Article

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Parallel Solution Phase Synthesis and Preliminary Biological Activity of a 5'-Substituted Cytidine Analog Library

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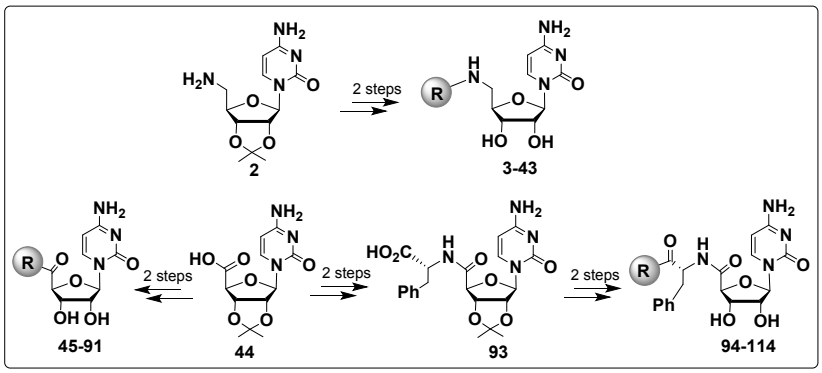
ABSTRACT: A 109-membered library of 5'-substituted cytidine analogs was synthesized, via funding through the NIH Roadmap Initiative and the Pilot Scale Library (PSL) Program. Reaction core compounds contained -NH_2 (**2)** and -COOH (**44** and **93)** groups that were coupled to a diversity of reactants in a parallel, solution phase format to produce the target library. The assorted reactants included -NH_2, -CHO, -SO_2Cl, and –COOH functional groups, and condensation with the intermediate core materials **2** and **44** followed by acidic hydrolysis, produced **3-91** in good yields and high purity. Linkage of the amino terminus of D-phenylalanine methyl ester to the free 5'-COOH of **44** and NaOH treatment led to core library –COOH precursor **93**. In a libraries from libraries approach, compound **93** served as the vital building block for our unique library of dipeptidyl cytidine analogs **94-114** through amide coupling of the –COOH group with numerous commercial amines followed by acidic deprotection. Initial screening of the complete final library through the MLPCN program revealed a modest number of hits over diverse biological processes. These hits might be considered as starting points for hit-to-lead optimization and development studies.



KEYWORDS: *cytidine nucleoside analogs, solution phase, diversity libraries, biological activities.*

INTRODUCTION

Nucleosides are fundamental biological components that interact with a significant portion of the human proteome, including peptide ligases, tRNA synthetases, polymerases, kinases, reductases, motor proteins, membrane receptors, and structural proteins. With advances in both high throughput chemical synthesis and screening, these key building blocks are increasingly relevant today, and they are finding utility in the discipline of chemical biology as well as modern drug discovery. As privileged and biologically relevant scaffolds, nucleosides continue to be useful as probes for novel and essential nucleoside-dependent processes. In fact, over the past few decades, new nucleoside-based agents have been discovered that are active for viral infections, cancers, cardiovascular disorders, pain, etc. A number of drugs have resulted from targeting nucleoside metabolism, including potent antiviral and anticancer drugs.[1-3] As with many drug classes, however, the increasing resistance of pathogens and cancers to current nucleoside-based drugs, the severe side effects sometimes produced by nucleoside antimetabolites, and the plethora of new nucleoside-dependent targets resulting from genetic sequencing highlight the continued need for new and unique nucleoside diversity sets relevant to this fundamental slice of the proteome. Such diverse nucleoside-based libraries will be potentially useful not only as probes for new chemical biology approaches to assess fundamental pathways and the role of target proteins in metabolism, growth, and drug resistance, but may also serve as a reservoir for new drug leads. Nucleosides are relatively low molecular weight compounds that can serve as superb starting components for diversity-oriented synthesis to prepare interesting and highly diversified small molecules with various groups having unique and varied spatial orientation for probing biological diversity space. In spite of their rich history and utility, however, nucleoside analogs are not commonly present in research or commercial chemical libraries.

