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Antiproliferative activities of halogenated thieno[3,2-*d*]pyrimidines

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ABSTRACT

The in vitro evaluation of thieno[3,2-*d*]pyrimidines identified halogenated compounds **1** and **2** with antiproliferative activity against three different cancer cell lines. A structure activity relationship study indicated the necessity of the chlorine at the C4-position for biological activity. The two most active compounds **1** and **2** were found to induce apoptosis in the leukemia L1210 cell line. Additionally, the compounds were screened against a variety of other microbial targets and as a result, selective activity against several fungi was also observed. The synthesis and preliminary biological results are reported herein.

1. Introduction

Fused bicyclic pyrimidines, such as the thieno- and pyrrolopyrimidines, are attractive scaffolds for drug design due to their close resemblance to the purines, arguably the most biologically significant class of bicyclic heterocyclic compounds.[1–5] Previously, Klein et al. explored the properties of the thieno[3,2-*d*]pyrimidine scaffold as nucleoside isosteres. Compounds containing the thienopyrimidine moiety exhibited moderate activity against tumor cell proliferation in vitro.[3,6] In the ensuing years, various thienopyrimidine analogues attracted additional attention due to the broad spectrum of biological properties they exhibited.[7–20] With a variety of annulations and functional group manipulations possible, many thieno[3,2-*d*]pyrimidine derivatives have shown interesting biological activity including as kinase[17–20] and phosphodiesterase[9] inhibitors (Fig. 1), among other uses.[21–23] Moreover, the thiophene ring itself has served as an isostere for benzene-fused pyrimidines in the design of molecules that possess antimalarial[13] and kinase inhibitory[14] activity.

Our laboratory has long been interested in the design, synthesis and medicinal properties of sulfur-containing tricyclic heterocycles whereby a thiophene ring has been introduced as a spacer between the imidazole and pyrimidine of the purine scaffold (Fig. 2).[11,24–27] Notably, several thiophene-expanded purine tricyclic analogues related to those shown in Figure 2, exhibited inhibitory activity against colorectal cancer cell proliferation and trypanosomes.[11,27] As an extension of this early work, the synthesis and biological evaluation of several thiophene 'extended' pyrimidine nucleosides was pursued.[28,29]

During the course of the investigation, key intermediates, including the heterocyclic bases, were subjected to broad screen biological testing. Although the original purpose was to explore the biological properties of the nucleosides,[28] the halogenated thieno[3,2-*d*]pyrimidine intermediates **1** and **2** were found to exhibit antiproliferative properties against several cancer cell lines. Furthermore, the cell cycle and apoptosis studies revealed their ability to induce apoptosis independent of cell cycle. In addition, evaluations of their antimicrobial activity lead to selective inhibition of clinical strains of *Cryptococcus neoformans*. Since **1** and **2** are reaction intermediates, their syntheses had been reported previously,[23] however a search of the literature revealed that surprisingly, there were no reports of biological activity, including against cancer. Herein we describe a structure activity relationship study for a series of thieno[3,2-*d*]pyrimidine analogues that facilitated identification of core functional groups responsible for their biological activity.

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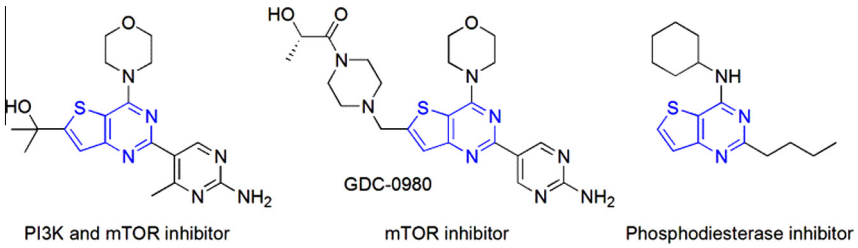


Figure 1. The thieno[3,2-*d*]pyrimidine scaffold in drug design.

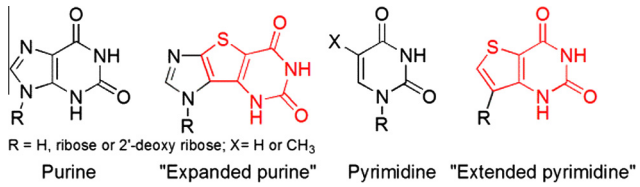


Figure 2. Thieno[3,2-*d*]pyrimidine as a component of the nucleobase scaffold.

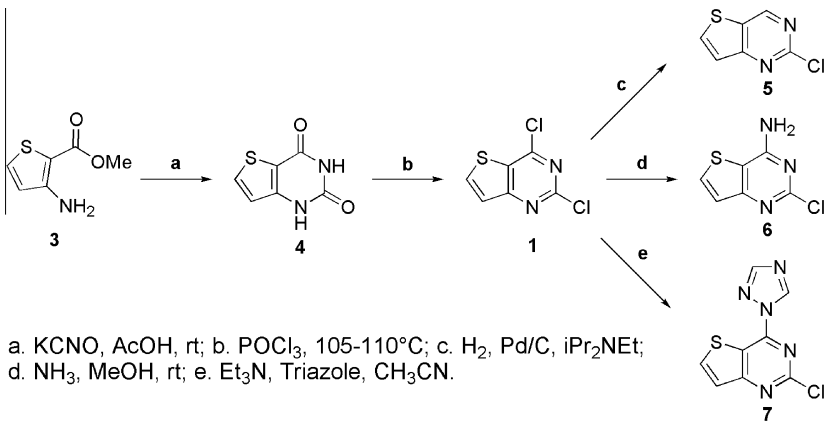
2. Results

2.1. Chemistry

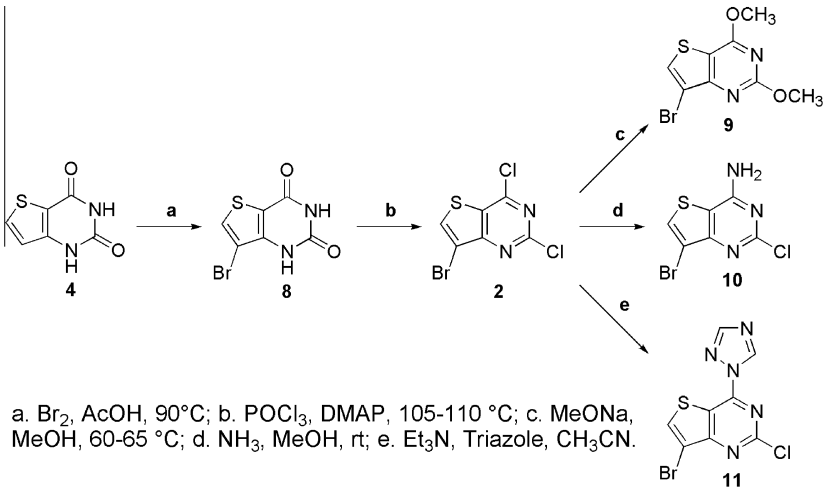
The thieno[3,2-*d*]pyrimidines were realized by synthesizing thieno[3,2-*d*]pyrimidin-2,4-dione **4** through a ring-cyclization of 2-methyl-3-aminothiophene carboxylate **3** (Scheme 1).[21–23] Subsequently, thieno[3,2-*d*]pyrimidin-2,4-dione **4** was subjected to chlorination by refluxing in phosphorous oxychloride ($POCl_3$)[30] for 20–24 h, at which point the $POCl_3$ was removed and the reaction mixture neutralized using saturated sodium bicarbonate ($NaHCO_3$) followed by crystallization of **1** from ethyl acetate (EtOAc). The chlorinated intermediate **1** was then subjected to various nucleophilic substitutions as shown in Scheme 1. Hydrogenation of **1** under basic conditions afforded the dehalogenated product **5** in 80% yield.[31] Interestingly, the rate of dehalogenation in the presence of *N*,*N*-diisopropylethylamine (DIPEA) was faster when compared to sodium bicarbonate ($NaHCO_3$). It should be noted that even with prolonged hydrogenation conditions, loss of the 2-Cl did not occur, implying that the C-2 position is fairly unreactive.

