Antiproliferative activities of halogenated pyrrolo[3,2-d]pyrimidines.

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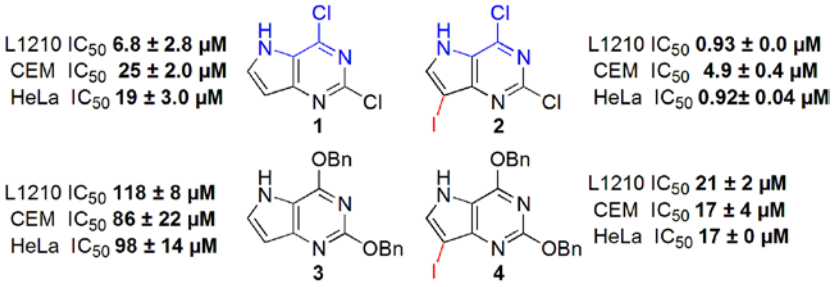
KEYWORDS . Pyrrolo[3,2-*d*]pyrimidine, heterocyclic chemistry, cytostatic, apoptosis, halogen.



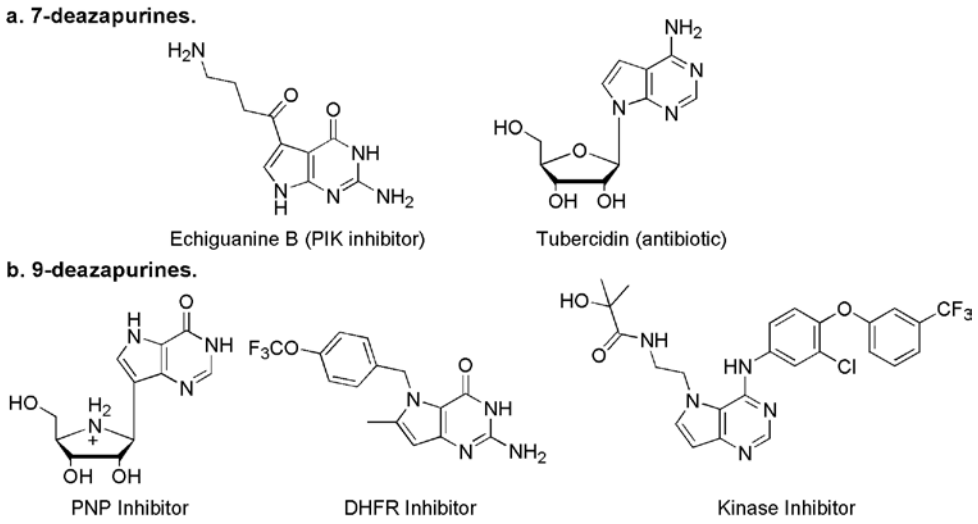
ABSTRACT: As a continuation of our previous report on the cytostatic activity for several halogenated thieno[3,2-*d*]pyrimidines, subsequent i*n vitro* evaluation of the corresponding halogenated pyrrolo[3,2-*d*]pyrimidines identified compounds **1** and **2** with antiproliferative activity against three different cancer cell lines. Upon screening of a

series of pyrrolo[3,2-*d*]pyrimidines, the 2,4-Cl compound **1** was found to exhibit antiproliferative activity in the lower micromolar concentrations. However introduction of iodine at C7 resulted in significant enhancement of potency by reducing the IC_{50} into sub-micromolar levels, thereby suggesting the importance of the halogen at C7. This finding was further supported by a markedly lower activity for **3**, which lack the 2,4-Cl substituents but an increased antiproliferative effect again when **3** was substituted by an halogen at C7 (**4**). Cell-cycle and apoptosis studies conducted on the two potent compounds **1** and **2** showed differences in their activity, wherein compound **1** induced accumulation of the G2/M stage in triple negative breast cancer MDA-MB-231 cells with little evidence of apoptotic death while compound **2** robustly induced apoptosis of MDA-MB-231 cells concomitant with G2/M cell cycle arrest.

