Bioorganic & Medicinal Chemistry Letters 22 (2012) 308–312

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis, cytostatic activity and ADME properties of C-5 substituted and N-acyclic pyrimidine derivatives

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ARTICLE INFO

Article history:
Received 3 October 2011
Revised 31 October 2011
Accepted 2 November 2011
Available online 10 November 2011

Keywords:
5-Alkyl pyrimidine derivatives
Cytostatic evaluations
Cell cycle analysis
ADME properties

ABSTRACT

The synthesis of the novel 5-alkyl pyrimidine derivatives, 5,6-dihydrofuro[2,3-*d*]pyrimidines and 5-alkyl *N*-methoxymethyl pyrimidine derivatives and evaluation of their cytostatic activities are described. The mechanism of antiproliferative effect of 5-(2-chloroethyl)-substituted pyrimidine **3** that exerted the pronounced cytostatic activity was studied in further details on colon carcinoma (HCT116) cells. The cell cycle perturbation analysis demonstrated severe DNA damage (G2/M arrest) pointing to a potential DNA alkylating ability of **3**. Preliminary ADME data of **3** and its 6-methylated structural congener (**6-Me-3**) showed their high permeability and good metabolic stability.

Synthetic nucleoside mimics with modified nucleobase and/or sugar moieties are of considerable importance in the search for new structural leads exhibiting biologically interesting activity.[1] Some nucleoside analogues substituted at various positions on the heterocycle, are known to have potent biological properties and have been investigated, for instance, as antiviral agents (against HSV, VZV, CMV, HIV, HBV and HCV), non-radioactive fluorescent labels for DNA and as anticancer drugs.[2] Extensive studies have been carried out on 5-substituted uracil analogues for use in cancer and viral chemotherapy, as enzyme inhibitors.[3] Notable among them are 5-fluorouracil (5-FU) and the corresponding 2′-deoxyribonucleoside (FdU) which have been used in cancer chemotherapy for decades.[4] Furthermore, bicyclic furo[2,3-*d*]pyrimidine nucleosides have demonstrated antiviral and antileukemic activity which has led to increased interest in preparation of corresponding nucleoside analogues.[5] Among fluorinated pyrimidine nucleoside analogues, 2′-deoxy-2′-fluoro-5-methyl-1-β-D-arabinofuranosyluracil (FMAU) and other small side-chain 5-substituted derivatives are phosphorylated with different efficacy by human and other mammalian nucleoside kinases including thymidine kinases TK1 and/or TK2; viral kinases such as herpes simplex virus type 1 and 2 (HSV1-TK and HSV2-TK).[6] Thymine derivatives with 6-(1,3-dihydroxyisobutenyl) and 6-(1,3-dihydroxyisobutyl) side-chain, as ligands for HSV1-TK, were developed as leads for gene expression imaging by positron emission tomography (PET).[7] In view of the importance of 5-substituted uracil derivatives in cancer chemotherapy[8] and as antiviral agents[9] we became interested in the synthesis of the novel C-5 and/or C-6 substituted pyrimidine derivatives.[10]

As part of our ongoing research in drug discovery we initiated the preparation of 5-alkyl pyrimidine derivatives (**3**–**6**, **8**, **9**, **11** and **17**), 5,6-dihydrofuro[2,3-*d*]pyrimidines (**7** and **10**) and 5-alkyl *N*-methoxymethyl pyrimidine derivatives (**12**–**16**). Construction of the pyrimidine ring was performed using the classical intramolecular cyclization of α-(1-carbamyliminomethylene)-γ-butyrolactone (**1**) with sodium ethoxide to afford 5-(2-hydroxyethyl)uracil (**2**) following a literature method.[11] Initially, the sodium α-hydroxymethylene-γ-butyrolactone was prepared by reaction of γ-butyrolactone and methylformate in dry ether with the presence of sodium methoxide, which was subsequently reacted with urea to give **2**. Transformation of hydroxyl and carbonyl functionalities to chlorine was performed using phosphoryl chloride affording chlorinated pyrimidine derivative **3** (Scheme 1).[11] The primary hydroxyl group of compound **2** was protected to give 5-(2-acetoxyethyl) substituted uracil **4**. Chlorination of **4** with phosphoryl chloride and *N*,*N*-diethylaniline yielded 2,4-dichloropyrimidines with 2-acetoxyethyl (**5**) and 2-hydroxyethyl (**6**) side-chain as well as bicyclic 5,6-dihydrofuro[2,3-*d*]pyrimidine derivative (**7**). Methoxylation of **5** gave desired 2,4-dimethoxypyrimidine (**8**) with 2-hydroxyethyl side-chain in moderate yield. 2-Chloro-4-methoxypyrimidine (**9**) and 2-methoxy-5,6-dihydrofuro[2,3-*d*]pyrimidine (**10**) were also isolated as minor products. Fluorination of **9**

