylcytosine (**21**).* Yield 65–75%, 130–150 mg. MS (ESI, pos.) *m/z*: 220 $[M + H]^+$; 242 $[M + Na]^+$; 461 $[2M + Na]^+$. ^{1}H NMR: δ 4.6 (d, $J = 3.4$ Hz, 2H, H-8); 6.3 (s, 1H, H-10); 6.4 (s, 1H, H-9); 7.6 (s, 1H, H-11); 8.4 (s, 1H, H-6); 8.7 (t, $J = 5.6$, 1H, NH-7), 9.4 (s, 1H, COH). ^{13}C NMR (100 MHz, MeOH): δ 38.07; 106.76; 108.93; 111.61; 143.83; 152.20; 157.26; 157.91; 163.16; 190.14.

*5.1.9.2. 4-N-Benzyl-5-formylcytosine (**22**).* Yield 60–70%, 125–146 mg. MS (ESI, pos.) *m/z*: 230 $[M + H]^+$; 252 $[M + Na]^+$; 481 $[2M + Na]^+$. ^{1}H NMR: δ 4.6 (d, $J = 5.6$ Hz, 2H, H-8); 7.3–7.2 (m, 5H, Ar); 8.4 (s, 1H, H-6); 8.8 (t, $J = 5.2$ Hz, 1H, NH-7); 9.5 (s, 1H, COH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 45.35; 104.13; 126.85; 127.01; 127.37; 128.16; 128.46; 138.52; 154.09; 157.02; 161.10; 189.04.

5.1.10. 4-N-Furfuryl-5-(n-butylamine)methylcytosine

5.1.10.1. Method A. To flask with solution of 4-N-furfuryl-5-hydroxymethylocytosine (**15**) (300 mg, 1.36 mmol) in pyridine (20 mL) the methanesulfonyl chloride (0.16 mL, 232 mg, 2.04 mmol, 1.5 equiv) was added. The reaction was kept at ambient temperature for 30 min, after that the appropriate amine (4.07 mmol, 3 equiv) was added and mixture was kept in oven at temperature 55 °C for 1 h. After that to reaction flask water was added. Then all solvents were evaporated and product was isolated by silanized silica gel column chromatography and was eluted with water and acetone.

5.1.10.2. Method B. 4-N-Furfuryl-5-formylcytosine (**21**) (300 mg, 1.36 mmol) and appropriate amine (6.38 mmol, with methanol (50 mL)) were refluxed for 8 h. After cooling of the mixture sodium borohydride (103 mg, 2.72 mmol, 2 equiv) was added and then refluxed for 1 h. After that the reaction mixture was adjusted to pH 7 with hydrochloric acid, solvents were evaporated under reduced pressure and a product was isolated by silica gel column silanized chromatography and was eluted with water and acetone.

*5.1.10.3. 4-N-Furfuryl-5-(n-butylamine-)methylcytosine (**23**).* Yield 60–75%, 225–282 (method A) mg. MS (ESI, pos.) *m/z*: 277 $[M + H]^+$; 299 $[M + Na]^+$; 575 $[2M + Na]^+$. ^{1}H NMR: 0.89 (t, $J = 7.2$ Hz, 3H, CH_3-18); 1.26 (m, 2H, CH_2-17); 1.45 (m, 2H, CH_2-16); 3.1 (t, $J = 7.5$ Hz, 1H, NH-14); 3.5 (m, 2H, H-15); 3.8 (m, 2H, H-13); 4.5 (s, 2H, H-8); 6.2 (m, 1H, H-11); 6.4 (m, 1H, H-10); 7.5 (s, 1H, H-6); 7.6 (m, 1H, H-12), 8.1 (m, 1H, NH-7). ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.45; 19.136; 28.93; 36.66; 42.72; 45.95; 96.16; 107.02; 110.43; 128.08; 141.77; 152.18; 155.89; 162.49.

5.1.11. Derivatives of 5-azacytosine with aryl substituent at 4-N

5-Azacytosine (500 mg, 4.46 mmol, 1 equiv) and appropriate aromatic aldehyde (26.77 mmol, 6 equiv) were added to methanol (*ca.* 40 mL) containing magnesium methylate (521.80 mg, 22.30 mmol, 5 equiv). The reaction was carried for 3 h at 55 °C. Then sodium borohydride (1.525 mg, 40.14 mmol, 9 equiv) was slowly added to the mixture and the reduction reaction was carried for 30 min at room temperature. After that, the pH of the mixture was adjusted to 7 with conc. HCl and then the solvents were removed by

evaporation under the reduced pressure. Methanol (30–40 mL) was added to the residue and the mixture was filtered. Then solvents were evaporated and product was isolated by silica gel column chromatography and was eluted with ethyl acetate and methanol. The yields range: 60%.