Introduction

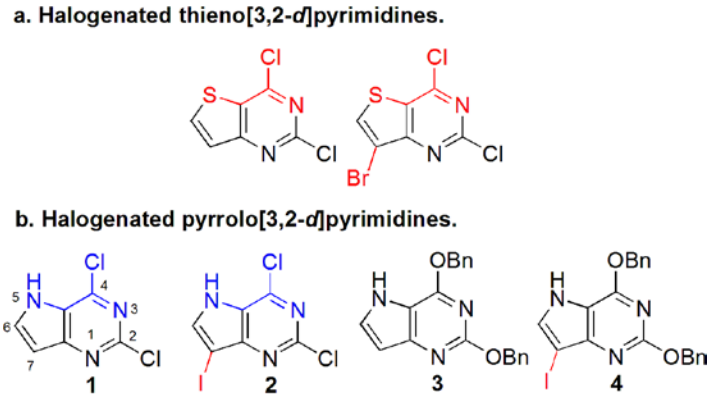
Pyrrolopyrimidines exist as two regioisomers, specifically pyrrolo[2,3-*d*]pyrimidine and pyrrolo[3,2-*d*]pyrimidine and are more commonly referred to as 7-deazapurine and 9-deazapurine respectively (Figure 1).[1] Due to a close resemblance to the purines, the pyrrolopyrimidine scaffold has found pharmacological utility as a purine isostere. [2-13] The 7-deazapurines (Figure 1a) are naturally occurring and widely used in drug design primarily due to their propensity to be ribosylated. Echiguanine B and Tubercidin are two examples of naturally occurring 7-deazapurines exhibiting anticancer properties (Figure 1a).

Figure 1: Deazapurines and their biological properties.



In contrast, the pyrrolo[3,2-*d*]pyrimidines do not occur naturally and must be synthetically prepared. The 9-deazapurines have been explored in the past as nucleoside isosteres (Figure 2b)[8] and these efforts have resulted in the design of purine nucleoside phosphorylase (PNP) inibitors[8,14-23], dihydrofolate reductase (DHFR) inhibitors[1,24] and more recently for kinase inhibition (Figure 2b).[25-27]

Figure 2: Exploration antiproliferative activities of halogenated fused pyrimidines.



Our laboratory's interest in exploring the biological properties of fused heterocyclic compounds[28-32] has resulted in the identification of antiproliferative properties of halogenated thieno[3,2-*d*]pyrimidines (Figure 2a). The halogenated thieno[3,2-*d*]pyrimidines were found to induce apoptosis in L1210 cells independent of cell cycle arrest. However, they showed remarkable differences in their cytotoxic mechanisms in the triple negative breast cancer (TNBC) cell line MDA-MB-231. In this cell model, cell death was accompanied by dramatic arrest at the G2/M transition, but only in the analogue lacking bromine at C7 (Figure 2a, *right*).[33] In an effort to further explore these findings with an alternate scaffold, we opted to synthesize the analogous series of halogenated pyrrolo[3,2-*d*]pyrimidines. Once in hand, the toxicity of each pyrrolo[3,2-*d*]pyrimidine was then tested against three cancer cell lines (L1210, CEM and HeLa). This examination revealed significant antiproliferative activities for compounds **1-4** (Figure 2b) but also indicated that this function was enhanced by inclusion of iodine at C7. Subsequent testing of compounds **1** and **2** across the NCI-60 cancer cell line panel further supported the impact of iodine at C7 on the cytostatic/cytotoxic activity of halogenated pyrrolo[3,2-*d*]pyrimidines. The consequences of halogenated pyrrolo[3,2-*d*]pyrimidines **1** and **2** were then evaluated on cell cycle distributions and apoptosis of

MDA-MB-231 cells to explore their mechanism of antiproliferative activity. The results indicated that both compounds trigger arrest at G2/M, and this and other findings will be discussed herein.