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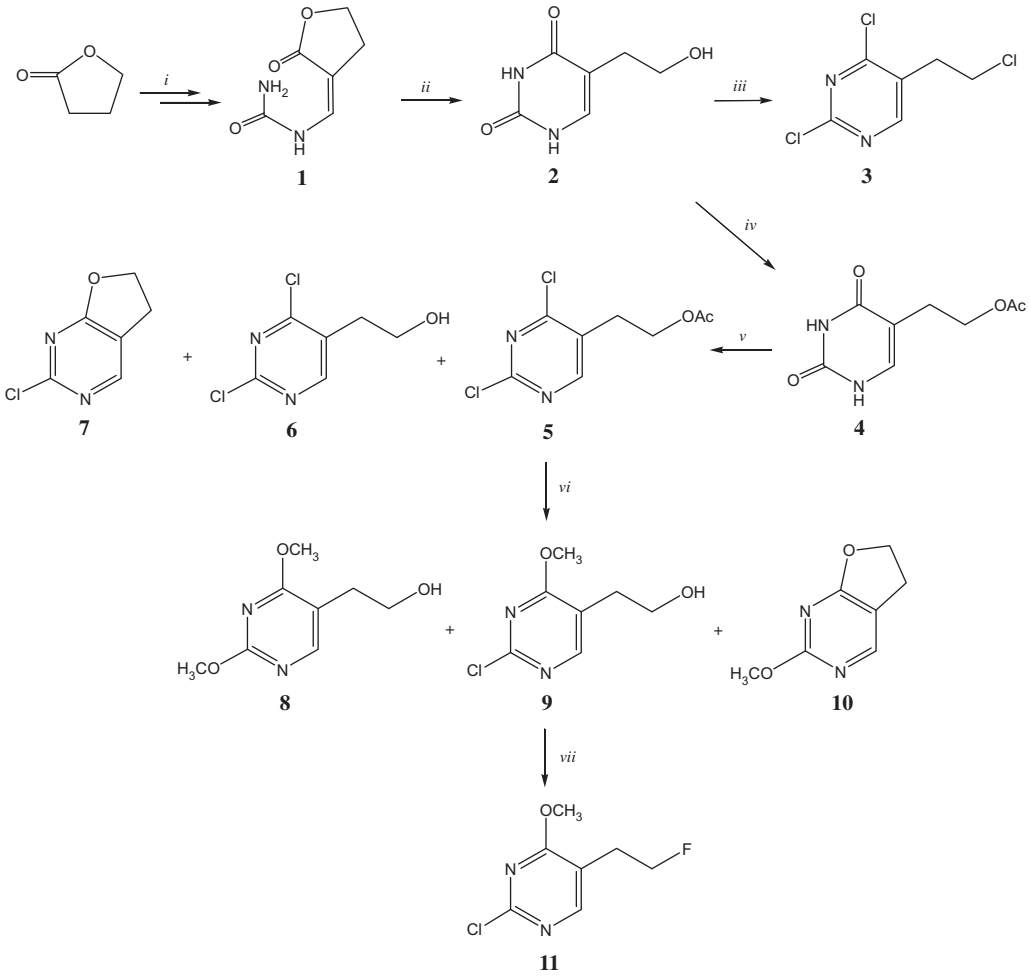
doi:10.1016/j.bmcl.2011.11.009



