Discovery of New Acid Ceramidase-Targeted Acyclic 5-Alkynyl and 5-Heteroaryl Uracil Nucleosides

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Supporting Information

ABSTRACT: A series of novel *N*-acyclic uracil analogs with linear, branched, aromatic, and cyclopropyl-alkynyl as well as heteroaryl moieties at C-5 were prepared using palladium catalyzed Sonogashira and Stille cross-coupling and evaluated against malignant tumor cell lines. C-5-Furan-2-yl uracil derivative **6** was shown to be more potent against MCF-7 than the reference drug 5-fluorouracil (5-FU), while C-5-alkynyl uracil derivatives **9c** and **9e** exhibited antibreast cancer activities comparable to 5-FU. Selected compounds induced cell death, partially due to apoptosis, of MCF-7 breast cancer cells. Abrogation of acid ceramidase (ASAHI) expression of **9c** and **9e** indicated that these compounds could perturb ASAHI-mediated sphingolipid signaling. The selective activity of **9c** and **9e** against breast cancer cells via the ASAHI-mediated signaling, as a molecular target, might have a great advantage for potential future therapeutic use.

KEYWORDS: Nucleosides, pyrimidine, apoptosis, breast cancer, acid ceramidase (ASAHI), sphingolipid signaling

Worldwide, breast cancer is the second most common cancer, after lung cancer, and the most frequent cause of death from cancer in women.^{1–3} Breast cancer is a complex and heterogeneous disease, characterized by deregulation of multiple cellular pathways, different morphology, and variable sensitivity to various treatments.⁴ However, despite significant progress in detection and treatment of breast cancer, metastatic breast cancer (MBC) remains incurable. In recent years, the addition of targeted therapy to chemotherapy or endocrine therapy using human epidermal growth factor receptor 2 (HER2) and hormone receptor (HR) status (positive or negative) significantly improved overall survival in patients with MBC.¹ Compounds used to treat either primary or MBC are limited, and there is a considerable demand for development of a new potent antibreast cancer agent with novel mechanism of action to overcome toxicity and drug resistance problems associated with current chemotherapeutics. Sphingosine-containing lipids are essential structural components of cell membranes with important signaling functions in the control of cell growth and differentiation.⁵ The ceramides, amides of sphingosine with long-chain fatty acids, have attracted attention for their roles in the replication and differentiation of normal

and neoplastic cells. Stress-related signals, such as tumor necrosis factor α (TNF- α), may stimulate ceramides production in cells, which in turn causes replicative senescence and apoptosis.⁶ Acid ceramidase (ASAHI) plays a pivotal role in regulating the intracellular concentration of sphingosine (SPH) and sphingosine-1-phosphate (S1P) by hydrolyzing ceramide into sphingosine, which is rapidly phosphorylated by sphingosine kinase 1 (SK1) to form S1P (Figure 1).⁷

Figure 1. Simplified overview of the biosynthetic pathway leading from ceramide to sphingosine-1-phosphate (S1P) and structure of carmofur.

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This enzyme pathway involved in controlling intracellular ceramide levels has emerged as a potential new target for antitumor therapy. In particular, it has been shown that regulation of sphingolipid levels by SK1 and ASAH1 are known components of carcinogenesis in hormone-dependent breast cancer MCF-7 cells⁸ and that inhibition of SK1 or ASAH1 in MCF-7 cells might substantially diminish their proliferative potential.⁹ Carmofur, an antineoplastic drug used in the clinic to treat colorectal cancers, was identified as the first highly potent inhibitor of intracellular ASAH1 activity (Figure 1).¹⁰

Among numerous modified nucleosides that are known to exhibit interesting biological properties,^{11–14} C-5-substituted uracil nucleosides have been investigated as anticancer and antiviral agents.^{15–18} Alkynyl modifications of the uracil base have been further explored for diverse biological properties.^{19–23}

As a part of our program to search for new antitumor and antiviral agents, we have previously reported the synthesis of a series of C-5-unsaturated N-1-substituted pyrimidine derivatives which proved to be efficient as tumor cell growth inhibitors (Figure 2).^{24–26}

Figure 2. Design and synthesis of C-5-alkynyl and -aryl uracil nucleoside analogs containing a PCV-like side chain at N-1.