Next, stirring **1** in methanolic ammonia at room temperature resulted in substitution of the 4-Cl group to afford the amino-substituted thieno[3,2-*d*]pyrimidine **6**. The progress of the reaction can be monitored by the gradual dissolution of **1** in methanol (MeOH) indicating the successful substitution of the 4-Cl by ammonia. Not surprisingly given our previous observations with the hydrogenation reactions, substitution at the 2-Cl was not observed even at elevated temperatures (80–100 °C) and extended reaction times. Upon stirring **1** with triazole under basic conditions at room temperature, the 4-Cl was successfully substituted by the triazole to give **7** in 86% yield.[32]

A second series of 7-bromo compounds (Scheme 2) was realized in a similar manner as depicted in Scheme 1. Although the bromine at C7 was originally introduced to enable subsequent Heck coupling and formation of the *C*-nucleoside targets,[28,29] presence of the bromine serendipitously resulted in antiproliferative activity against several cancer cell lines as discussed in the next section. The syntheses of **2**, **8**, and **9** have been previously reported by Tor et al. for the study of fluorescent nucleoside analogues.[23] During the course of our studies however, modifications to their approach[23] were undertaken—the bromination was carried out at 90 °C instead of 120 °C and the bromine was added in two portions instead of one, at an interval of 24 h between the additions, to obtain 7-bromothieno[3,2-*d*]pyrimidin-2,4-dione **8** in 90–95% yields. Additionally, the chlorination reaction was conducted using $POCl_3$ in presence of dimethylaminopyridine (DMAP) instead of previously reported[23] *N*,*N*-dimethylaniline (DMA). This change was introduced to avoid the formation of intractable emulsions that were observed when DMA was used. Furthermore the reflux was stopped after 2 h to obtain **2** in a 60% yield. Substitution of the 4-Cl with a methoxy group was then achieved by refluxing **2** with sodium methoxide in MeOH as reported previously.[23] The amine (**10**) and triazole (**11**) substitutions were achieved at room temperature in a similar manner as reported for **6** and **7** in Scheme 1, respectively. It should be noted that substitution of the 4-Cl group was quite facile, however the 2-Cl remained unreactive in all cases.[33]



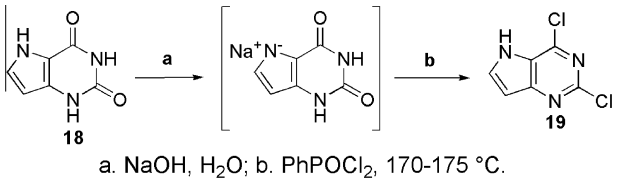
Scheme 1. Synthesis of substituted thieno[3,2-*d*]pyrimidines.



Scheme 2. Synthesis of 7-bromo analogues.

Next, the preparation of the *C*-nucleosides was accomplished via Heck coupling of 7-bromo-2,4-dimethoxythieno[3,2-*d*]pyrimidine **9** with glycal **12**[29] (Scheme 3) similar to a previous report by Tor et al.[23] Several additional steps were added, however, to facilitate purification, thus the syntheses of **15** and **16** were also accomplished. The Heck coupling afforded keto intermediate **13** in 40–55% yield, and subsequent stereoselective reduction of the ketone using sodium triacetoxyborohydride gave 2′-deoxy **14** in 72% yield. Deprotection of the methoxy of **14** using sodium iodide in AcOH resulted in a highly polar product, which was difficult to purify, thus **14** was instead acylated using acetic anhydride[32] to afford **15** which was then subjected to hydrolysis of the methoxy groups using sodium iodide in AcOH to afford **16**.[23] The subsequent purification was facilitated by the presence of acetyl groups on the sugar. Removal of the acetyl groups by methanolic ammonia then afforded nucleoside **17** in a 75% yield (Scheme 3).

Based on the promising biological activity of halogenated thieno[3,2-*d*]pyrimidines **1** and **2**, 2,4-dichloropyrrolo[3,2-*d*]pyrimidine **19** was synthesized (Scheme 4) to evaluate the effect of sulfur and nitrogen in the fused five-membered ring on the biological activity. This was accomplished by preparation of the sodium salt of pyrrolo[3,2-*d*]pyrimidin-2,4-dione **18**[30,34–36] by stirring with 1 N NaOH at 40 °C followed by heating with phenylphosphonic dichloride ($PhPOCl_2$) at 170–175 °C for 5 h. The hot reaction mixture was poured on ice and upon purification by column chromatography **19** was obtained in 55–60% yield.[37]

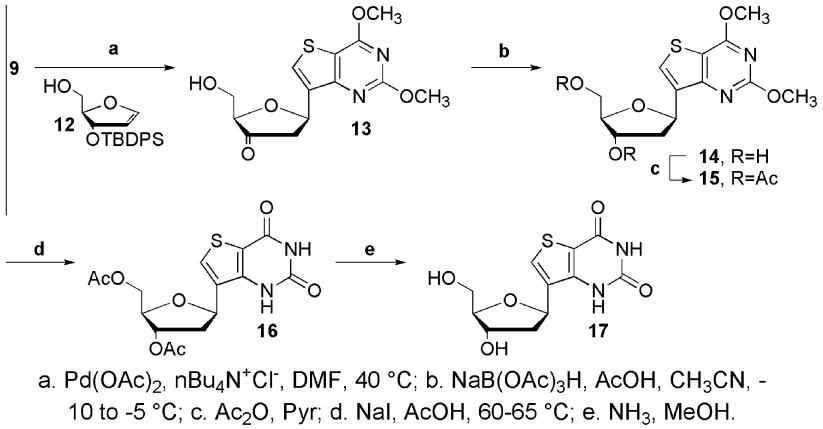


Scheme 4. Synthesis of halogenated pyrrolo[3,2-*d*]pyrimidine.

2.2. In vitro tumor cell growth inhibition

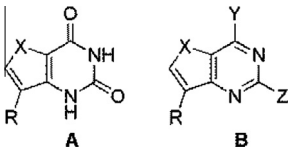
The ability of selected compounds to inhibit tumor cell proliferation were tested using L1210, a mouse lymphocytic leukemia cell line[38], CCRF-CEM[39], an acute lymphoblastic leukemia cell line, and HeLa, a cancer cell line derived from a human cervical adenocarcinoma.[40,41] Cell counting assays (Table 1) indicate that the dichloro compounds **1** and **2** are the most active compounds against all three cell lines. As an orthogonal approach, the effects of compounds **1** and **2** on cell viability were also measured using MTT assays in L1210 cells.

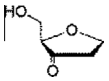
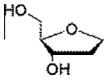
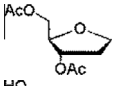
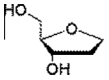
These assays monitor cellular metabolic activity rather than cell number, but yielded very similar IC_{50} values for both compounds (cf. Fig. 3 versus Table 1). Replacement of 4-Cl with hydrogen (**5**) leads to complete loss of cytotoxic activity indicating the necessity of 4-Cl for activity (Table 1). Similarly, substitution of 4-Cl by an



Scheme 3. Synthesis of the thieno[3,2-*d*]pyrimidine C-nucleosides.

Table 1
Anti-tumor cell activity of test compounds.