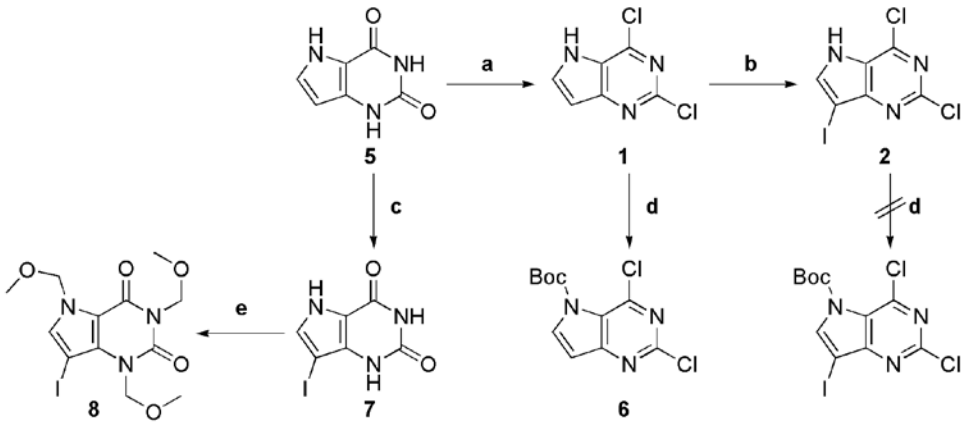
Results and Discussion

Chemistry. In order to explore the antiproliferative properties of the halogenated pyrrolo[3,2-*d*]pyrimidines as well as the effect of the halogen at C7, two sets of compounds were synthesized (Schemes 1 and 2). The target compounds in Scheme 1 were used to explore the anticancer properties of the pyrrolo[3,2-*d*]pyrimidines with a chlorine at C4. A second set of compounds illustrated in Scheme 2 possess *O*-benzyl groups on the C2 and C4 positions. These compounds, which are also intermediates in the syntheses of the corresponding 2'-deoxy-9-deaza-nucleosides, were tested against cancer cell lines concurrent with the testing of the halogenated pyrrolo[3,2-*d*]pyrimidines in Scheme 1. This latter exercise helped indentify the impact of halogens on the anticancer properties of pyrrolo[3,2-*d*]pyrimidines. Finally the corresponding *C*-nucleosides (Figure 3) reported previously by our group[34] were also examined for their antiproliferative properties.

The synthesis of halogenated pyrrolo[3,2-*d*]pyrimidines (Scheme 1) began with chlorination of the known pyrrolo[3,2-*d*]pyrimidin-2,4-dione **5**[35-39]. Preparation of the sodium salt, followed by heating with phenylphosphonic dichloride ($PhPOCl_2$) at 170-175 ˚C gave **1** in a 60% yield.[35-39] Iodination of **1** using N-iodisuccinimide (NIS) in tetrahydrofuran (THF) at room temperature yielded the iodo analogue **2** in a 54% yield.[40]

Attempts to protect the 5-NH of **2** with *tert*-butoxycarbonyl (Boc) in order to evaluate the effect of the NH on the biological activity of **2** failed due to the instability of the Boc protecting group during the subsequent silica gel purification. Alternatively, Boc protection of **1** was attempted using Boc_2O in the presence of 4-dimethylaminopyridine (DMAP) at room temperature to give **6** in 65% yield.[41] Iodination of pyrrolo[3,2-*d*]pyrimidin-2,4-dione **5** by NIS in DMF followed by crystallization from ethanol gave **7** in 87% yield.[42] Next, 7-iodo pyrrolo[3,2-*d*]pyrimidin-2,4-dione **7** was protected with the methoxymethyl (MOM) group by addition of MOMCl to a stirred solution of **7** and 60% sodium hydride (NaH) suspension under an inert atmosphere, which resulted in **8** with all the three NH groups MOM protected in a 40% yield.

Scheme 1: Synthesis of halogenated pyrrolo[3,2-*d*]pyrimidines.