Taking into consideration aforementioned results, the aim of the present study was to synthesize nucleoside mimetics in order to further explore the structure activity relationship of the C-5-unsaturated substituent and the penciclovir-like (PCV) side chain as structural features at N-1 of the pyrimidine scaffold.

In the synthesis of a series of C-5-substituted alkynyl and heteroaryl *N*-acyclic uracil derivatives, the N-3 of uracil was selectively protected with a benzoyl group to avoid both *N*-1- and *N*-1,*N*-3-dialkylation (Scheme 1).

Direct iodination at the C-5 of uracil **1** was subsequently performed by abstraction of a hydrogen atom through the base-promoted reaction using molecular iodine as an iodine donor and ammonium cerium(IV) nitrate (CAN) for activation of the uracil base to afford 5-iodinated pyrimidine derivative **2**. Alkylation of compound **2** with the corresponding alkyl iodide **3** in the presence of K₂CO₃ gave the 5-iodouracil **4**, containing PCV-like side chain. N-1 alkylation was followed by the introduction of a substituent at C-5 of uracil via palladium-catalyzed reactions. Thus, the furyl and thienyl substituents were introduced at C-5 of *N*-acyclic uracil **4** via Stille coupling of the precursor **4** and the corresponding organostannane in the presence of tetrakis(triphenylphosphine)palladium(0) [Pd-

Scheme 1. Synthesis of Novel *N*-Acyclic C-5-Heteroaryl Uracil Derivatives (**6** and **8**) via Stille Cross-coupling^a

^aReagents and conditions: (a) Benzoyl chloride, pyridine, acetonitrile, Ar, 24 h, r.t.; (b) I₂, CAN, acetonitrile, 2 h, reflux; (c) K₂CO₃, DMF, 24 h; (d) Pd(PPh₃)₄, anhydrous toluene, 2 h, reflux; (e) 0.1 M NaOCH₃/CH₃OH, CH₃OH, 24 h, r.t.

(PPh₃)₄] to give **5** and **7**. Both acetyl and benzoyl protecting groups were subsequently removed under basic conditions with 0.1 M NaOCH₃/CH₃OH, and the target *N*-acyclic C-5-heteroaryl uracil derivatives **6** and **8** were obtained (Scheme 1). A variety of alkynyl substituents were introduced at the C-5 position of pyrimidine via Sonogashira coupling of *N*-acyclic 5-iodouracil nucleoside **4** with a series of terminal alkynes in the presence of the palladium catalyst Pd(PPh₃)₄, triethylamine (Et₃N), and Cu(I) iodide to afford the C-5-alkynyl uracil acyclonucleosides **9a–9i** (Scheme 2).

Scheme 2. Synthesis of *N*-Acyclic C-5-Alkynyl Uracil Derivatives (**9a–9i**, **10a–10i**) via Sonogashira Cross-coupling Reaction^a

^aReagents and conditions: (a) Pd(PPh₃)₄, CuI, Et₃N, anhydrous toluene, Ar, 24 h, r.t.; (b) 0.1 M NaOCH₃/CH₃OH, CH₃OH, 24 h, r.t.

Deprotection of acetyl groups in the side chain and the N-3-benzoyl group in **9a–9i** was performed under basic conditions by treatment with 0.1 M NaOCH₃/CH₃OH at room temperature to yield the unprotected target compounds **10a–10i** in moderate yields. Crystal structures of target C-5-heteroaryl **6** and C-5-alkynyl **10g** uracil acyclonucleosides were determined by a single crystal X-ray diffraction method (Figure S3, Supporting Information). It should be noted that only one structure of 5-furan-2-yl-pyrimidine-2,4-dione has been published until now²⁷ and that the bond lengths and angles in that structure agree well with the equivalent ones in **6**. The furan and pyrimidine rings are almost coplanar (Figure S3a), with the dihedral angle between them being 5.87(10)°, whereas C7/C8/