Compound	Structure	m	Y	Z	R	IC_{50}[a] (μM)			MIC[b] (μM)		
						L1210	CEM	HeLa	HEL	Vero	HeLa
1	B	S	Cl	Cl	H	0.67 ± 0.50	5.2 ± 1.3	3.9 ± 0.5	100	20	4
2	B	S	Cl	Cl	Br	5.4 ± 1.2	14 ± 9	4.3 ± 0.2	20	20	20
4	A	S	—	—	H	>250	>250	>250	NT[c]	NT	NT
5	B	S	H	Cl	H	>250	>250	>250	NT	NT	NT
6	B	S	NH_2	Cl	H	>250	>250	>250	NT	NT	NT
7	B	S		Cl	H	>250	>250	>250	NT	NT	NT
8	A	S	—	—	Br	>250	>250	>250	NT	NT	NT
9	B	S	OCH_3	OCH_3	Br	>250	⩾ 250	203 ± 61	NT	NT	NT
10	B	S	NH_2	Cl	Br	150 ± 14.0	109 ± 15.0	96 ± 6.0	NT	NT	NT
11	B	S		Cl	Br	157 ± 0	84 ± 12.0	116 ± 8.0	NT	NT	NT
13	B	S	OCH_3	OCH_3		>250	>250	>250	NT	NT	NT
14	B	S	OCH_3	OCH_3		>250	>250	>250	NT	NT	NT
15	B	S	OCH_3	OCH_3		>250	>250	>250	NT	NT	NT
17	A	S	—	—		>250	>250	>250	NT	NT	NT
19	B	NH	Cl	Cl	H	6.8 ± 2.8	25 ± 2.0	19 ± 3.0	20	⩾20	20
5FU	—	—	—	—	—	0.33 ± 0.17	18 ± 5	0.54 ± 0.12	NT	NT	NT

[a] 50% inhibitory concentration.
[b] Minimum concentration for inhibition by 99%. 5FU-5-fluoro Uracil.
[c] NT - Not tested.

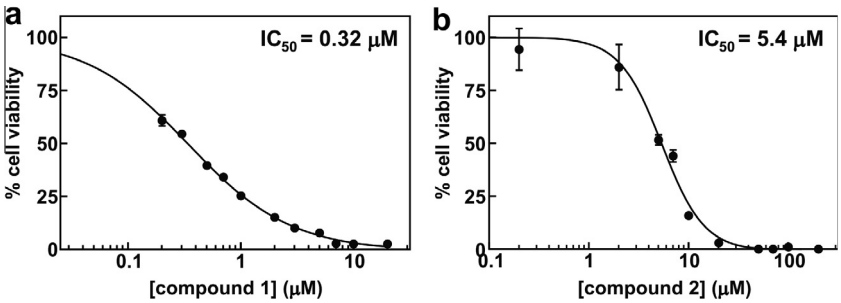


Figure 3. MTT assays of cytotoxicity by **1** and **2** in L1210 cells. L1210 cells were treated with selected concentrations of compounds **1** (a) and **2** (b). After 48 h, cell viability was measured using MTT assays. Compound cytotoxicity was calculated by non-linear regression to a sigmoidal dose response function and is quoted as the IC_{50}.

amine or triazole leads to complete loss of activity as in the case of **6** and **7**. However, with a 7-Br group, the amino **10** and triazolo **11** derivatives retained some activity, albeit modest. The presence of a carbonyl group as in the case of **4**, **8**, and **17** also did not result in antiproliferative activity. The nucleoside analogues **13**–**15**, and **17** were also tested, but none inhibited the growth of the cancer cell lines. These results lend credence to the importance of the 4-Cl group on the nucleobase (Fig. 4).

Furthermore, replacement of the sulfur with nitrogen in the fused five-membered ring (**19**) leads to loss of activity by a factor of 5-10 although the dichloropyrrolo[3,2-*d*]pyrimidine retained some activity, thereby further alluding to the role of the 4-Cl group. Thus, preliminary conclusions were (i) that the 4-Cl appears to be critical for cytostatic activity; (ii) that the presence of the sulfur enhances the activity by a factor of 5–10 and (iii) bromine imparts modest cytotoxic properties in the absence of the 4-Cl group (Fig. 4).

As previously mentioned, the thieno[3,2-*d*]pyrimidine scaffold has been used extensively in the design of kinase inhibitors, and has also been associated with excellent anticancer properties.[17–20] As a result compounds **1** and **2** were screened against twenty kinases at 5 μM using Invitrogen's Select Screen® kinase profiling[42]

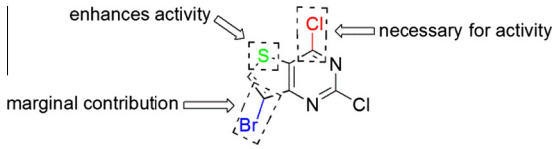


Figure 4. Structure–activity relationship (SAR) for halogenated thieno[3,2-*d*]pyrimidines.

service (S1) to explore the possibility of kinase inhibition by the halogenated thieno[3,2-*d*]pyrimidines. Surprisingly they did not exhibit inhibitory activity against any of the twenty tested kinases, suggesting that the antiproliferative activity may be due to other mechanisms. As a result, the lack of inhibitory activity against the kinases opened up an array of alternative possibilities for the mechanism of action of the antiproliferative activity of **1** and **2**. In that regard, to gain further insight into the molecular mechanism(s) of action, cell-cycle analysis and apoptosis studies were undertaken.

Many antineoplastic compounds arrest cell proliferation by activating specific checkpoints that block progression through the cell cycle.[43] To test whether compounds **1** or **2** inhibited cell proliferation by this mechanism, we monitored their effects on L1210 cell cycle distributions using propidium iodide staining and flow cytometry (Fig. 5). Vehicle-treated cells were largely confined to G1 and S phases, with very small (<5%) subpopulations in the G2/M phase or showing sub-(<G1) or super-genomic (>G2) DNA content. Surprisingly, 24 h or 48 h treatment with IC_{50} concentrations of either compound **1** or **2** yielded no substantial changes in L1210 cell cycle distribution, indicating that these reagents do not suppress cell growth by activating cell cycle checkpoint mechanisms.

Subsequently, unfixed L1210 cell samples were analyzed by staining with Annexin V and 7-amino-actinomycin D (7-AAD) to identify potential cell death pathways that may be activated by either compound. Flow cytometric analyses of cells treated with vehicle for 48 h indicated that the vast majority were non-apoptotic and non-necrotic (Fig. 6a and c, Annexin V negative, 7-AAD negative). However, after incubation with 1 μM compound **1** for 48 h, 60% of the cells were observed undergoing apoptotic cell death, with the vast majority of these in early apoptosis (Fig. 6b, Annexin V positive, 7-AAD negative). Cell death consistent with apoptosis was also evident in L1210 cells treated with 5.4 μM solution of **2** where 55% of cells were Annexin V positive (Fig. 6d), although a larger proportion of these (40%) were also 7-AAD positive, consistent with late apoptosis. Together, the cell cycle and apoptosis assays indicate that both compounds **1** and **2** can induce cell death by an apoptotic-like pathway, but in a manner that does not require arrest at a specific stage of the cell cycle.

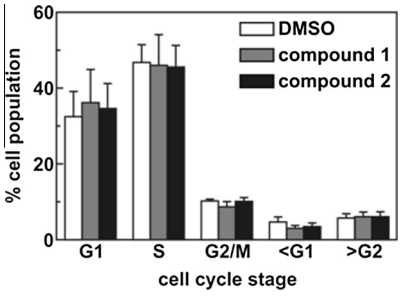


Figure 5. Cell cycle analyses of L1210 cells following treatment with **1** and **2**. Cell cycle distributions of L1210 cells after 48 h in the presence of 0.32 μM compound 1, 5.4 μM compound **2**, or vehicle control (DMSO) analyzed by flow cytometry of fixed, propidium iodide-stained cells. A minimum of 3000 cells were analyzed per cell population. Each bar represents the mean ± SD across 4 independent cell samples.

2.3. Evaluation of antimicrobial activity in vitro

Several of the compounds were tested for inhibitory activity against a variety of DNA and RNA viral strains. However, the antiviral activity of the halogenated compounds **1** and **2** could often not be fully evaluated because of their pronounced cytotoxicity against the mammalian host cell cultures while the other compounds did not exhibit significant antiviral activity.