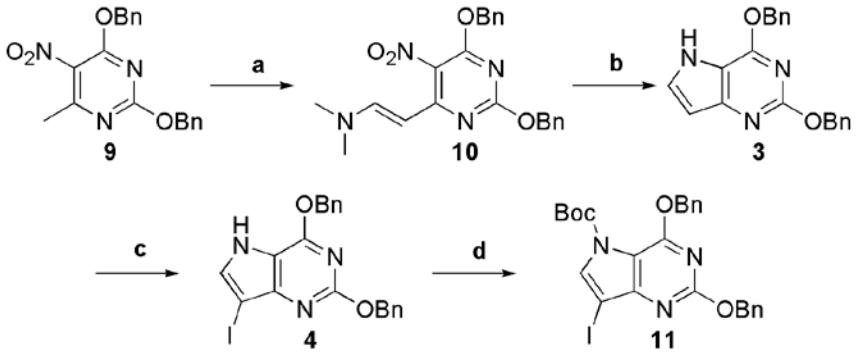
a. $PhPOCl_2$, 170-175 °C, b. NIS, THF, rt, c. NIS, DMF, rt, d. Boc_2O, DMAP, CH_2Cl_2, e. NaH, CH_3OCH_2Cl, DMF.

In addition to the compounds shown in Scheme 1, several bis-*O*-benzylated pyrrolo[3,2-*d*]pyrimidines were synthesized (Scheme 2) in order to evaluate the impact of the iodine at C7 on the cytostatic properties of the pyrrolo[3,2-*d*]pyrimidines in the absence of the C4 chloro group. The 2,4–bis-*O*-benzylated compounds were chosen for

this evaluation due to the facile synthesis and our past experience with these compounds for the preparation of 2'-deoxy-9-deaza-nucleosides *via* Heck coupling.[34]

As shown in Scheme 2, synthesis of the bis-*O*-benzylated pyrrolo[3,2-*d*]pyrimidines was initiated by formylation of the 6-methyl group on **9** by heating with DMF-dimethylacetal in DMF at 60-65 °C, to obtain **10** in 70% yield (Scheme 2).[37] Subsequent ring cyclization using Batcho-Leimgruber pyrrole methodology was accomplished by stirring **10** with zinc (Zn) in acetic acid (AcOH) to give 2,4-OBn pyrrolo[3,2-*d*]pyrimidine **3** in 90% yield.[37,43-45] Iodination of **3** using NIS in methylene chloride (CH_2Cl_2) afforded **4** in 90% yield. Protection of 5-NH using Boc in the presence of DMAP afforded **11** in 53% yield.[34]

Scheme 2: Synthesis of the bis-O-benzyl analogues.

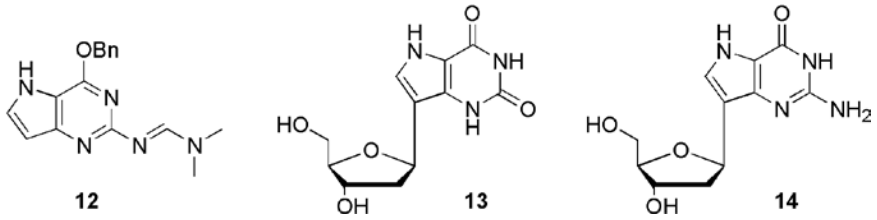


a. $(CH_3)_2NCH(OMe)_2$, DMF, 60-65 °C, b. Zn, AcOH, c. NIS, CH_2Cl_2, rt, d. Boc_2O, DMAP, CH_2Cl_2

Along with compounds in Scheme 1 and Scheme 2, three additional compounds, namely 2-[(dimethylamino)methyleneimino]-4-benzyloxy-5*H*-pyrrolo[3,2-*d*]pyrimidine **12** and 9-deaza-2′-deoxynucleosides **13** and **14** (Figure 3) were also tested. While **12** helped evaluate the effect of a substituent other than OBn at C2, the *C*-nucleosides[34] **13** and **14** were tested owing to past[2-8,46-56] and recent[57] interest in the pharmacological properties of 9-deazanucleosides. The syntheses of compounds shown in Figure 3 have

been reported by our group but their antiproliferative properties had not been previously evaluated.[34,58]

Figure 3: Intermediate 12 and 9-deaza-2'-deoxynucleosides 13 and 14.[34]