Table 1. Growth-Inhibition Effects *in Vitro* of Compounds 6–8, 9a–9i, and 10a–10i on Selected Tumor Cell Lines and Normal Fibroblasts

6, 8: X = O; S

9a–9i: R¹ = Ac; R² = Bz
10a–10i: R¹ = R³ = H

Compd	R ² /X	IC ₅₀ ^a (μM)					CLogP ^c	CLogP ^d	Yield/%
		MCF-7	SW-620	HeLa	BJ/3T3				
6	X = O	< 0.01	> 100	> 100	0.5±0.01	-1.23	-1.26	17	
8	X = S	6.0±0.01	> 100	7.8±0.01	38.7±0.01 ^b	-0.38	0.1	34	
9a		28.9±0.03	> 100	64.5±0.11	46.6±0.03	3.06	3.01	58	
9b		28.9±0.01	> 100	47.2±0.25	37.6±0.01	3.54	3.52	42	
9c		0.1±0.01	> 100	79.1±0.03	17.6±0.01	5.06	4.85	81	
9d		26.8±0.01	36.4±0.02	29.9±0.09	26.89±0.01 ^b	3.21	3.11	70	
9e		0.1±0.01	> 100	> 100	0.3±0.08	1.90	1.75	71	
9f		62.2±0.02	40.7±0.03	53.7±0.03	88.8±0.01 ^b	3.41	3.17	72	
9g		28.9±0.01	65.8±0.03	32.4±0.08	> 100	4.75	4.42	67	
9h		21±0.06	> 100	52.8±0.11	> 100 ^b	2.44	2.37	57	
9i		67.1±0.01	> 100	71.5±0.03	36.9±0.01	2.85	2.72	83	
10a		63.1±0.03	> 100	31.2±0.09	> 100	0.33	0.70	13	
10b		46±21.25	> 100	67.7±0.11	> 100	0.81	1.22	24	
10c		26.8±0.01	> 100	48.2±0.05	> 100 ^b	2.34	2.54	40	
10d		> 100	72.4±0.01	63.8±0.05	> 100	0.49	0.80	52	
10e		> 100	> 100	> 100	35.9±0.01 ^b	-0.82	-0.55	21	
10f		37.8±0.02	> 100	76.3±0.01	39.8±0.03 ^b	0.69	0.87	70	
10g		40.3±0.01	> 100	58.9±0.05	39.6±0.03 ^b	2.02	2.12	80	
10h		35.2±0.01	> 100	> 100	> 100 ^b	-0.28	0.06	91	
10i		95.9±0.01	> 100	> 100	58.2±0.01	0.13	0.42	51	
5-FU		0.09±0.01	0.79±0.67	> 100	28.3±0.01 ^b	-	-	-	

^a50% inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%. ^bCompounds 8, 9d, 9f, 9h, 10c, 10e, 10f, 10g, 10h, and 5-FU were tested on the 3T3 cell line. Values of *n*-octanol/water partition coefficients logP for synthesized compounds were calculated by two algorithms with their default settings. ^cChemAxon algorithm available within MarvinView Ver. 5.2.6. ^dACD/logP model available within ChemBioDraw Ultra 11.0.

C9/C11 atoms form a plane which is almost perpendicular with respect to the pyrimidine ring [dihedral angle 84.53(15)°].

The dihedral angle between the same moiety atoms and the pyrimidine ring in 10g is very similar and amounts to 89.7(2)°. Descriptions of the molecular structures of intermediates 1 and 2, as well as the crystal structures of all four compounds are given in the [Supporting Information](#) (Figures S2–S5).