In the subsequent antimicrobial testing, **1**, **2**, **5** and **19** were screened for growth inhibition activity against a panel of bacteria and fungi at a concentration of 100 μM as shown in Table 2. Compound **5** was included to test the importance of the 4-Cl substituent for activity across a broader range of biological systems. Compounds **1** and **2** exhibited activity against several pathogenic yeast strains (55–99% growth inhibition) and compound **1** also showed weak activity against *Bacillus subtilis*. In addition, **1** displayed a higher potency than **2** against several clinical strains of *Cryptococcus neoformans*.

Compounds **1** and **2** were further tested against the susceptible fungal strains to determine the MIC_{95} values (Table 2). Although the spectrum of anti-microbial activity for **1** is broad (Table 3), the bromo analogue **2** is much more selective towards fungi and 2–5 times more potent than **1** (Table 3).

3. Discussion

Traditionally, halogens have been introduced on organic molecules to facilitate functional group conversions for nucleophilic substitution reactions.[44] In that regard, the halogens on **1** and **2** were originally intended to be replaced by an amine (**6**, **10**), triazole (**7**, **11**) or methoxide (**9**) group thus making them key reaction intermediates. The serendipitous discovery that these intermediates exhibited antiproliferative properties is an important new lead for drug design and the study of alternative cell-death pathways. From the studies discussed herein, it is clear that the presence of the halogens imparts important antiproliferative properties to the thieno[3,2-*d*]pyrimidine scaffold. This may be due to the electrophilic nature of the 4-Cl group.

Another interesting observation for the active thieno[3,2-*d*]pyrimidines **1** and **2** is that they induce cell-cycle independent apoptosis. Owing to the 4-Cl rendering the C-4 electrophilic, we expected these compounds to exhibit cytotoxic properties similar to DNA alkylating agents.[43,45] Typically, DNA damage leads to arresting the cell in a specific cycle that may lead to apoptosis depending upon the nature of the damage.[46] To our surprise, **1** and **2** did not lead to cell-cycle arrest but still induced apoptosis. Apoptosis without cell cycle arrest generally occurs in the event of cellular stress affecting cytoplasmic p53.[47] However, p53 is mutated in L1210 cells,[48,49] suggesting that these compounds likely induce apoptosis by a p53-independent mechanism. Alternatively, autophagy and necrotic cell death are also possibilities,[50] however, the absence of a signal in the upper left quadrant of the apoptosis graph (Fig. 6, Annexin V negative, 7-AAD positive) minimizes the likelihood that the cytotoxic effects of **1** and **2** on L1210 cells is mediated by a necrotic mechanism. Given the intense clinical interest in identifying new therapeutic strategies that can circumvent the profound inhibition of apoptotic signaling common among aggressive cancers,[51–54] these compounds may prove to be interesting leads to further study the biological targets of cytotoxicity and cell death.

Furthermore, both **1** and **2** also selectively inhibited the growth of several strains of yeast (*C. albicans* and *C. neoformans*) which are important fungal pathogens, particularly among immunocompromised patients. Thus these compounds may provide a new lead for the development of antifungal therapeutics with potentially novel mechanisms of action.

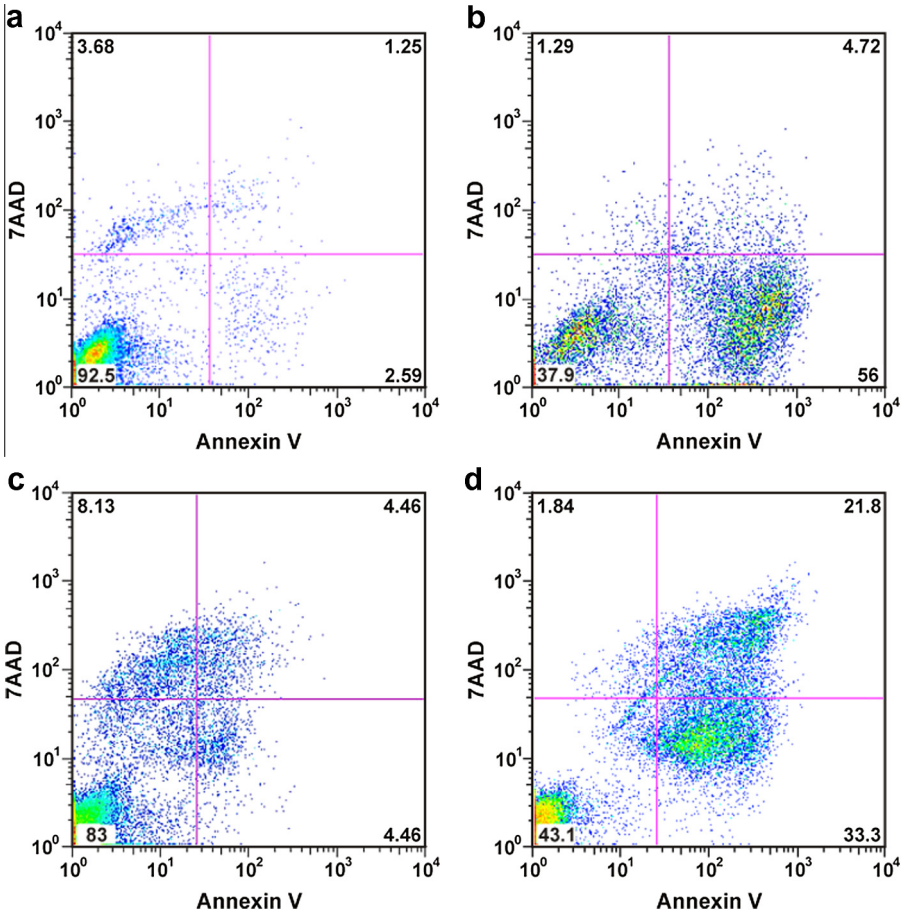
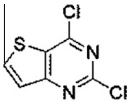
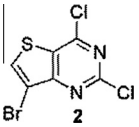
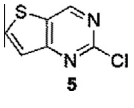
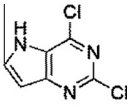


Figure 6. Apoptosis study on L1210 cell line. L1210 cells treated with (a) an equal volume of vehicle or (b) 1 μM compound **1**. Similarly, L1210 cells were treated with (c) an equal volume of vehicle or (d) 5.4 μM compound **2**. After 48 h, cells were stained using Annexin V and 7-ADD and analyzed by flow cytometry. A minimum of 10,000 cells were analyzed per condition, and subdivided into four categories: non-apoptotic/necrotic (*bottom left*), early apoptotic (*bottom right*), late apoptotic/necrotic (*top right*), and necrotic (*top left*). Percentages of total cells detected in each quadrant are indicated.

Table 2
Antimicrobial screening of thieno[3,2-*d*]pyrimidines.

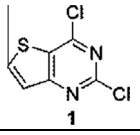
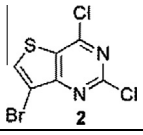
Microbial strain	Strain designation	% inhibition at 100 μM			
		 **1**	 **2**	 **5**	 **19**
Bacterial strains					
Escherichia coli	ATCC 25922	NA	NA	NA	NA
Bacillus subtilis	ATCC 6633	43%	NA	NA	NA
Staphylococcus aureus subsp. aureus (MRSA)	ATCC 43300	NA	NA	NA	NA
Enterococcus faecalis (VRE)	ATCC 51299	NA	NA	NT	NT
Pseudomonas aeruginosa	ATCC 27853	NA	NA	NT	NT
Fungal strains					
Candida albicans	ATCC 10231	99%	99%	NA	NA
Cryptococcus neoformans	ATCC 66031	101%	55%	NA	NA
C. neoformans	JEC20	100%	91%	NA	NA
C. neoformans	VANC-R265	93%	67%	NA	NA
C. neoformans	B4546	96%	82%	NA	NA

NA: No activity, NT: Not tested.