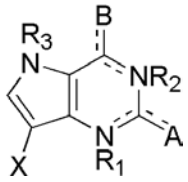


In vitro cell growth inhibition. The cytotoxic activities of several compounds described above were first assessed in three cultured tumor cell models: L1210, a mouse lymphocytic leukemia cell line[59], CCRF-CEM[60], an acute lymphoblastic leukemia cell line, and HeLa, a human cancer cell line derived from a human cervical adenocarcinoma.[61-62] Comparisons of resolved IC_{50} values (Table 1) indicate that the dichloro compounds **1** and **2** show measureable cytostatic activity in all three tumor cell lines. The presence of iodine at C7 on the 2,4-dichloro pyrrolo[3,2-*d*] (**2**) increases the antiproliferative activity by a factor of 5 to 20, when compared to **1**. A analogues increase in cytotoxicity for bis-*O*-benzylated pyrrolo[3,2-*d*]pyrimidine (**3**) was observed upon introduction of C7 iodine (**4**). Replacement of the 2,4-chloro moieties with O-benzyl groups led to a loss of activity by a factor of 3-15, however the activity of **4** was comparable to **1**. This suggests a compensation for the loss of activity for the O-benzyl groups by the presence of iodine at C7.

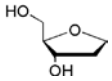
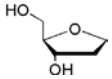
Introduction of the Boc group on 2,4-dichloro pyrrolo[3,2-*d*]pyrimidine **6** did not lead to any loss in activity against CEM and HeLa cells, while activity against L1210 was weakened by a factor of 3. On the other hand, the presence of the Boc group on the bis-

O-benzyl analogue (**11**) led to a complete loss in activity. These data support: a) the importance of 7-NH in the cytostatic role of the 2,4-O-benzylated analogues (**3** and **4**) and b) a potentially different mode of action for the 2,4-O-benzylated analogues as compared to 2,4-dichloro analogues (**1** and **2**). Additionally, replacement of the 2-*O*-benzyl on **3** with the aminovinyl dimethylamine group of **12**[34] led to a marginal loss of activity indicating that an OBn group at C2 is not crucial for cytostatic activity and can be replaced. In order to evaluate the effect of the C7 iodine, the iodo analogue of **12** was sought, however all attempts at iodination failed due to the presence of 5-NH.[34] The ketone or amide groups, as found in **5**, **7** and **8**, do not impart activity against any cancer cell line tested, which is consistent with our previous studies on the thieno[3,2-*d*]pyrimidines. Similarly, the *C*-nucleosides **13** and **14** were both inactive and exhibited no cytotoxicity.[34] However, an interesting discovery resulting from examining various intermediates was the very modest cytotoxicity observed for the 2,4-bis-*O*-benzyl-5-nitro-6-dimethylaminovinyl pyrimidine (**11**).

Table 1: Anti-tumor cell activity of test compounds.



Compound	A	B	R1	R2	R3	X	IC_{50}* (μM)		
							L1210	CEM	HeLa
1	Cl	Cl	-	-	H	H	6.8 ± 2.8	25 ± 2	19 ± 3
2	Cl	Cl	-	-	H	I	0.93±0.0	4.9±0.4	0.92±0.04
3	OBn	OBn	-	-	H	H	118±8	86±22	98±14