Advancements in high-throughput chemistry have rarely been applied to nucleoside synthesis but may allow rapid preparation of diverse and biologically relevant libraries featuring this privileged scaffold. Thus, the goal of our PSL grant was to prepare novel and diverse nucleosides not only for our own use, but also as a public resource for access via government-sponsored chemical libraries and screening programs. It is notable that standard nucleosides can be promiscuous toxins via phosphorylation of the 5'-hydroxyl group and entry into numerous crucial metabolic pathways. As such, the perception that nucleoside drugs are generally nonselective and potently toxic through cross reactivity through multifarious DNA and RNA pathways has led to the stigma associated with this class. In sharp contrast, however, there are abundant cases of relatively discriminating nucleosides, both simple and complex, that exhibit specific biological activities.[5,6] The naturally occurring nucleoside antibiotics, are one such example. As a class, they show widely varying and compelling activities against numerous specific targets such as the protein synthesis machinery, glycosyltransferases, and methyltransferases as well as many other critical protein targets.[4-7] Of particular interest are the 5'-substituted peptidyl nucleoside analogs containing a diversity of amino acids and amino sugars, leading to the vigorous research to isolate and prepare new amino acid and perptide-substituted nucleosides.[7-11] Herein, we report the preparation of a focused set of cytidine compounds inspired by the broad class of natural peptidyl nucleosides[6,12,13] including capuramycin, the polyoxins (tunicamycin and nikkomycin), the muramycins and the mureidomycins.

EXPERIMENTAL DESIGN AND DISCUSSION

We recently described the preparation and testing of a variety of adenosine and uridine analogs that demonstrated diverse biological activities in early MLPCN assays.[14] We now report the

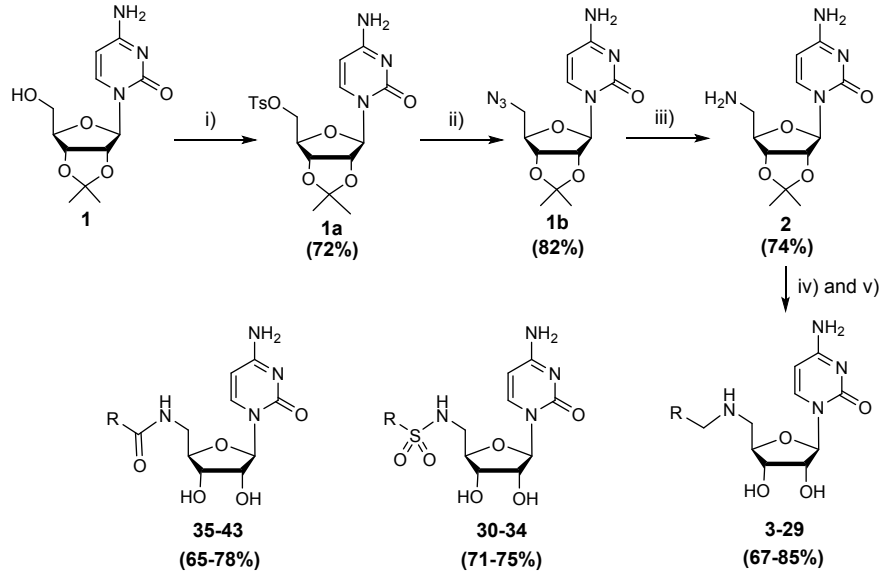
synthesis of a cytidine analog library derived from intermediates **2** (-NH_2 reactant) and **44** and **93** (-COOH reactants) (Schemes 1 & 2) using solution phase, parallel chemistry.