4. Summary

We have identified a set of halogenated thieno[3,2-*d*]pyrimidines that possess selective antiproliferative properties against cancer cells and fungi, as well as to induce cell-cycle-independent apoptosis. These results render them of interest to further study their cytotoxicity against cancer cells and to elucidate alternative cell death pathways. Finally, although the role of the 4-Cl group for these compounds has been established herein, the role of the 2-Cl and 7-Br groups remains unclear. Thus, future efforts will

Table 3
Antifungal activity of thieno[3,2-*d*]pyrimidines **1** and **2**

Strain	MIC_{95} (μM)	
	 **1**	 **2**
C. albicans ATCC 10231	23.4	10.7
C. neoformans JEC20	16.6	5.8
C. neoformans B4546	34.6	7.0

MIC_{95}: minimum concentration for inhibition by 95%. The positive control antibiotic for the antibacterial assays was tetracycline (50 μg/mL for *P. aeruginosa* and 10 μg/mL for all other strains). The control antifungal compound was amphotericin B (2 μg/mL). All positive control experiments exhibited 93–100% cell death.

investigate the role, if any, the 2-Cl and 7-Br groups play in the biological properties of the thienopyrimidine scaffold.

5. Experimental section

5.1. Synthetic methods

5.1.1. General

All chemicals and reagents listed in this section were purchased through commercially available sources unless otherwise noted. All reactions run in CH_2Cl_2, CH_3CN, and THF were obtained from a solvent purification system (SPS, Model: mBraun Labmaster 130). All reactions run in anhydrous DMF, MeOH and pyridine were obtained from Sigma–Aldrich or Acros Organics. All ^1H and ^{13}C NMR spectra were obtained from a JEOL ECX 400 MHz NMR. All ^1H and ^{13}C NMR spectra were referenced to internal tetramethylsilane (TMS) at 0.0 ppm. The spin multiplicities are indicated by the symbols s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), and br (broad). All NMR solvents were obtained from Cambridge Isotope Laboratories. All reactions were monitored by thin layer chromatography (TLC) on 0.25 mm precoated glass plates. All column chromatography was run on 32–63 μ silica gel obtained from Dynamic Adsorptions Inc. (Norcross, GA, USA). Melting points are uncorrected. Yields refer to chromatographically and spectroscopically (^1H and ^{13}C NMR) homogeneous materials. All mass spectra (MS) were recorded and obtained from the University of Maryland Baltimore County Mass Spectrometry Facility and Johns Hopkins Mass Spectrometry Facility. The FAB mass spectra were obtained using double focusing magnetic sector mass spectrometer equipped with a Cs ion gun and fourier transform ion cyclotron resonance equipped with ESI source.

5.1.1.1. Thieno[3,2-*d*]pyrimidin-2,4(1H,3H)-dione (4). In a dry flask methyl-3-amino-2-thiophene carboxylate **3** (5.00 g, 31.80 mmol) was dissolved in acetic acid (100 mL) to obtain a yellow solution to which potassium cyanate (10.31 g, 127.30 mmol) dissolved in water (80 mL) was added dropwise over 3 h. The resultant suspension was stirred overnight (16 h) at which point the suspension was filtered. The white solid residue was dissolved in 2 N NaOH (80 mL) by warming to 70 °C. The clear solution was then acidified by AcOH (pH 4–5), the resulting white precipitate was filtered, washed with water then acetone and dried to obtain **4** as a white solid (3.80 g, 22.60 mmol, 71%). Spectroscopy data agrees with literature.[21–23]

5.1.1.2. 2,4-Dichlorothieno[3,2-*d*]pyrimidine (1). In a dry flask, thieno[3,2-*d*]pyrimidin-2,4(1H,3H)-dione **4** (4.00 g, 23.78 mmol) was refluxed in freshly distilled $POCl_3$ (50 mL) under nitrogen overnight (16 h) at which point the $POCl_3$ was evaporated and the residue extracted with CH_2Cl_2 (50 mL). The organic layer was washed with saturated $NaHCO_3$ solution (50 mL), brine (50 mL), dried over $MgSO_4$ and concentrated. The residue was crystallized from EtOAc to obtain **1** as a pale green-yellow solid (4.00 g, 19.50 mmol, 82%). Mp: 135–137 °C. ^1H NMR (400 MHz, $CDCl_3$): δ 7.55 (d, 1H, ***J*** = 5.0 Hz), 8.12 (d, 1H, ***J*** = 5.5 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 124.6, 129.4, 139.3, 155.8, 156.3, 163.5. FAB-MS ***m/z*** for $C_6H_2Cl_2N_2S$ calculated $[M+H]^+$ 204.9388, found 204.9400 ($2x^{35}Cl$), 206.9366 (^{35}Cl ^{37}Cl).

5.1.1.3. 2-Chlorothieno[3,2-*d*]pyrimidine (5). To a solution of 2,4-dichloro-thieno[3,2-*d*]pyrimidine **1** (300 mg, 1.46 mmol) in EtOAc, DIPEA (0.52 mL, 2.92 mmol) was added, followed by 10% Pd/C (300 mg) and the suspension shaken in a Parr hydrogenator at 45 psi of H_2 pressure for 5 h. The reaction mixture was filtered over celite, the filtrate evaporated and the crude material loaded onto silica. The crude reaction mixture was purified using column chromatography eluting with 9:1 hexanes/EtOAc to obtain **5** as an off-white solid (200 mg, 1.17 mmol, 80%). R_f 0.1 in 9:1 hexanes/EtOAc. Mp 166.3–169.6 °C. ^1H NMR (400 MHz, $CDCl_3$): δ 7.47 (d, 1H, ***J*** = 5.5 Hz), 8.00 (d, 1H, ***J*** = 5.0 Hz), 9.11 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 123.8, 129.8, 139.1, 153.6, 157.5, 163.1. FAB-MS ***m/z*** for $C_6H_3ClN_2S$ calculated $[M+H]^+$ 170.9778, found 170.9784 (^{35}Cl), 170.9766 (^{37}Cl).

5.1.1.4. 4-Amino-2-chlorothieno[3,2-*d*]pyrimidine (6). In a glass tube 2,4-dichlorothieno[3,2-*d*]pyrimidine (800 mg, 3.90 mmol) was suspended in MeOH (10 mL) and cooled to −60 to −70 °C upon which ammonia was bubbled in the suspension. The glass tube was sealed and the suspension stirred at room temperature overnight to obtain a clear solution. The solvent was evaporated and the resulting crude compound was purified using column chromatography eluting first with 9:1 hexanes/EtOAc, followed by 8:2 hexanes/EtOAc to obtain **6** as an off-white solid (575 mg, 3.10 mmol, 80%). R_f 0.2 in 3:1 hexanes/EtOAc. Mp 272.3–274.2 °C. ^1H NMR (400 MHz, DMSO-***d*$_6$**): δ 7.73 (d, 1H, ***J*** = 5.0 Hz), 8.38 (br s, 2H, **NH_2**), 8.60 (d, 1H, ***J*** = 5.5 Hz). ^{13}C NMR (100 MHz, DMSO-***d*$_6$**): δ 114.2, 124.7, 136.6, 157.7, 160.6, 162.4. FAB-MS ***m/z*** for $C_6H_4ClN_3S$ calculated $[M+H]^+$ 185.9887, found 185.9895 (^{35}Cl), 187.9861 (^{37}Cl).

5.1.1.5. 2-Chloro-4-[1,2,4-triazolo]thieno[3,2-*d*]pyrimidine (7). To solution of 2,4-dichlorothieno[3,2-*d*]pyrimidine **1** (250 mg, 1.22 mmol) in CH_3CN, 1,2,4-triazole (255 mg, 3.69 mmol), Et_3N (1 mL, 7.38 mmol) were added and stirred overnight upon which a thick white precipitate was obtained. The reaction mixture was loaded onto silica and product purified using column chromatography eluting with 19:1, followed by 9:1 and and 8:2 hexanes/EtOAc to obtain **7** as a white solid (250 mg, 1.05 mmol, 86%). R_f 0.4 in 4:1 hexanes/EtOAc. Mp 205.6–207.1 °C. ^1H NMR (400 MHz, $CDCl_3$): δ 7.21 (d, 1H, ***J*** = 5.5 Hz), 7.87 (d, 1H, ***J*** = 5.5 Hz), 7.92 (s, 1H), 9.05 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 117.1, 124.0, 142.6, 143.1, 150.7, 154.0, 156.0, 166.3. FAB-MS ***m/z*** for $C_8H_4ClN_5S$ calculated $[M+H]^+$ 237.9948, found 237.9954 (^{35}Cl), 239.9926 (^{37}Cl).