*5.1.11.1. 4-N-Furfuryl-5,6-dihydro-5-azacytosine (**24**).* Yield 60%, 520 mg. MS (ESI, pos.) *m/z*: 195 $[M + H]^+$. ^{1}H NMR: 4.2 (s, 2H, H-6); 4.5 (s, 2H, H-8); 6.2 (m, 1H, H-11); 6.4 (m, 1H, H-10); 7.1 (s, 1H, NH-3); 7.5 (m, 1H, H-9). ^{13}C NMR (100 MHz, DMSO-d_6): δ 37.14; 56.14; 106.72; 110.44; 142.05; 152.46; 154.63; 167.43.

*5.1.11.2. 4-N-Benzyl-5,6-dihydro-5-azacytosine (**25**).* Yield 63%, 573 mg. MS (ESI, pos.) *m/z*: 205 $[M + H]^+$. ^{1}H NMR: 4.2 (s, 2H, H-6); 4.5 (s, 2H, H-8); 7.1 (s, 1H, NH-3); 7.3 (m, 5H, Ar). ^{13}C NMR (100 MHz, DMSO-d_6): δ 43.91; 55.69; 127.27; 127.61; 127.92; 128.72; 129.44; 139.78; 155.17; 165.37.

5.1.12. 4-N-Arylmethyl-5-methyl-2′-deoxycytidine

5-Methyl-4-(1,2,4-triazol-1-yl)-1-(β-D-3,5-di-O-acetyl-2-deoxyribofuranosyl)pyrimidin-2(1H)-one (**26**, 388 mg, 1.33 mmol, 1 equiv) and appropriate amine (1.99 mmol, 1.5 equiv) were dissolved in anhydrous acetonitrile (15 mL) [26]. The reaction flask was closed and kept at 50 °C for 2 h. The part of the product in the form of a beige solid was filtered off. To remove the acetyl groups from rest of the product, the filtrate was evaporated down and the residue was dissolved in methanol (15 mL) and 32% aqueous ammonia (15 mL). The mixture was heated till boiling for an hour. Then the solvents were evaporated under reduced pressure. The residue was extracted with dichloromethane (20 mL) and water (3 × 20 mL). The aqueous layer (with product) was evaporated down and the product as a white foam was obtained.

*5.1.12.1. 4-N-Furfuryl-5-methyl-2′-deoxycytidine (**27**).* Yield 94%, 399 mg. MS (ESI, pos.) *m/z*: 322 $[M + H]^+$, 360 $[M + K]^+$. ^{1}H NMR: δ 1.8 (s, 3H, CH_3); 1.9 (m, 1H, H-2′); 2 (m, 1H, H-2″); 3.5 (d, J = 2.4 Hz, 2H, H-5′); 3.7 (q, J = 3.8 Hz, J = 6.7 Hz, 1H, H-4′); 4.2 (t, J = 2.9 Hz, 1H, H-3′); 4.5 (d, J = 5.8 Hz, 2H, H-8); 5.0 (m, 1H, 5′-OH); 5.1 (m, 1H, 3′-OH); 6.1 (t, J = 5.8 Hz, 1H, H-1′); 6.2 (d, J = 2.9 Hz, 1H, H-9); 6.3 (m, 1H, H-10); 7.3 (m, 1H, H-11); 7.8 (s, 1H, H-6); 8.0 (m, 1H, NH-7). ^{13}C NMR (100 MHz, DMSO-d_6): δ 15.22; 40.24; 42.10; 64.23; 73.51; 88.63; 89.39; 108.49; 110.89; 113.56; 140.20; 145.50; 153.97; 160.28; 166.23.

*5.1.12.2. 4-N-Benzyl-5-methyl-2′-deoxycytidyne (**28**).* Yield 96%, 423 mg. MS (ESI, pos.) *m/z*: 332 $[M + H]^+$; 357 $[M + Na]^+$; 685 $[2M + Na]^+$.