Our goal was to target variations around the 5'-position to mimic structures represented by the natural nucleoside antibiotics that demonstrate a variety of interesting activities. This task was accomplished through robust coupling chemistry either utilizing 5'-aminocytidine (**2**) or the 4'-COOH analog of the nucleoside to produce amines (*via* reductive amination), amides (peptide coupling), or sulfonamides (*via* diverse commercial R-SO_2Cl reagents). In a libraries-from-libraries approach, D-phenylalanine was randomly chosen to be coupled to **44** to give the chiral amino acid intermediate. Further coupling through the free D-Phe carboxylic acid yielded a diverse array of amide-linked analogs potentially relevant to the natural nucleoside antibiotics, since the more complex natural products oftentimes contain chiral linkages (amino acids, carbohydrates, etc.) that are considered critical for biological uptake and targeting. It was essential to demonstrate that our new library was unique in order to obtain PSL funding. Hence, we first selected a commercial set of -CHO, -NH_2, -COOH, and –SO_2Cl containing reactants that were obtainable in satisfactory amounts and at an acceptable cost to drive downstream synthesis and diversity. This set was further filtered by eye for diversity and desired reactivity to work in the designed coupling reactions and provide the expected diversity of nucleoside products. Finally, the 3D representations were further assessed using Tanimoto structural similarity relative to the current MLSCN library set at that time. Tanimoto similarity is one measure to determine similarity between compounds and compound sets using compound fingerprints. The score ranges from 0 to 1 with 0 (no similarity) and 1 (100% similarity). A Tanimoto value of 0.7 to 1.0 suggests that two molecules are highly similar to identical. Every proposed structure was compared with the total MLSCN library (as of the year 2010 - 197,873 discrete molecules). Only 9.3% of our total

proposed set had a Tanimoto value greater than 0.7, indicating that the new materials were significantly divergent from the MLSCN library available in 2010. Furthermore, 36% of the new nucleosides had Tanimoto values below 0.5, indicating significant diversity relative to the current MLSCN library.

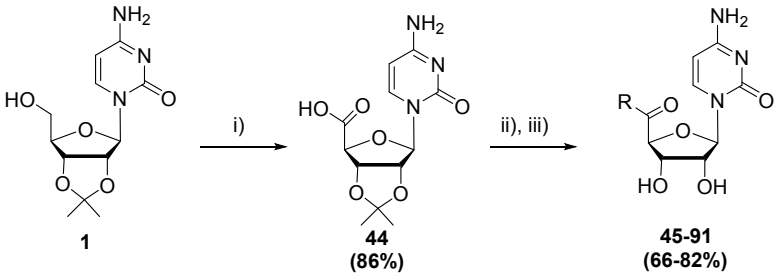
5'-Amino and 5'-carboxylic nucleosides are common staring points to prepare diverse nucleosides for screening.[12-20] Consequently, the nucleoside **2** and nucleosides **44** and **93** were chosen as three diversification reagents for robust reductive aminations, sulfonation reactions or amidations in order to synthesize our unique cytidine-based library as depicted in Schemes 1-3.

Scheme 1: Synthesis of Analogs 3-43



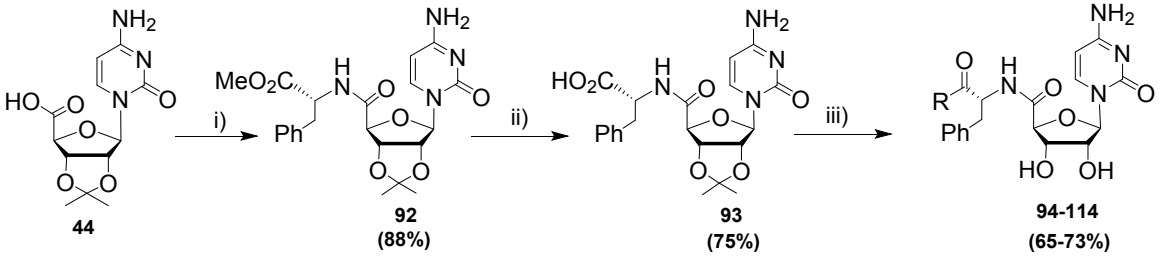
Reagents and conditions: i) TsCl, pyridine; ii): NaN_3, DMF, 50 ^oC. iii): NH_4OOCH, Pd-C 10%, MeOH. iv) aldehyde derivative MeOH, 0-40 ^oC, $NaBH_4$; sulfonyl chloride derivative, DMF, Cs_2CO_3 or carboxylic acid derivative, HATU, DIEA, CH_3CN; v) 50% HCO_2H, 70 ^oC.