5.1.1.6. 7-Bromo-thieno[3,2-*d*]pyrimidin-2,4 (1H, 3H)-dione (8). To 100 mL glass tube containing thieno[3,2-*d*]pyrimidin-2,4-dione **1** (5.88 g, 34.86 mmol), AcOH (60 mL) and bromine (3.6 mL, 69.72 mmol) were added and the tube capped. The sealed tube was stirred in a preheated oil bath at 90 °C for 24 h. An additional portion of bromine (3.6 mL, 69.72 mmol) was added to the sealed tube and mixture stirred for another 24 h at 90 °C. The AcOH was evaporated to obtain a solid residue to which water was added (200 mL) and the suspension filtered and residue washed repeatedly with water and dried under vacuum to obtain **8** as an off-white solid (8.07 g, 32.67 mmol, 94%). Mp 251.0–252.9 °C. ^1H

NMR (400 MHz, DMSO-d_6): δ 8.22 (s, 1H), 11.40 (br s, 1H, ***NH***), 11.58 (br s, 1H, ***NH***). ^{13}C NMR (100 MHz, DMSO-d_6): δ 99.6, 112.1, 133.4, 145.1, 152.1, 159.0. FAB-MS ***m/z*** for $C_6H_3BrN_2O_2S$ calculated $[M+H]^+$ 246.9171, found 246.9175 (^{79}Br), 248.9161 (^{81}Br).

5.1.1.7. 7-Bromo-2,4-dichlorothieno[3,2-*d*]pyrimidine (2). To a round bottom flask containing 7-bromothieno[3,2-*d*]pyrimidin-2,4-dione **8** (4.07 g, 16.47 mmol), DMAP (8.38 g, 68.76 mmol) and freshly distilled $POCl_3$ were added and the suspension stirred at 105–110 °C for 2 h under nitrogen. The $POCl_3$ was evaporated and the residue extracted with CH_2Cl_2 (300 mL). The organic layer was washed with aq $NaHCO_3$ (300 mL), brine (200 mL) and dried over $MgSO_4$. The dried organic layer was concentrated, loaded on silica and the product purified using column chromatography eluting with 19:1 hexanes:EtOAc to obtain **2** as a white solid (3.41 g, 12.00 mmol, 73%). R_f 0.5 in 9:1 hexanes/EtOAc. Mp 180.2–183.0 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.13 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.0, 158.0, 156.0, 135.0, 128.0, 109.5. FAB-MS ***m/z*** for $C_6HBrCl_2N_2S$ calculated $[M+H]^+$ 282.8493, found 282.8495 (2x^{35}Cl, ^{79}Br), 284.8468 (2x^{35}Cl, ^{81}Br), 286.8443 (^{35}Cl, ^{37}Cl, ^{81}Br), 288.8419 (2x^{37}Cl, ^{81}Br).

5.1.1.8. 7-Bromo-2,4-dimethoxythieno[3,2-*d*]pyrimidine (9). To a solution of 7-bromo-2,4-dichlorothieno[3,2-*d*]pyrimidine **2** (3.6 g, 12.6 mmol) in anhydrous MeOH, 30% NaOMe solution (10 mL, 2.90 g, 54.5 mmol) was added and the reaction mixture was refluxed overnight (16 h). TLC indicated the absence of starting material upon which the reaction mixture was neutralized (pH 7–8) using 2 M HCl followed by removal of MeOH in vacuo. The product was extracted with CH_2Cl_2 (300 mL) and the organic layer washed with water (300 mL) and brine (300 mL). The CH_2Cl_2 was evaporated completely and to the residue EtOAc was added to obtain slurry that was heated to reflux and cooled to get a white precipitate. The precipitate was filtered to obtain **9** as a white granular solid (2.40 g, 8.72 mmol, 69%). R_f 0.5 in 9:1 hexanes/EtOAc. Mp 193–195 °C. The spectral data was in agreement with the reported data.[23] ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.10 (s, 3H), 4.14 (s, 3H), 7.76 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.9, 55.4, 108.7, 111.4, 131.2, 159.8, 164.9, 166.2. FAB-MS ***m/z*** for $C_8H_7BrN_2O_2S$ calculated $[M+H]^+$ 274.9484, found 274.9490 (^{79}Br), 276.9473 (^{81}Br).

5.1.1.9. 4-Amino-7-bromo-2-chlorothieno[3,2-*d*]pyrimidine (10). In a glass tube 7-bromo-2,4-dichloro thieno[3,2-*d*]pyrimidine **2** (100 mg, 0.35 mmol) was suspended in MeOH (10 mL) and cooled to −60 to −70 °C at which point ammonia was bubbled in the suspension. The glass tube was sealed and the suspension stirred at room temperature overnight (16 h) to obtain a clear solution. The solvent was evaporated and the resulting crude compound was purified using column chromatography eluting first with 9:1 hexanes/EtOAc, followed by 8:2 hexanes/EtOAc to obtain **10** as an off-white solid (65 mg, 0.24 mmol, 68%). R_f 0.3 in 3:1 hexanes/EtOAc. Mp 286–287.9 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.97 (br s, 2H, ***NH₂***), 9.14 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 108.6, 114.0, 133.5, 158.6, 158.8, 160.8. FAB-MS ***m/z*** for $C_6H_3BrClN_3S$ calculated $[M+H]^+$ 263.8992, found 263.9001 (^{35}Cl, ^{79}Br), 265.8974 (^{35}Cl, ^{81}Br), 269.8948 (^{37}Cl, ^{81}Br).

5.1.1.10. 7-Bromo-2-chloro-4-[1,2,4-triazolo]thieno[3,2-*d*] pyrimidine (11). To solution of 7-bromo-2,4-dichlorothieno[3,2-*d*]pyrimidine **2** (100 mg, 0.35 mmol) in CH_3CN, 1,2,4-triazole (73 mg, 1.05 mmol), Et_3N (0.18 mL, 1.26 mmol) were added and stirred overnight at which point a thick white precipitate was obtained. The reaction mixture was loaded onto silica and the product purified using column chromatography eluting with 19:1, then 9:1 and finally 4:1 hexanes/EtOAc to obtain **11** as a white solid (77 mg, 0.24 mmol, 69%). R_f 0.2 in 19:1 hexanes/EtOAc. Mp 224.6–227.9 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (s, 1H), 8.29 (s, 1H), 9.42 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 109.1, 116.5, 139.4, 143.2, 150.5, 154.1, 156.2, 162.8. FAB-MS ***m/z*** for $C_8H_3BrClN_5S$ calculated $[M+H]^+$ 315.9053, found 315.9059 (^{35}Cl, ^{81}Br), 317.9036 (^{35}Cl, ^{81}Br), 319.9009 (^{37}Cl, ^{81}Br).