Scheme 1. Reagents and conditions: (i) NaOMe, ether, $HCOOCH_3$, rt, 24 h; urea, 3 M HCl, 4 °C, 24 h; (ii) NaOEt, EtOH, reflux, 6 h; (iii) $POCl_3$, *N*,*N*-diethylaniline, reflux, 1 h; (iv) Ac_2O, pyridine, reflux, 1 h; (v) $POCl_3$, reflux, 1 h; (vi) NaOMe, reflux, 1 h; (vii) DAST, CH_2Cl_2, −75 °C, 0.5 h.

with diethylaminosulfur trifluoride (DAST) as the fluorinating reagent afforded 2-chloro-4-methoxypyrimidine **11** with 2-fluoroethyl substituent at C-5.

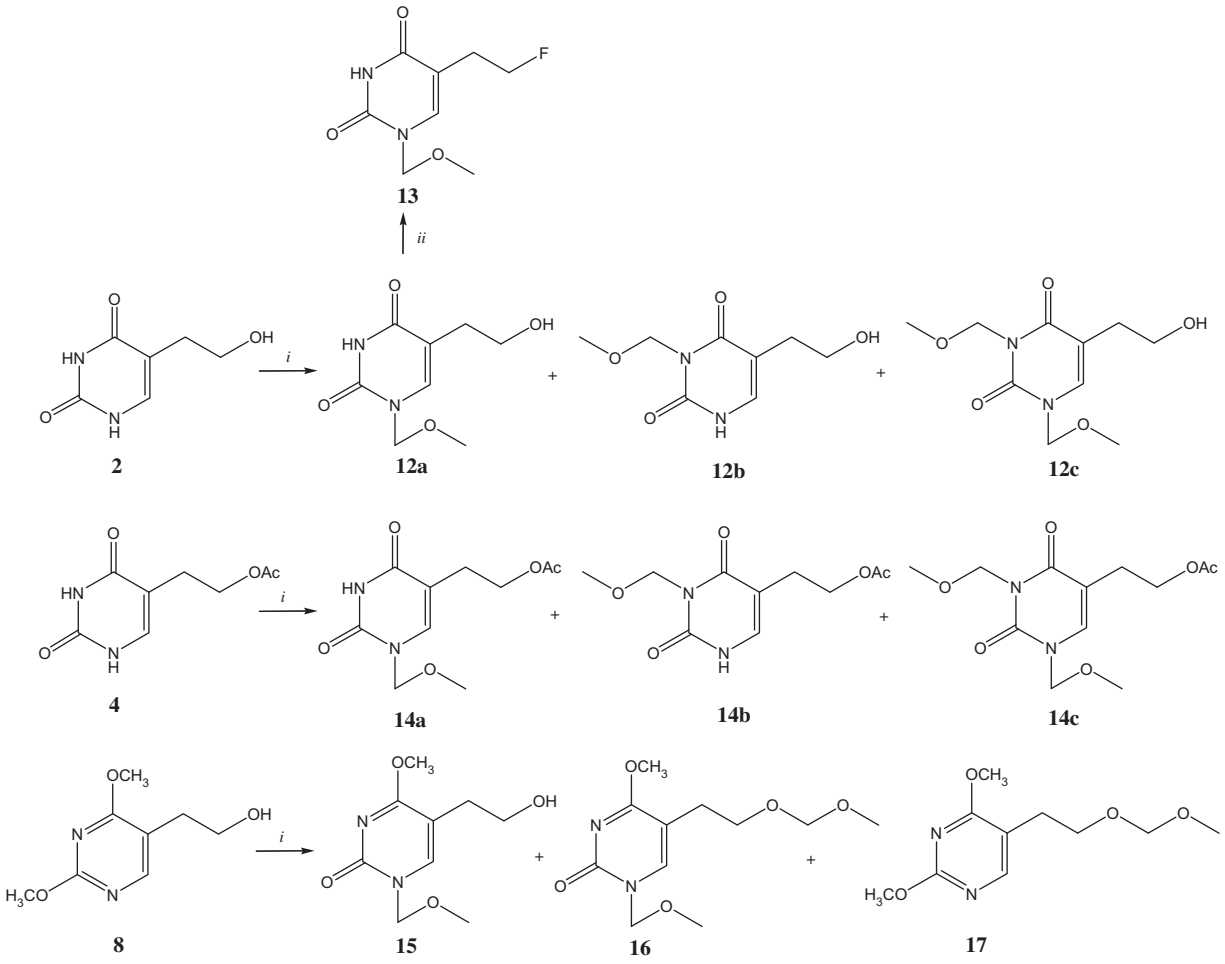
N-methoxymethylation of **2** was accomplished with methoxymethyl chloride (MOMCl) as alkylating reagent and potassium carbonate to yield *N*-1-MOM (**12a**), *N*-3-MOM (**12b**) and *N*,*N*-1,3-diMOM (**12c**) 5-(2-hydroxyethyl)pyrimidine derivatives in 24%, 15% and 3% yields, respectively (Scheme 2). To the best of our knowledge there are only a few reports describing the N-methoxymethylated pyrimidine derivatives.[12]