Scheme 2: Synthesis of Analogs 45-91



***Reagents and conditions**:* i) cat. TEMPO, BAIB, $NaHCO_3$, MeCN:H_2O, r.t.; ii): R = amine derivative, HATU, DIEA, CH_3CN.; iii) 50% HCO_2H, 70 ^oC.

Scheme 3: Synthesis of Analogs 94-114



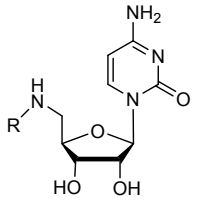
***Reagents and conditions**:* i): Methyl (*R*)-2-amino-3-phenylpropanoate, HATU, DIEA, MeCN. r.t.; ii): 1N NaOH, dioxane, r.t.; iii): a: R = amine derivatives, HATU, DIEA, MeCN, r.t.; b: 50% HCO_2H, 70 ^oC, 2 h.

5'-Amino-5'deoxycytidine (**2**) was prepared from **1** in three steps *via* a reported procedure and was identical in all respects to the reported material.[20] Reductive amination is efficient and easily adaptable to parallel chemistry, and we utilized this approach to couple pure compound **2** with a variety of diverse, commercially available -CHO containing reagents. Coupling was accomplished in MeOH over molecular sieves to drive intermediate imine formation through water removal; the drying reagent was critical for higher yields. A Radleys 12-place carousel reaction station was used for parallel production of the libraries. Chemistry was carried out at room temperature unless indicated as certain reactions with poorly soluble aldehydes required warming to 40 ^oC for 10 minutes. The aldimine intermediates were treated *in situ* by careful addition of solid $NaBH_4$. After 30 minutes, the reaction was adsorbed onto silica gel without further workup and dried followed

by automated flash chromatography purification. Acid catalyzed removal of the acetonide protecting group in 50% formic acid yielded the final compounds **3**-**29** (Scheme 1) in 67-85% yields.

Target compounds are presented in Charts 1-3.

Chart 1: Structures of Analogs 3-43



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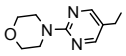
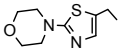
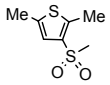
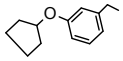
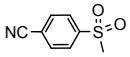
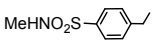
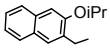
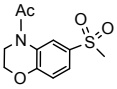
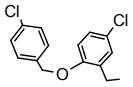
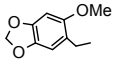
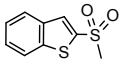
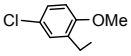
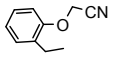
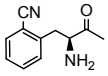
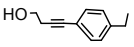
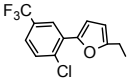
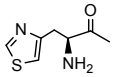
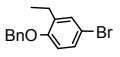
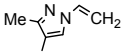
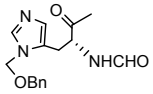
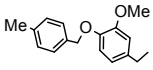
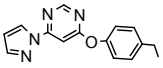
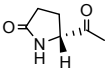
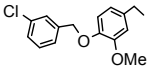
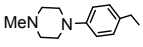
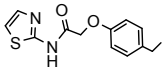
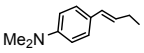
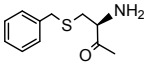
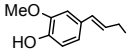
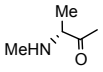
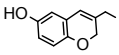
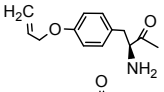
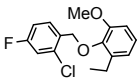
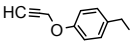
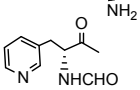
3		17		31	
4		18		32	
5		19		33	
6		20		34	
7		21		35	
8		22		36	
9		23		37	
10		24		38	
11		25		39	
12		26		40	
13		27		41	
14		28		42	
15		29		43	
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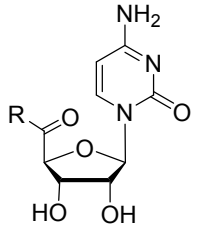
Chart 2: Structures of Analogs 45-91