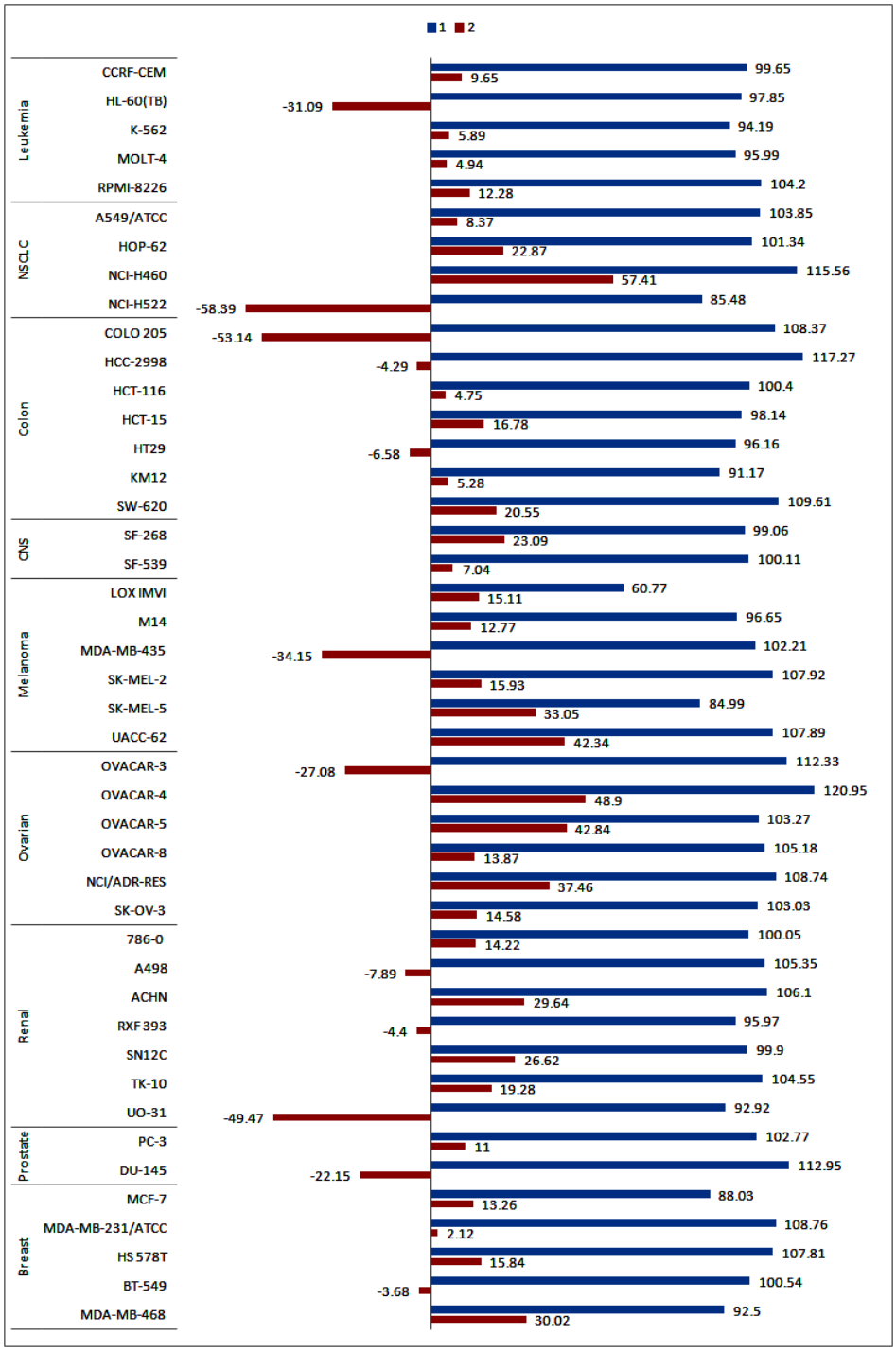
4	OBn	OBn	-	-	H	I	21 ± 2	17 ± 4	17 ± 0
5	O	O	H	H	H	H	>250	>250	>250
6	Cl	Cl	-	-	Boc	H	21±1	26±2	18±3
7	O	O	H	H	H	I	>250	>250	>250
8	O	O	MOM	MOM	MOM	I	>250	>250	>250
10	OBn	OBn	-	-	-[¶]	-[¶]	74±49	59±41	100±38
11	OBn	OBn	-	-	Boc	I	247 ± 4	193 ± 81	72 ± 47
12[37]	OBn		-	-	H	H	170 ± 24	164 ± 68	126 ± 32
13[34]	O	O	H	H	H		>250	>250	>250
14[34]	O	NH_2	-	H	H		>250	>250	>250
5FU	-	-	-	-	-	-	0.33 ± 0.17	18 ± 5	0.54 ± 0.12

*50% inhibitory concentration. 5FU- 5-fluoro Uracil. [¶] See Scheme 2 for structure.