Results of antiproliferative evaluations of compounds 6, 8, 9a–9i, and 10a–10i *in vitro* on selected tumor cell lines: breast epithelial adenocarcinoma (MCF-7), colorectal adenocarcinoma (SW620), and cervical carcinoma (HeLa), as well as normal diploid human fibroblasts (BJ) and mouse embryonic fibroblast (3T3) cells are presented in [Table 1](#). 5-Fluorouracil was used as the reference drug. It can be observed that the most pronounced and selective effects were found on the breast epithelial adenocarcinoma (MCF-7) and normal diploid human fibroblasts (BJ). *N*-Acyclic 5-furyluracil 6 was shown to be the most potent against MCF-7 cells, with IC₅₀ value in the low

nanomolar range (IC₅₀ < 0.01 μM). However, this compound also showed cytotoxic effects on normal fibroblasts (IC₅₀ = 0.5 μM). Moreover, C-5-heteroaryl uracil derivative 8, containing sulfur instead of oxygen in the five-membered ring, inhibited the growth of MCF-7 (IC₅₀ = 6 μM) and exerted lower cytotoxicity on normal fibroblasts (IC₅₀ = 38.7 μM) than its C-5-furyl structural congener 6. The selectivity index for 6 is 50, and that of 8 is 6.45. Within the group of internal alkynes (9a–9i), pyrimidine acyclonucleosides containing aromatic and cyclopropyl rings attached to the alkyne gave better antiproliferative activities against MCF-7 cells than those with aliphatic substituents. Thus, *p*-pentylphenyl- (9c) and cyclopropyl-substituted (9e) alkynes showed marked cytostatic effects against MCF-7 cells (IC₅₀ = 0.1 μM). C-5-(*p*-Pentylphenyl)ethynyl (9c) was shown to be more selective than cyclopropylethynyl (9e) acyclonucleoside, whose activity was accompanied by cytotoxicity on normal human fibroblasts. Among the internal aliphatic substituted alkyne uracil

derivatives, **9h** had the highest potency on MCF-7 cells (IC_{50} = 21 μ M). Generally, we can say that comparison of activities for a series of compounds (**9a–9i**) with protected hydroxyl groups in the side chain and 3–N–H of the pyrimidine ring to those of corresponding analogs containing free functionalities (**10a–10i**) revealed that protected functionalities in compounds led to improvements in activities against MCF-7 cells relative to unprotected counterparts. The only exception was C-5-heptynyl acyclonucleoside **9f**, whose unprotected structural analog **10f** exhibited higher antiproliferative effect (IC_{50} = 37.8 μ M) than the protected one (IC_{50} = 62.2 μ M). Furthermore, compounds exerted no or weak nonspecific antiproliferative effects on the growth of the metastatic SW620 cells. Moreover, no detectable potency was observed against cervical carcinoma (HeLa) except for *N*-acyclic 5-(thiophene-2-yl)uracil derivative **8**, that showed considerable antiproliferative effect (IC_{50} = 7.8 μ M). CLogP values, as an index of molecular hydrophobicity and a parameter which affects the compound bioavailability and the interaction with the biological targets, amounted to between ca. –1.2 and ca. 5 for the most active compounds **6**, **8**, **9c**, and **9e**. Two acetoxy and *N*-3-benzoyl groups in **9c** (CLogP $\sim$ 5) and **9e** (CLogP $\sim$ 2) typically increased the lipophilicity in comparison to their unprotected analogs **10c** (CLogP $\sim$ 2) and **10e** (CLogP $\sim$ –0.7). The corresponding hydrolyzed compounds **10c** and **10e** showed only marginal or no antiproliferative effects, that was in agreement with findings that protecting groups increased the rate of cellular delivery by enhancing the lipophilicity of compounds and that they were subsequently cleaved by carboxylesterase within the cell.²⁸ Due to the excellent selective cytostatic activities against breast epithelial adenocarcinoma (MCF-7), acyclonucleosides **6**, **8**, **9c**, and **9e** were chosen to be further investigated regarding their mechanism of action. In order to characterize the mode of cell death induced by selected compounds, the apoptotic analysis was performed (Table 2).