The fluorine in side-chain of **12a** was introduced using DAST affording *N*-MOM pyrimidine derivative **13** containing 2-fluoroethyl at C-5 position. N-methoxymethylation of acetylated analog **4** also gave *N*-1-MOM (**14a**) and *N*-3-MOM (**14b**) regioisomers as well as *N*,*N*-1,3-diMOM (**14c**) in 22%, 2% and 17% yields, respectively. We can deduce that N-alkylation of uracil with both hydroxyethyl and acetoxyethyl substituents gave target *N*-1-MOM uracil derivatives in rather low yield (∼20%). Thus, we applied a more efficient N-methoxymethylation using same reaction conditions but starting from 2,4-dimethoxy-5-(2-hydroxyethyl)uracil (**8**) to give *N*-1-MOM (**15**) in improved yield of 46%. Compounds **16** and **17** with 2-(methoxymethoxy)ethyl substituent at C-5 were also isolated as byproducts.

Compounds **3**–**17** were evaluated for their cytostatic activities against three human malignant tumor cell lines: breast carcinoma (MCF-7), colon carcinoma (HCT116), lung carcinoma (H 460) and compared to the reference compound 5-fluorouracil. In general, compounds **4**, **6** and **8**–**16** showed no inhibitory activity on tumor-cell growth up to 100 μM (data not shown). However, moderate inhibition of cell growth was obtained with compounds **5**, **7** and to a certain extent **17** (Table 1).

While 2,4-dichloropyrimidine (**6**) with 5-(2-hydroxyethyl) side chain did not show any inhibitory effect, its acetylated structural analog **5** exhibited cytostatic activity (IC_{50} ∼30 μM). Similar activity (IC_{50} ∼27 μM) was found for bicyclic 2-chloro-5,6-dihydro furo[2,3-*d*]pyrimidine (**7**). The 2,4-dimethoxypyrimidine (**17**) with 5-(2-(methoxymethoxy)ethyl) side chain showed only slight inhibitory effect against colon carcinoma (HCT116). Of all compounds tested, 5-(2-chloroethyl)-2,4-dichloropyrimidine (**3**) exerted the most potent antiproliferative activity (in the low micromolar range), especially and rather selectively towards HCT116 colon cancer cell line (IC_{50} = 0.8 μM) (Table 1 and Fig. 1). Similar results showing significant impact of chlorine atoms on biological activity were described previously.[13] Interestingly, the increase in the chlorine content also enhanced the antiproliferative effects of 2,4-dichloro-6-methylpyrimidine structural congener bearing the same 5-(2-chloroethyl)-substituent.[13]

In order to shed more light on the mechanism of action of the most active compound **3**, we attempted the flow cytometry analysis of potential cell cycle perturbations. The compound was tested at two concentrations being close or slightly higher than its IC_{50} (1 and 5 μM) on HCT116 cells (Table 2). Interestingly, the results



Scheme 2. Reagents and conditions: (i) K_2CO_3, DMF, MOMCl, −15 °C, 0.5 h, rt, overnight; (ii) DAST, CH_2Cl_2, −75 °C, 0.5 h.