NCI-60 DTP Human Tumor Cell Line Screen. Cytostatic/toxic activities of the 2,4-dichloro pyrrolo[3,2-*d*]pyrimidines **1** and **2** were tested at 10 μM concentrations against the NCI-60 DTP Human Tumor Cell Line screen. These cell lines are derived from many different cancer types including leukemia, non-small cell lung cancer (NSCLC), colon, CNS cancer, melanoma, ovarian, renal, prostate, and breast cancer (Figure 4). In this screen, 10 μM of each compound is added to cells and incubated for 48 hrs before chemical fixation and measurement of cellular protein content using the sulphorhodamine B (SRB) dye. This assay enables differentiation between cytostatic and cytotoxic activities based on the magnitude and direction of endpoint deviations from initial cell density.[63] Compound **1** did not show meaningful inhibition of growth of the cancer cell lines at 10 μM concentration (Figure 4) however introduction of iodine at C7

(compound **2**) led to an increase in the ability of the halogenated pyrrolo[3,2-*d*]pyrimidine to inhibit the growth of all cancer cell lines tested. The results of single-dose testing suggests that compound **2** predominately exhibits cytostatic activity (growth percent 0-50%), but in some instances led to a cytotoxic effect (growth percent <0%). The latter was observed in HL-60(TB), NCI-H522, COLO 205, MDA-MB-435, OVACAR-3, UO-31 and DU-145 (Figure 4). These results, in addition to the earlier testing, verify that the iodine at C7 plays a key role in increasing the potential anticancer properties of the C7-iodinated compound **2**.

Figure 4: Growth Inhibition of cancer cell lines by 1 and 2 at 10 µM.



Compound 1 and 2 tested at 10µM concentration against NCI-60 DTP Human Tumor Cell Line Screen. The blue bar indicates growth percent of cell line upon incubation with **1** at 10 µM, and the red bar indicates cell line upon incubation with **2** at 10 µM. The number on each bar indicates the % growth.

Cell cycle and apoptosis studies. Previously, 2,4-dichloro thieno[3,2-*d*]pyrimidines (see Figure 2a) were shown to induce apoptosis independent of cell cycle arrest in L1210 cells. Our recent findings show that 2,4-dichloro thieno[3,2-*d*]pyrimidine can induce cell cycle arrest at G2/M in the MDA-MB-231 breast cancer cell line. Subsequently, we chose to study the effects of 2,4-dichloro pyrrolo[3,2-*d*]pyrimidines **1** and **2** on cell cycle progression and apoptosis in MDA-MB-231 cells. MTT cytotoxicity assays resolved IC_{50} values of 6.0 ± 1.3 μM and 0.51 ± 0.10 μM for compounds **1** and **2**, respectively, in MDA-MB-231 cell cultures (Figure 5). These data provided dosage information critical for subsequent cell cycle and apoptosis assays, but also further confirm that introduction of iodine at C7 increases the potency by at least a factor of 10.

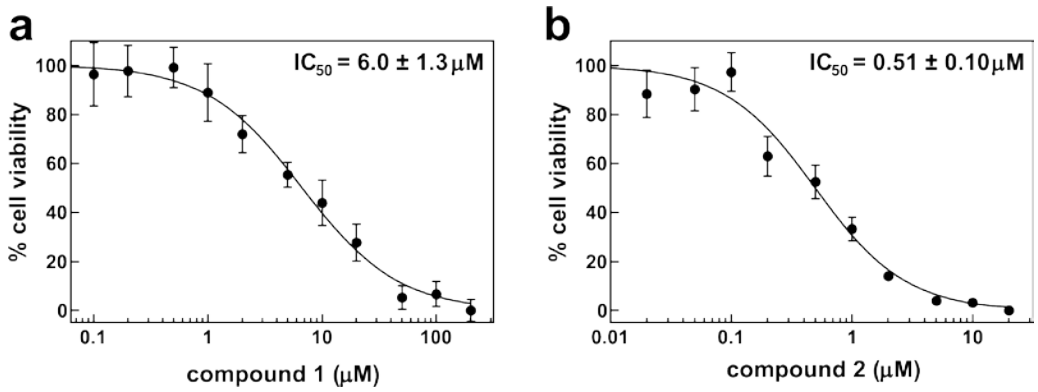


Figure 5. MTT assays for cytotoxicity by 1 and 2 in MDA-MB-231 cells. Cells were treated with select concentrations of compounds 1 (a) and 2 (b). After 48 h, cell viability was measured using MTT assays. Compound cytotoxicity was calculated by non-linear regression to a sigmoidal dose response function. Quoted IC_{50} values represent the mean ± SD of three independent experiments.