^{1}H NMR: δ (MeOH) 1.9 (s, 3H, CH_3); 2.1 (m, 1H, H-2′); 2.2 (m, 1H, H-2″); 3.9 (q, J_A = 6.8 Hz, J_B = 3.2 Hz, 1H, H-3′); 4.1 (s, 2H, H-5′); 4.3 (m, 1H, H-4′); 4.6 (s, 2H, H-8); 6.2 (t, J = 6.8 Hz, 1H, H-1′); 7.4 (m, 6H, Ar); 7.8 (s, 1H, H-6). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.34; 35.56; 53.31; 62.54; 77.76; 79.23; 95.53; 118.54; 119.02; 119.91; 120.40; 120.66; 129.40; 130.75; 149.20; 155.39.

5.1.13. 4-N-Arylmetyl-5-methylcytosine

4-N-Arylmethyl-5-methyl-2′-deoxycytidine (**27**, **28**) (**10**) (1.25 mmol, 1 equiv) was dissolved in the mixture of water:methanol (15 mL, 2:1, v/v) and (115 μL) concentrated hydrochloric acid (3 equiv) was added. The resulted mixture was refluxed for *ca.* 4 h and after cooling to room temperature the pH of the mixture was adjusted to *ca.* 7 with 1 M methanol solution of potassium hydroxide. Then the solvents were removed by evaporation under the reduced pressure. The residue was partitioned between water (15 mL) and n-butanol (3 × 15 mL), and then the active carbon (300 mg, Norit A, 4–7 μm) was added to the organic layer. The filtered organic layer was evaporated to give a product as a pale yellow solid.

*5.1.13.1. 4-N-Furfuryl-5-methylcytosine (**29**).* Yield 74%, 189 mg. MS (ESI, pos.) *m/z*: 206 $[M + H]^+$, 228 $[M + Na]^+$, 244 $[M + K]^+$. ^{1}H NMR: δ 1.3 (s, 3H, CH_3); 4.6 (s, 2H, H-8); 6.2 (m, 1H, H-9); 6.3 (m, 1H, H-10); 7.3 (m, 1H, H-11); 7.4 (s, 1H, H-6). ^{13}C NMR (75 MHz, DMSO-d_6): δ 13.40; 37.38; 104.66; 108.82; 111.97; 140.57; 143.53; 153.76; 161.01; 166.39.

*5.1.13.2. 4-N-Benzyl-5-methylcytosine (**30**).* Yield 74%, 199 mg. MS (ESI, pos.) *m/z*: 216 $[M + H]^+$; 238 $[M + Na]^+$; 453 $[2M + Na]^+$. ^{1}H NMR: δ 1.9 (s, 3H, CH_3); 4.5 (d, J = 6.0 Hz, 2H, H-8); 7.1 (s, 1H, H-6); 7.2–7.3 (m, 5H, Ar); 7.5 (t, J = 6.0 Hz, 1H, NH-7). ^{13}C NMR (75 MHz, DMSO-d_6): δ 12.75; 42.97; 100.21; 126.60; 127.10; 128.14; 128.20; 128.45; 139.78; 156.73; 163.10.

5.2. Biology

5.2.1. Plasmid DNA in vitro methylation assay

SssI, CpG methyltransferase, isolated from a strain of *Escherichia coli* transfected with the *SssI* gene from *Spiroplasma* sp. strain MQ1 was purchased from New England Biolabs. [^{3}H]CH_3-adenosyl-L-methionine (SAM) (specific activity 10 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences. The scintillation cocktail ingredients: toluene, 2,5-diphenyloxazole (PPO), 2,2′-*p*-Phenylene-bis(5-phenyloxazole) (POPOP) were purchased from Sigma–Aldrich. pUC18 plasmid was isolated using Qiagen Plasmid Plus Maxi Kit. The purity of DNA was estimated by UV spectroscopic measurement and the A_{260}/A_{280} ratio was 1.9–2.0.