Table 2. Results of Annexin V assay^a

Cell line	Duration	Compd.	Early apoptosis	Late apoptosis	Necrosis
MCF-7	24h	Control ^b	8.7%	7.5%	9.4%
		6	20.4%	19.7%	34.0%
		8	32.8%	33.6%	26.2%
		9c	41.7%	28.3%	34.6%
		9e	10.9%	36.6%	34.1%
	48h	Control ^b	36.1%	27.7%	25.4%
		6	39.3%	18.0%	28.7%
		8	54.9%	22.9%	13.1%
		9c	28.2%	24.1%	37.9%
		9e	20.9%	24.4%	31.6%

^aResults are presented as percentages of apoptotic/necrotic cells. IC_{50} values calculated by MTT-assay were used for treatment of MCF-7 cells for 24 and 48 h. ^bControl—untreated cells.

Obtained results confirmed the induction of apoptosis in MCF-7 cells treated with tested compounds (Table 2). All tested compounds, however, induced necrosis as well, which is partially expected due to intrinsic errors in the cascade of MCF-7 cells. Indeed, these cells did not express caspase 10, important in activation of extrinsic apoptosis and caspase 3, a central effector capsase for both extrinsic and intrinsic apoptosis pathways. This was in line with previous findings which also revealed that although caspase-3 deficient MCF-7 cells were

still sensitive to cell death induction by several stimuli, including tumor necrosis factor (TNF) and various DNA damaging agents, death of these cells occurred in the absence of DNA fragmentation.²⁹ Therefore, we hypothesized that parallel activation of other cell death mechanisms, such as necrosis, might be additionally driven by other signaling molecules besides caspases, i.e. sphingolipids that have been identified as important bioactive signaling molecules in cancer, including breast cancer.³⁰ Since sphingosine kinase 1 (SK1) and acid ceramidase 1 (ASAH1) were recently recognized as novel targets for intervention with antibreast agents,⁹ we have assessed the effect of potent cytostatic compounds **6**, **8**, **9c**, and **9e** on SK1 and ASAH1 expression in MCF-7 breast cancer cells. Acyclonucleosides **6**, **8**, **9c**, and **9e** can be considered as structural analogs of antineoplastic drug carmofur (Figure 1), that was identified as a highly potent inhibitor of intracellular ASAH1 activity.¹⁰ While compounds **6**, **8**, **9c**, and **9e** contain a PCV-like side chain, carmofur is an acyclic 5-fluorouracil analog bearing 1-hexylcarbamoyl at N-1. We found that C-5-heteroaryl acyclonucleosides **6** and **8** did not affect substantially the expression of SK1 and ASAH1 (Figure 3).

Figure 3. (a) Analysis of sphingosine kinase 1 (SK1) and (b) acid ceramidase 1 (ASAH1) expression in MCF-7 cells treated with compounds **6**, **8**, **9c** and **9e** at IC_{50} concentrations for 24 h. C – control, untreated cells.

Contrarily, C-5-alkynyl acyclonucleosides **9c** and **9e** completely abrogated the expression of ASAH1, leaving the levels of SK1 the same as in control, untreated cells (Figure 3). Our results are supported by previous findings^{6,10} showing that structural modification at C-5 of the pyrimidine scaffold had a major influence on ASAH1 activity. Therefore, replacement of the C-5-heteroaryl ring (in **6** and **8**) with the alkynyl moiety (in **9c** and **9e**) resulted in the blocking of ASAH1 expression. In addition, we presumed that the mechanism for growth inhibition of **6** and **8** might be via a different target. Therefore, potential biological targets for **6** and **8** were estimated using the program PASS (Prediction of Activity Spectra for Substances), that is widely utilized for prediction of pharmacotherapeutic effects and biological targets for drug-like compounds based on the SAR analysis of known compounds with experimentally established biological activity spectra.³¹ Thus, the PASS prediction for compounds **6** and **8** indicated that the observed antibreast cancer activity of compounds **6** and **8** might be attributed to the inhibition of the 17 β -hydroxysteroid dehydrogenase (17 β -HSD1) ($P_a \geq 0.5$, Table S1, Supporting Information). It has been confirmed that a high level of