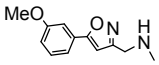
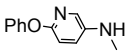
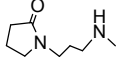
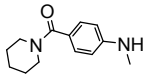
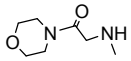
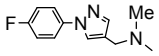
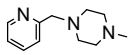
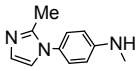
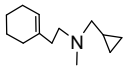
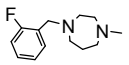
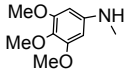
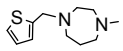
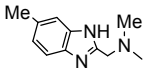
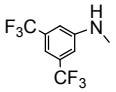
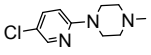
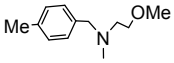
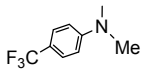
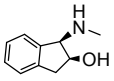
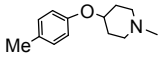
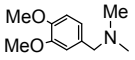
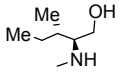
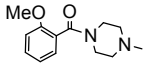
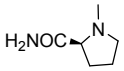
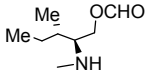
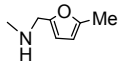
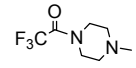
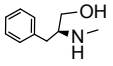
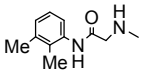
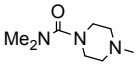
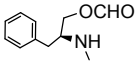
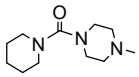
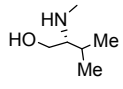
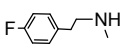
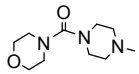
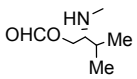
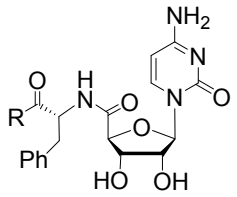
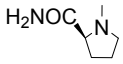
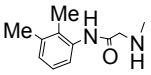
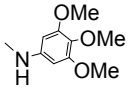
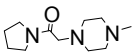
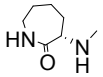
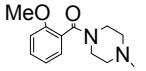
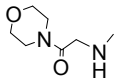
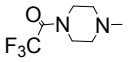
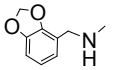
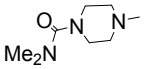
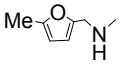
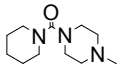
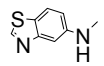
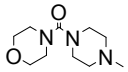
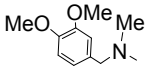
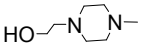
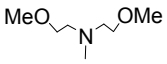
Compound	R	Compound	R	Compound	R
45		61		77	
46		62		78	
47		63		79	
48		64		80	
49		65		81	
50		66		82	
51		67		83	
52		68		84	
53		69		85	
54		70		86	
55		71		87	
56		72		88	
57		73		89	
58		74		90	
59		75		91	
60		76			

Chart 3: Structures of Analogs 94-114



Compound	R	Compound	R
94		**105**	
95		**106**	
96		**107**	
97		**108**	
98		**109**	
99		**110**	
100		**111**	
101		**112**	
102		**113**	
103		**114**	
104			