5.1.1.11. 2,4-Dimethoxy-7-(β-D-glycero-pentofuran-3′-ulos-1′-yl)thieno[3,2-*d*]pyrimidine (13). In a dry flask 7-bromo-2,4-dimethoxythieno[3,2-d]pyrimidine 9 (1.10 g, 4.01 mmol) was co-evaporated with CH3CN (10 mL) and dried under high vacuum. To this tetra-*n*-butyl ammonium chloride (7.81 g, 28.1 mmol) was added and the mixture dissolved in DMF (10 mL). To this solution 47.5% Pd(OAc)2 (378 mg, 0.8 mmol) was added followed by NaHCO3 (674 mg, 8.02 mmol) under nitrogen. In a separate flask, glycal (2.13 g, 6.01 mmol) was co-evaporated with CH3CN (10 mL) and dissolved in DMF (10 mL). A solution of glycal in DMF was added to the previous solution and the mixture stirred at 40 °C for 48 h under nitrogen until the TLC indicated the absence of starting materials. The reaction mixture was evaporated, the residue dissolved in CH2Cl2 (50 mL) and the Pd filtered over celite. The organic layer was loaded on silica and the product purified using column chromatography eluting with 3:1 hexanes/EtOAc to afford 13 as a pale yellow solid (650 mg, 2.09 mmol, 52%). Mp 123.2–126.9 °C. R_f 0.4 in 1:1 hexanes/EtOAc. The spectral data was in agreement with literature values.[23] 1H NMR (400 MHz, CDCl3): δ 2.80 (dd, 1H, ***J*** = 6.4 Hz, ***J*** = 17.6 Hz), 3.23 (dd, 1H, ***J*** = 11.0 Hz, ***J*** = 17.6 Hz), 3.96–3.98 (br m, 2H), 4.03 (s, 3H), 4.10 (t, 1H, ***J*** = 2.3 Hz), 4.16 (s, 3H), 4.89 (br d, 1H, OH), 5.50 (dd, 1H, ***J*** = 6.4 Hz, ***J*** = 11.0 Hz), 7.80 (s, 1H). 13C NMR (100 MHz, CDCl3): δ 49.3, 54.5, 55.4, 62.7, 74.0, 82.3, 113.3, 133.4, 133.9, 160.3, 164.0, 166.1, 213.8. FAB-MS ***m/z*** for C13H14N2O5S calculated $[M+H]^+$ 311.0696, found 311.0695.

5.1.1.12. 1′-β-[7-(2,4-Dimethoxythieno[3,2-*d*]pyrimidine)]-2′-deoxyribofuranose (14). 2,4-Dimethoxy-7-(β-D-glycero-pentofuran-3′-ulos-1′-yl)thieno[3,2-*d*]pyrimidine **13** (650 mg, 2.09 mmol) was dissolved in a mixture of CH_3CN (5 mL) and AcOH (5 mL) under nitrogen. The solution was cooled to −10 to −5 °C after which $NaB(OAc)_3H$ (555 mg, 2.62 mmol) was added and stirred at −10 to −5 °C for 1 h. The reaction mixture warmed to room temperature and stirred until the TLC indicated the absence of the starting material. The reaction mixture was concentrated, loaded on silica and purified using column chromatography eluting with 49:1 CH_2Cl_2/MeOH to afford **14** as yellow solid (470 mg, 1.50 mmol, 72%). Mp 129.7–132.4 °C. R_f 0.4 in 19:1 CH_2Cl_2/MeOH. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.011 (dd, 1H, ***J*** = 5.3 Hz, ***J*** = 13.0 Hz), 2.76–2.84 (m, 1H), 3.80 (dd, 1H, ***J*** = 1.4 Hz, ***J*** = 12.4 Hz), 3.95 (dd, 1H, ***J*** = 2.1 Hz, ***J*** = 12.1 Hz), 4.06 (s, 3H), 4.18–4.15 (m, 1H), 4.16 (s, 3H), 4.72 (d, 1H, ***J*** = 5.0 Hz), 5.46 (dd, 1H, ***J*** = 5.5 Hz, ***J*** = 11.4 Hz), 7.73 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) 41.9, 54.3, 55.3, 64.0, 75.2, 88.9, 113.2, 133.0, 135.1, 160.6, 163.7, 166.0. FAB-MS ***m/z*** for $C_{13}H_{16}N_2O_5S$ calculated $[M+H]^+$ 313.0853, found 313.0862.

5.1.1.13. 1′-β-[7-(2,4-Dimethoxythieno[3,2-*d*]pyrimidine)]-3′,5′-acetoxy-2′-deoxyribofuranose (15). To a solution of 1′-β-[7-(2,4-dimethoxythieno[3,2-*d*]pyrimidine)]-2′-deoxyribofuranose **14** (470 mg, 1.50 mmol) in pyridine (5 mL), Ac_2O (0.28 mL, 3.01 mmol) was added and stirred overnight (16 h) at which point the TLC indicated the absence of starting material. The pyridine was evaporated and the residue co-evaporated with toluene. The residue was then loaded on silica and the product purified using column chromatography eluting with 19:1 then 9:1 hexanes/EtOAc to give product **15** as a pale yellow solid (570 mg, 1.43 mmol, 95%). Mp 79.6–83.2 °C. R_f 0.2 in 3:1 hexanes/ EtOAc. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.03 (s, 3H), 2.12 (s, 3H), 2.15–2.23 (m, 1H), 2.64 (ddd, 1H, ***J*** = 1.4 Hz, ***J*** = 5.5 Hz, ***J*** = 13.7 Hz), 4.04 (s, 3H), 4.12 (s, 3H), 4.23–4.26 (m, 2H), 4.42 (dd, 1H, ***J*** = 3.2,

J = 10.3), 5.26 (d, 1H, ***J*** = 6.4), 5.46 (dd, 1H, ***J*** = 5.5, ***J*** = 10.4), 7.73 (d, 1H, ***J*** = 1.4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.9, 21.2, 38.8, 54.3, 55.0, 64.3, 76.1, 76.5, 82.4, 112.3, 130.1, 136.4, 160.3, 163.7, 165.8, 170.7, 170.8. FAB-MS ***m/z*** for $C_{17}H_{20}N_2O_7S$ calculated $[M+H]^+$ 397.1064, found 397.1063.

5.1.1.14. 1′-β-[7-(Thieno[3,2-*d*]pyrimidin-2,4-dione)]-3′, 5′-acetoxy-2′-deoxyribofuranose (16). To a solution of 1′-β-[7-(2,4-dimethoxythieno[3,2-*d*]pyrimidine)]-3′,4′-acetoxy-2′-deoxyribofuranose **15** (570 mg, 1.43 mmol) in AcOH, sodium iodide (862 mg, 5.75 mmol) was added and the mixture stirred for 3 h at 58–62 °C during which time the reaction mixture turned a maroon color. The AcOH was evaporated and the residue was extracted with CH_2Cl_2 (50 mL) and the organic layer washed with water (50 mL), aq $NaHCO_3$ (50 mL), aq $Na_2S_2O_3$ (25 mL), aq NaCl (50 mL) and then dried over $MgSO_4$. The organic layer was loaded on silica and the product purified by column chromatography eluting with 1:1 hexanes/EtOAc to give **16** as a white solid (500 mg, 1.35 mmol, 95%). Mp 157–162.4 °C. R_f 0.2 in 1:1 hexanes/EtOAc. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.13 (s, 3H), 2.16–2.21 (m, 1H), 2.22 (s, 3H), 2.37 (dd, 1H, ***J*** = 5.0, ***J*** = 13.7 Hz), 4.26–4.29 (m, 2H), 4.60 (dd, 1H, ***J*** = 3.7 Hz, ***J*** = 12.8 Hz), 5.20–5.26 (m, 2H), 7.50 (s, 1H), 8.09 (br s, 1H, ***NH***), 9.11 (br s, 1H, ***NH***). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.0, 21.1, 39.7, 64.0, 75.9, 76.5, 83.4, 114.1, 128.8, 131.3, 143.7, 151.4, 159.0, 170.7, 170.8. FAB-MS ***m/z*** for $C_{15}H_{16}N_2O_7S$ calculated $[M+H]^+$ 369.0751, found 369.0791.