Table 1
Inhibitory effects of **6-Me-3**, **3**, **5**, **7** and **17** on the growth of malignant tumor cell lines

Compd	IC_{50}[a] (μM)		
	HCT116	MCF-7	H 460
6-Me-3[13]	2 ± 0.2	8 ± 7	3 ± 0.01
3	0.8 ± 0.2	10 ± 1	3 ± 0.001
5	29 ± 13	30 ± 5	31 ± 0.0
7	13 ± 10	45 ± 6	22 ± 0.05
17	75 ± 17	>100	>100
5-FU[b]	4 ± 0.8	14 ± 0.3	3 ± 0.3

[a] IC_{50}: 50% inhibitory concentration, or compound concentration required to inhibit tumor cell proliferation by 50%.
[b] 5-FU: 5-fluorouracil.

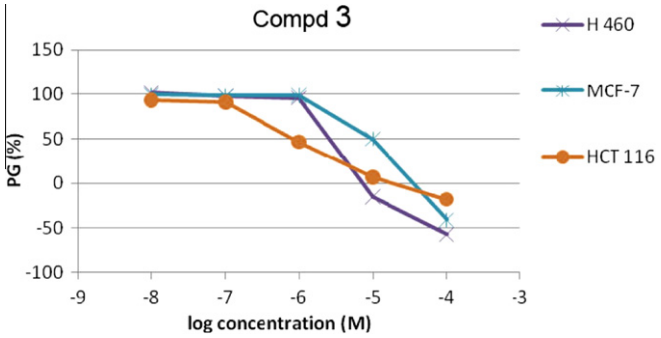


Figure 1. Concentration–response profiles for compound **3** tested on tumor cell lines in vitro. PG = percentage of growth.

Table 2
The effects of compound **3** at 1 and 5 μM on the cell cycle distribution of HCT116 cells after the 24- and 48-hour treatments

Treatment		Percentage of cells[a] (%)			
		SubG1	G1	S	G2/M
24 h	Control	1.8	32.5	50.0	17.4
	1 μM	2.5	33.5	48.8	17.2
	5 μM	8.1	7.8	14.6	77.6
48 h	Control	1.5	42.3	31.0	26.6
	1 μM	1.7	41.7	31.5	26.7
	5 μM	8.5	24.6	14.8	60.7

[a] The numbers represent the percentages of cells in respective cell cycle phase (G1, S and G2/M), along with the percentage of cells in the subG1 (dead cells) obtained by flow cytometry.

demonstrated that **3** at the higher concentration (5 μM) induced strong G2/M phase arrest at both time points, that is the majority of cells could not progress through mitosis and eventually died by apoptosis, as evidenced by accumulation of subG1 cells, representing apoptotic cells. Accumulation of cells in G2/M phases (a G2/M phase arrest) points to a damage in the cell, which occurs during the S phase (DNA replication), or before mitosis (aberrant mitotic spindle formation). The G2 checkpoint senses unreplicated DNA, generates a signal leading to cell cycle arrest and therefore preventing the initiation of M phase before completion of S phase. Thus, it is usually triggered by agents that induce DNA- or mitotic spindle-damage. Since compound **3** is structurally similar to a

Table 3
The apparent permeability (P_{app}) values for **3** and **6-Me-3** (with and without P-gp inhibitor)

Compd	P_{app} (AB) (cm/s)	P_{app} (BA) (cm/s)	Efflux ratio	P_{app} (AB) (cm/s)	P_{app} (BA) (cm/s)	Efflux ratio
	−Elacridar[a]			+Elacridar		
3	20.8×10^{-6}	25.8×10^{-6}	1.2	25.6×10^{-6}	29.6×10^{-6}	1.2
6-Me-3	35.8×10^{-6}	20.9×10^{-6}	0.6	21.6×10^{-6}	31.1×10^{-6}	1.4

[a] Elacridar (GF120918) as P-gp inhibitor.[16]

Table 4
Stability of **3** and **6-Me-3** in plasma and liver microsomes of various species