A 24-position MiniBlock XT vessel was employed to prepare our library members **30-43** (Chart 1), **45-91** (Chart 2) and **94-114** (Chart 3) in solution phase. A Tecan liquid handling system was used for dispensation, retraction, and aspiration with a compatible 17 × 110 mm test tube carousel and a Genevac evaporation system. In this format, five sulfonyl chlorides were reacted with **2** to prepare the sulfonylamide cytidine analogs **30-34** (Scheme 1) using the solvent DMF and CsCl (base) for coupling. The isopropylidene blocking groups were removed as described above. HATU (1 equiv) was used for amide coupling of **2** with nine -COOH compounds catalyzed by DIEA (1.5 equiv) in acetonitrile (three hours) to prepare amide-linked compounds **35-43** in 65-78% yields (Scheme 1) after acid mediated deprotection.. These conditions with intermediate **44** gave amides **45-91** in 66-82% yields (Scheme 2). Compound **44** was prepared by reported methods using catalytic amounts of TEMPO and BAI in acetonitrile-water (1:1).[12a]

Compound **44** was coupled to the free amino group of D-phenylalanine-COOMe **92** (Scheme 3) in 88% yield. Hydrolysis with 1N NaOH in dioxane at 25 ^{o}C gave the -COOH intermediate **93** in 75% yield. Deprotection with 50% formic acid at 70 ^{o}C afforded a high yield of nucleoside **94**. Target **93** was designed from a "libraries from libraries" standpoint, and produced a site of further diversity expansion through another amide coupling with diverse amines to produce targets **95-114** (Scheme 2). Coupling of **93** with 20 diverse amines and HATU/DIEA produced the desired targets in 65-73% yield and high purity after acid catalyzed removal of the isopropylidene protecting group and purification.

Analysis of all products involved ^{1}H NMR, HPLC, and mass analysis. Purity ranged from 90-100%, and the average purity was 97%. Comparison of the NMR spectra of crude **93** as its carboxylate salt with spectra of the corresponding crude *epi*-**93** carboxylate salt -prepared by amide coupling of **44** with methyl (*S*)-2-amino-3-phenylpropanate to form *epi*-**92**, purification, then

saponification using 1N LiOH in dioxane - established that the hydrolysis of **92** to **93** proceeds without epimerization.[22]

BIOLOGICAL EVALUATION

All biological screening was performed via government grants and contracts to external and independent academic laboratories through the MLPCN program, and all results can be readily accessed by a specific assay name in PubChem Assay or through the compound database via www.ncbi.nlm.nih.gov/pcsubstance (search term Robert Reynolds). Certain nucleosides (see Table 1) afforded a variety of modest activities in the primary screens.

Table 1: Selected Results from PubChem Bioactivity Analysis

Compound	Assay Title in PubChem	Biological results
54, 59, 65, 97, 101, 103, 107 and **112**	"High-Throughput-Screening (HTS) to identify inhibitors against Sialic Acid Acetyl Esterase (SIAE)"	26%, 52%, 26% 45%, 17%, 47%, 45% and 23%, respectively at 9.6 μM
28	"Discovery of small molecule inhibitors of the oncogenic and cytokinetic protein MgcRacGAP."	34% inhibition at 40 μM
35	"HTS to identify agonists of the mouse 5-hydroxytryptamine (serotonin) receptor 2A (HTR2A)"	29% inhibition at 7.6 μM
39	"HTS to identify inhibitors of phospholipase C isozymes (PLC-gamma1)."	10% inhibition at 12.2 μM
42	"Small Molecule Inhibitors of FGF22-Mediated Excitatory Synaptogenesis & Epilepsy Measured in Biochemical System Using RT-PCR."	43% inhibition at 9.9 μM
54	"HTS to identify positive allosteric modulators (PAMs) of the human cholinergic receptor, muscarinic 4 (CHRM4)."	20% activation at 3 μM
59 and **85**	"THS to identify modulators of interaction between CendR and NRP-1."	40% inhibition at 25 μM
65	"Counterscreen for inhibitors of 5-meCpG-binding domain protein 2 (MBD2)."	43% inhibition at 4.4 μM