5.1.1.15. 1′-β-[7-(Thieno[3,2-*d*] pyrimidin-2,4-dione)]-2′-deoxyribofuranose (17). A solution of 1′-β-[7-(thieno[3,2-*d*]pyrimidin-2,4-dione)]-3′,5′-acetoxy-2′-deoxyribofuranose **16** (500 mg, 1.35 mmol) in MeOH (10 mL) was cooled to −60 to −70 °C at which point ammonia was bubbled into the solution. The glass tube was sealed and the reaction mixture stirred at room temperature overnight (16 h). The reaction mixture was concentrated and the residue loaded onto silica. The product was purified by column chromatography eluting with 19:1 CH_2Cl_2/MeOH to obtain **17** as a white solid (293 mg, 1.03 mmol, 76%). R_f 0.1 in 19:1 CH_2Cl_2/MeOH. ^{1}H NMR (400 MHz,DMSO-***d*****$_6$**): δ 1.96–2.01 (m, 2H), 3.16 (d, 1H, *J* = 5.0 Hz), 3.57–3.65 (m, 2H), 3.84 (d, 1H, ***J*** = 1.8 Hz), 4.26–4.27 (m, 1H), 5.13–5.14 (d, 1H, ***J*** = 3.2 Hz, *OH*), 5.22 (dd, 1H, ***J*** = 6.4 Hz, ***J*** = 9.4 Hz), 5.76–5.82 (br s, 1H, *OH*), 7.99 (s, 1H), 11.24–11.26 (br s, 2H, *2* ***NH***). ^{13}C NMR (100 MHz,DMSO-***d*****$_6$**): δ 43.0, 62.4, 73.4, 75.7, 88.2, 113.0, 132.0, 132.2, 144.5, 152.0, 159.5. FAB-MS ***m/z*** for $C_{11}H_{12}N_2O_5S$ calculated $[M+H]^+$ 285.0540, found 285.0548.

5.1.1.16. 2,4-Dichloropyrrolo[3,2-*d*]pyrimidine (19). To pyrrolo[3,2-*d*]pyrimidin-2,4-dione **18** (2.00 g, 13.2 mmol), 1 N NaOH (15 mL), and 0.60 g NaOH in 15 mL H_2O was added and the mixture stirred at 40 °C until a solution was formed. The solution was then cooled to room temperature (21–25 °C) and then placed in an ice bath to obtain thick slurry. The slurry was then filtered to obtain a pale yellow solid. The solid was dissolved in 1 N NaOH (15 mL), and heated to 40 °C to obtain a clear solution that upon cooling provided white crystals. The crystals were washed with MeOH (20 mL) and acetone (20 mL), and then dried under vacuum. The dry solids were taken in phenylphosphonic dichloride (10 mL) and heated to 170–175 °C for 5 h during which the reaction mixture became a brown–black solution. After 5 h the hot reaction mixture was poured onto ice, extracted with EtOAc (200 mL) and the organic layer washed with sat. $NaHCO_3$ solution (3 × 100 mL) till all effervescence subsided. The organic layer was then washed with brine and dried over $MgSO_4$. The organic layer was concentrated in vacuo and loaded onto silica. The product was purified using column chromatography eluting with 9:1 then 3:1 hexanes/EtOAc to obtain **18** as an off-white solid (1.50 g, 7.9 mmol, 60%). R_f 0.5 in 3:1 hexanes/EtOAc. Mp 228.3–232.0 °C. ^{1}H NMR (400 MHz, DMSO-***d*****$_6$**): δ 6.71 (d, 1H, ***J*** = 3.2 Hz), 8.09 (d, 1H, ***J*** = 2.8 Hz), 12.75 (s, 1H, ***NH***). ^{13}C NMR (100 MHz, DMSO-***d*****$_6$**): δ 103.2, 124.3, 138.0, 143.5, 149.6, 153.9. ESI-MS ***m/z*** for $C_6H_3Cl_2N_3$ calculated $[M+H]^+$ 187.9776, found 187.9777.

5.2. Cytostatic assays

Murine Leukemia L1210, human lymphocytic CEM and human cervix carcinoma HeLa cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). All assays were performed in 96-well microtiter plates. To each well were added $(5-7.5) \times 10^4$ tumor cells and a given amount of the test compound. The cells were allowed to proliferate for 48 h (murine leukemia L1210 cells) or 72 h (human lymphocytic CEM and human cervix carcinoma HeLa cells) at 37 °C in a humidified CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter counter. The IC_{50} (50% inhibitory concentration) was defined as the concentration of the compound that inhibited cell proliferation by 50%. Orthogonally, cytotoxicity of selected compounds for L1210 cells was assessed by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Here, cells were seeded in 96-well plates at 5×10^3 cells per well and treated with a range of drug concentrations. After 48 h, cell viability was measured using the MTT Cell Proliferation Assay kit (ATCC) according to the manufacturer's instructions. Percent cell viability was analyzed as a function of drug concentrations using a sigmoidal dose response function with PRISM v3.03 software (GraphPad) to resolve IC_{50} values.

5.3. Cell cycle distribution and apoptosis assays

Cell cycle and apoptosis studies were conducted on L1210, a mouse lymphocytic leukemia cell line obtained from the American Type Culture Collection and grown in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal bovine serum (FBS). The cell cycle distributions of L1210 cells treated with compounds **1** or **2** were analyzed using propidium iodide staining and flow cytometry. 2×10^6 cells were seeded in 100 mm dishes and treated with vehicle alone or compounds **1** or **2** at the IC_{50} values determined by cell viability assays or an equivalent volume of DMSO. 48 h following treatment the cells were collected, fixed, and stained with propidium iodide (Sigma Aldrich) as described[54] immediately before analysis by flow cytometry. To analyze apoptotic cell death, L1210 cells were seeded as described above but then treated with vehicle alone or compounds **1** or **2** at 1 μM and 5.4 μM, respectively. After 48 h, the cells were then collected and the cellular fractions undergoing early apoptotic, late apoptotic/necrotic, and necrotic cell death were measured by staining with Annexin V and 7-aminoactinomycin D (7-AAD) using the BD Pharmingen PE Annexin V Apoptosis Detection Kit I (BD Pharmingen) as described.[55] All flow cytometry analyses required for cell cycle distribution and apoptosis assays were performed at the University of Maryland Greenebaum Cancer Center Shared Flow Cytometry Facility.

5.4. Antimicrobial assays

Compounds were tested against the following panel of bacteria and fungi purchased from the American Type Culture Collection (ATCC): Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, vancomycin-resistant *Enterococcus faecalis* (VRE) ATCC 51299, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Cryptococcus neoformans* ATCC 66031 and *Candida albicans* ATCC 10231. Three clinical isolates of *C. neoformans* (JEC20, VANC-R265 and B4546) were also tested. *S. aureus*, *B. subtilis*, *P. aeruginosa* and *E. coli* were grown in BBL™ Trypticase™ Soy Broth (BD) at 37 °C. VRE was grown in brain

heart infusion broth (Bacto) at 37 °C and *C. albicans* and *C. neoformans* were grown in yeast malt extract medium (Difco) and Sabouraud dextrose broth (Difco), respectively, at 30 °C.

Microbial susceptibility testing was performed using an adaptation of the standard microbroth dilution assay.[56] Briefly, bacteria were grown to mid-log phase, diluted with fresh medium to an optical density at 600 nm (OD_{600}) of 0.030–0.060 and then diluted again 1:10. This suspension (195 μL) was added to wells in a 96 well microtiter plate (Sarstedt) and 5 μL of compound dissolved in DMSO was added to give a final concentration of 100–0.1 μM at 2.5% DMSO by volume. A DMSO negative control and standard antibiotic positive controls were included in each plate. All compounds were tested in triplicate for each concentration. Plates were sealed with parafilm, placed in a Ziploc bag to prevent evaporation, and incubated at 30 °C (fungi) or 37 °C (bacteria) for 16–20 h (48 h for *C. neoformans*). The OD_{600} values for each well were determined with a plate reader (Biotek, EL800) and the data were standardized to the DMSO control wells after subtracting the background from blank medium. Initial single concentrations were tested at 100 μM and active compounds were further tested with at least nine concentrations for a full dose response. Dose response curves were generated using GraphPad Prism 5 software and used to determine the MIC_{95} concentrations (minimal concentration that inhibits 95% of growth).

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Supplementary data

Supplementary data (the ^{1}H, ^{13}C NMR spectra and High Resolution Mass Spectrometry (HRMS) and list of kinases tested for compounds **1**–**2**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.02.033.

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