Compd	Plasma stability % remaining (24 h) ($t_{1/2}$ (min))			Microsomal stability % remaining (60 min) ($t_{1/2}$ (min))		
	Human	Rat	Mouse	Human	Rat	Mouse
3	0	0	0	65	73	33
	(153)	(24)	(91)	(104)	(134)	(34)
6-Me-3	20	3	22	76	74	41
	(600)	(184)	(478)	(145)	(134)	(46)

pyrimidine antagonist 5-FU, this mechanism of cell cycle perturbation is quite peculiar. Namely, antimetabolite drugs, such as pyri midine antagonists arrest/delay the cell cycle in G1, or early S phase.[14] Nevertheless, the chloroethyl-substituent in **3**, which is present in other known alkylating agents (such as: nitrogen mustards, or uracil mustard—uramustine) might be responsible for alkylation of DNA, thus causing a more severe DNA damage, which induces G2/M arrest and apoptosis. More detailed analysis of DNA alkylation/cross linking/damage ability of **3** is underway.

Compound **3** and its 6-methylated structural analog (**6-Me-3**) with pronounced cytostatic activities were selected for preliminary ADME assays including permeability and P-glycoprotein (P-gp) substrate assessment, stability in plasma (rat, human and mouse), as well as metabolic stability in liver microsomes (rat, human and mouse). These properties are important for new molecules design and affect their bioavailability in a great manner. Permeability assay was designed to determine permeability of selected compounds through MDCKII-MDR1 cell monolayers, as well as for their identification as potential P-gp supstrate.[15] MDCKII-MDR1 cells are Madin–Darby canine kidney cells with overexpressed MDR1 (human multidrug resistance 1) gene. This gene encodes an integral membrane protein P-gp that functions as ATP-dependant efflux pump, which could have a significant influence on compounds permeability. Both compounds **3** and **6-Me-3** showed to be highly permeable compounds with *Papp* values above 20.0×10^{-6} cm/s (Table 3). Besides, efflux ratios did not exceed 2, indicating that both compounds are not P-gp substrates. Results of plasma stability showed that **6-Me-3** was more stable in all tested species comparing to **3** (Table 4). Moreover, this compound exhibited significantly higher (four to eightfold) half-life values than those of **3**, suggesting that methyl substituent at C-6 of pyri midine scaffold in **6-Me-3** has impact on plasma stability. On the contrary to this, determination of metabolic stability in liver microsomes, monitoring exclusively phase I metabolism, showed similar metabolic stability of tested compounds. In addition, both compounds exhibited higher stability in human and rat microsomes than in mouse microsomes.

In summary, 5-alkyl *N*-methoxymethyl pyrimidine derivatives (**12**–**16**) were prepared by intramolecular cyclization reaction of α-(1-carbamyliminomethylene)-γ-butyrolactone (**1**) with sodium ethoxide and subsequent chemical transformations of hydroxyl and carbonyl functionalities as well as N-methoxymethylation. While N-alkylation of uracil with both 5-(2-hydroxyethyl) (**2**) and 5-(2-acetoxyethyl) (**4**) substituents suffered from poor yield, N-alkylation of 5-(2-hydroxyethyl)-2,4-dimethoxypyrimidine (**8**) gave *N*-1-methoxymethyl pyrimidine **15** in improved yield. Among all evaluated compounds, pyrimidine derivative **3** that bears two aromatic and one aliphatic chlorine atoms showed the most potent inhibitory activity against malignant tumor cell lines tested, particularly against the colon cancer (HCT116) cell line. Interestingly, the cell cycle perturbation analysis demonstrated severe DNA damage (G2/M arrest) pointing to a potential DNA alkylating ability of chloroethyl-substituted pyrimidine **3**. Furthermore, this compound exhibited favourable in vitro ADME properties, that is high permeability and good metabolic stability in liver microsomes of human and rat.

Acknowledgments

Support of this study by the Ministry of Science, Education and Sports of the Republic of Croatia (projects #125-0982464-2925 and 098-0982464-2514) is gratefully acknowledged.

Supplementary data

Supplementary data (experimental procedures, compound characterization data, methods for antitumor activity assay, cell cycle analysis and ADME assay) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.11.009.

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