57 and **71**	"DENV2 CPE-Based HTS Measured in Cell-Based and Microorganism Combination System"	70% and 87 inhibition, respectively, at 9.99 μM
80	"HTS to identify D3 Dopamine Receptor Antagonist."	40% inhibition at 2.3 μM
90	"HTS to identify inhibitors of the interaction of nucleotide-binding oligomerization domain containing 2 (NOD2) and the receptor-interacting serine-threonine kinase 2 (RIPK2)."	28% inhibition at 5 μM
102	"HTS to identify inhibitors of protein arginine methyltransferase 1 (PRMT1)."	21% inhibition at 2.8 μM

For example: 5'-carboxamide nucleoside analogs **54**, **59** and **65**, and di-peptidyl compounds **97**, **103**, **107** and **112** showed modest, lead-like inhibitory activity against Sialic Acid Acetyl Esterase (SIAE) (Table 1). This enzyme could serve as a target for the pharmacological induction of immune activation to empower B cells that are specific for weak epitopes and to enhance T cell memory responses.

It has been shown that excitatory and inhibitory synapses can be organized in the brain by two fibroblast growth factor (FGF) family members, FGF22 (excitatory) and FGF7 (inhibitory). . Remarkably, FGF22-deficient mice are resistant to epileptic seizures, and FGF7-deficient mice are prone to them.[22] These results indicate that the identification of small molecules that inhibit FGF22-mediated excitatory synapse formation or those that can activate FGF7-mediated inhibitory synapse formation may lead to new treatment approaches for epilepsy. Analog **42** showed modest inhibition (43%) against FGF22 at 9.9 μM (Table 1) and may be used as a lead for developing potent FGF inhibitors due to its unique structure.

In addition, protein arginine methyltransferase 1 (PRMT1) activity has been linked with a number of human health conditions including cardiovascular disease, cancer, infectious disorders and autoimmune conditions.[23] Di-peptidyl analog **102** showed modest activity against PRMT1 with

21% inhibition at 2.8 μM (Table 1) and may warrant further investigation as this structure is unique from other reported inhibitors.[23]

CONCLUSION

Preparation and initial screening of a unique library of 109 cytidine-based compounds is reported. Application of newer higher throughput equipment and technologies were utilized in the preparation of this small-sized diversity set. Targets were sent to the MLPCN for screening, and early assay results showed modest activities against the targets SIAE, FGF, and PRMT1. The PRMT1 hit, while only modestly active, is unique relative to reported PRMT1 inhibitors, and we are currently preparing more material as well as analogs for confirmation of activity in advanced assays. All assay data and protocols can be viewed by visiting PubChem Substance.

ASSOCIATED CONTENT

Supporting Information detailing the general experimental procedures and analytical data for the libraries is available free of charge on the ACS Publications website at DOI:.

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Notes

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ABBREVIATIONS

PSL, Pilot Scale Library; NIH, National Institutes of Health USA; tRNA, transfer ribonucleic acid; MLPCN, Molecular Libraries Probe Production Centers Network; DNA , deoxyribonucleic acid; MLSCN, Molecular Libraries Screening Centers Network; DMF, *N,N*-dimethylformamide; HATU, [(2-(7-Aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]; DIEA, *N,N*-diisoproplyethylamine; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxyl; BAIB, [bis(acetoxy)iodo]benzene; NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography; FGF, fibroblast growth factor; PRMT1, Protein arginine methyltransferase 1; SIAE, Sialic Acid Acetyl Esterase; TLC, thin layer chromatography; TMS, tetramethylsilane.

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