estradiol (E2) is positively correlated to breast cancer risk, as this potent estrogen plays an important role in the proliferation of cancer cells.³² 17 β -HSD1 is considered to be the main enzyme involved in the conversion of estrogens or androgens, thus representing an attractive target for the development of new drugs against breast carcinoma. Therefore, C-5-heteroaryl acyclonucleosides **6** and **8** with free hydroxyl groups were evaluated for their inhibitory activities of 17 β -HSD1 at IC₅₀ and 5 $\times$ IC₅₀ concentration analyzed by MTT test in MCF-7 cells (Figure S1, Supporting Information). C-5-furyl acyclonucleoside **6** did not exhibit the anticipated inhibitory potency of 17 β -HSD1, while C-5-thienyl acyclonucleoside **8** showed a slight inhibitory effect of 17 β -HSD1 with no statistical relevance ($p > 0.05$) (Figure S1). Other possible modes of action of compounds **6** and **8** could be explained by their inhibition of DNA polymerase (DNA pols) that might affect cellular DNA synthesis. DNA pols that are involved in DNA replication and repair have been shown to serve as targets for known anticancer nucleosides.³³ Due to their similarity to natural nucleosides, compounds **6** and **8** could be metabolized by endogenous nucleoside kinases to their triphosphate forms within the cells and further could interact with the catalytic site of the DNA pols, competing with natural substrates and impeding the elongation of the DNA growing strand.

In conclusion, a series of novel C-5-substituted *N*-acyclic uracil derivatives **6**, **8**, **9a–9i**, and **10a–10i** was prepared and evaluated for their cytotoxic activity against three malignant tumor cell lines and normal fibroblasts. Linear, branched, aromatic, and cyclopropyl-alkynyl moieties were introduced at C-5 of the pyrimidine core using a palladium catalyzed cross-coupling Sonogashira reaction, while a C-5-heteroaryl moiety was introduced via Stille coupling reaction. Replacement of 5-iodine in the intermediate **4** with 5-furyl, 5-(*p*-pentylphenyl)-ethynyl, and 5-(cyclopropyl)ethynyl moieties led to new highly potent compounds **6**, **9c**, and **9e**, respectively, against MCF-7 cells. Compound **6** showed the most pronounced antibreast cancer activity (IC₅₀ < 0.01 μ M) with higher potency in comparison with the reference drug 5-fluorouracil (IC₅₀ = 0.09 μ M). Moreover, the cytostatic effects of **9c** and **9e** (IC₅₀ = 0.1 μ M) were comparable to the reference drug. However, compounds **6** and **9e** exerted cytotoxic effects on normal fibroblasts (IC₅₀ = 0.5 μ M for **6**; IC₅₀ = 0.3 μ M for **9e**), while compounds **8** and **9c** were less cytotoxic on normal fibroblasts. Further investigation of the mechanism of action for four selected compounds **6**, **8**, **9c**, and **9e** showed that these compounds induced cell death, partially due to apoptosis, of MCF-7 breast cancer cells. Furthermore, C-5-alkynyl substituted pyrimidines **9c** and **9e** could perturb ASAH1-mediated sphingolipid signaling by interfering with intracellular ASAH1 activity. Abrogation of ASAH1 expression of **9c** and **9e** may play an important role in their pronounced antibreast cancer activity. We can also conclude that 17 β -HSD1, estimated as a potential target by PASS prediction, cannot be recognized as a biological target for the strong antibreast cancer effect of C-5-furyl acyclonucleoside **6**. Hence, compounds **6**, **9c**, and **9e** could be considered as promising candidates for further lead optimization and development of a new potent and selective antibreast cancer agent. More extensive structure–activity relationship and precise mode of action for structurally related C-5-heteroaryl and C-5-alkynyl pyrimidine derivatives with diverse moieties at N-1 are envisaged and will be reported on in due course.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.5b00298.

In silico analysis of biological targets for C-5-heteroaryl pyrimidine derivatives **6** and **8**; evaluation of inhibitory potential of compounds **6** and **8** on 17 β -HSD1 activity; X-ray crystal structure analyses of compounds **1**, **2**, **6**, and **10g**; experimental procedures for the preparation of compounds, biological evaluation, and X-ray crystal structure determination; ¹H and ¹³C NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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