To optimize the assay, different concentrations of SAM (0.1–25 μM), pUC18 plasmid (0.1–1 μg), SssI enzyme (0.5–4 U) and reaction time (5–180 min) were evaluated. To determine the rate of methylation reaction (V_0 [nM/min]), the reaction was carried out in total volume of 20 μL containing 0.1–1 μg of pUC18 plasmid, 2 μM [^{3}H]CH_3-SAM, 1 U of SssI in 10 mM Tris–HCl pH 7.9 reaction buffer containing 50 mM NaCl, 10 mM DTT for 1, 3, 5 and 10 min at 37 °C. The reaction was stopped by loading 18 μL of reaction mixture on Whatmann GF/C filters, washed with 3 mL of 10% TCA, twice with 3 mL of 5% TCA, and 3 mL of ethanol. The amount of methylated DNA precipitated on dried filters was measured in 4 mL of scintillation cocktail in Beckman 5000TA counter. All reactions were repeated twice.

Optimized SssI methyltransferase inhibition activity assay was performed with three different amounts of substrate (pUC18 plasmid) – 0.1, 0.5 and 1 μg, 2 μM SAM, 1 U of SssI and increasing concentration of tested compounds in reaction buffer described above. Concentration of CpG in pUC18 is 4.4 μM in 1 μg (0.56 pmol) of plasmid. The reactions were carried out at 37 °C for 10 min. K_i values were determined from Dixon plot (1/V *vs* I – inhibitor concentration) [37]. K_i' value was calculated according to Cornish-Bowden method [38]. All reactions were repeated twice.

5.2.2. Inhibition of human DNMT1

Nuclear extracts containing human DNMT1 were prepared using EpiQuik Nuclear Extraction Kit (Epigentek) according to the manufacturer's protocol. HeLa cells were grown up to 80% confluency in 75 mm^2 culture flask. The protein concentration was determined by Bradford method [46]. The inhibitory activities of compounds were measured in *in vitro* enzymatic assay using EpiQuik DNMT1 Activity/Inhibitor Screening Assay Core Kit (Epigentek). Analysis is based on ELISA using anti-5-methycytosine antibodies to detect methylated substrate. According to manufacturer's protocol nuclear extract with substrate was incubated for 1 h in the presence or absence of inhibitor, then antibody was added to samples and analyzed colorimetrically in plate reader (BioTek Synergy 2) at 450 nm. Each experiment was repeated twice.

5.2.3. Inhibition of methylation process in mammalian cell based assay

HeLa, derived from a cervical carcinoma (Sigma, Munich, Germany) and HEK293 cells, human embryonic kidney cells derived from healthy fetus transformed with adenovirus (Sigma) were seeded at a density of 1.25×10^5 per well in 6-well tissue culture plates. The HeLa cells were grown in RPMI-1640 medium, HEK293 in DMEM medium (Sigma, Munich, Germany) supplemented with 10% fetal bovine serum (Gibco, Paisley, UK), 1% antibiotics (Sigma) and 1% RPMI vitamin mix (Sigma) at 37 °C under 5% CO_2 atmosphere. Cells were incubated for 72 h with inhibitors at 1, 10, 100 μM concentrations. DNA from HeLa and HEK293 cells was isolated with a commercial kit (A&A Biotechnology, Gdynia, Poland). The analysis of m^5C level in DNA was carried out as previously described [40]. Dried DNA (1 μg) was digested with 0.001 U of spleen II and 0.02 U of micrococcal nuclease in 20 mM succinate buffer containing 10 mM $CaCl_2$ for 6 h at 37 °C. After that the hydrolysate (0.3 μg) was labeled with 1.6 μCi [γ-^{32}P]ATP (6000 Ci/mmol Hartmann Analytic, Braunschweig, Germany) and 1.5 U T4 polynucleotide kinase in 10 mM bicine-NaOH pH 9.7 buffer containing 10 mM $MgCl_2$, 10 mM DTT and 1 mM spermidine. After incubation for 30 min at 37 °C, 0.03 U of apyrase in 10 mM bicine-NaOH buffer was added and incubated for 30 min. Next, 0.2 μg of RNase P1 in 500 mM ammonium acetate buffer pH 4.5 was used for 3′ phosphate cleavage. Analysis of [γ-^{32}P] m^5C was done by 2D TLC on cellulose plates (Merck) in isobutyric acid:-NH_4OH:H_2O (66:1:17) and 0.1 M sodium phosphate pH 6.8–ammonium sulfate–n-propanol (100 mL/60 g/1.5 mL). Radioactivity was measured with Fluoro Image Analyzer FLA-5100 with Multi Gauge 3.0 Software. Every analysis was repeated three times.

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