To determine whether the anticancer activities of pyrrolo[3,2-*d*]pyrimidine compounds **1** and **2** are associated with perturbation of the cell cycle, we monitored the effects of each compound on cell cycle distributions using propidium iodide staining and flow cytometry. At the IC_{80} concentration of 15 μM, compound **1** induced profound accumulation of the tumor cells at the G2/M stage after 48 hours (Figure 6a), consistent

with mitotic arrest and similar to the effect of the non-brominated 2,4-dichloro thieno[3,2-*d*]pyrimidine (Figure 2a, *left*) on MDA-MB-231 cells reported previously.[64] By contrast, treatment with an equitoxic concentration (1.75 μM) of compound **2** yielded a much smaller but still statistically significant enrichment in the G2/M fraction (Figure 6b, $P = 0.0006$ *vs*. vehicle control). The similarities in IC_{50} and the accumulation of the tumor cells in the G2/M phase observed for compound **1** and the non-brominated thieno[3,2-*d*]pyrimidine described previously[64] suggests that they may induce MDA-MB-231 tumor cell death via similar mechanisms. However, the dramatic enhancement in IC_{50} observed with compound **2** concomitant with its decreased impact on the cell cycle suggests that introduction of iodine at C7 might induce cytotoxicity by a distinct molecular mechanism. That such an impact might be directed by the iodine group on C7 is also supported by the abrogation of G2/M accumulation that was observed when 2,4-dichloro thieno[3,2-*d*]pyrimidine was brominated at the same site (Figure 2a, *right*).[64]

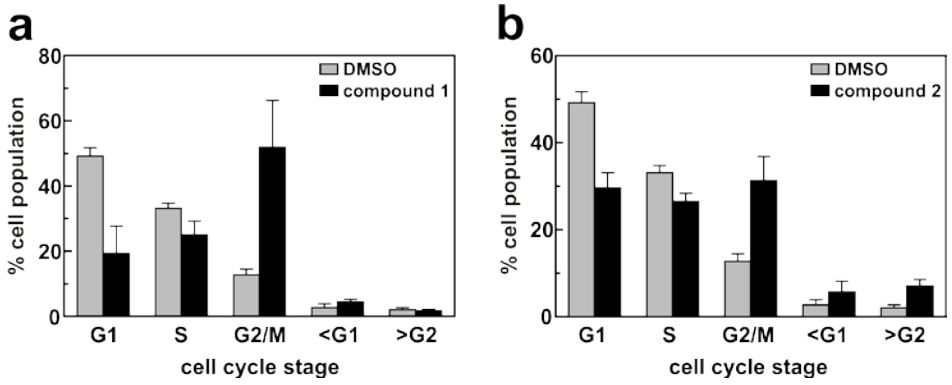


Figure 6. Cell cycle analyses of MDA-MB-231 cells following treatment with 1 and 2. Cells were treated with IC_{80} concentrations of compound 1 (a, 15 μM) or compound 2 (b, 1.75 μM) for 48 h. The cell cycle distributions of drug-treated cells and vehicle (DMSO) controls were analyzed by flow cytometry 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.

Next, we analyzed unfixed MDA-MB-231 cell samples by staining with annexin V and 7-amino-actinomycin D (7-AAD) to determine whether pyrrolo[3,2-*d*]pyrimidine

compounds **1** and **2** direct cell death using classical apoptotic or necrotic mechanisms. Vehicle (DMSO)-treated cells yielded very low proportions of apoptotic or necrotic cells after 48 h (Figure 7a). Curiously, treating cells with IC_{80} concentrations (15 µM) of compound **1** only slightly increased the proportion of cells recovered in early/late apoptosis (Figure 7b, d). By contrast, over 40% of MDA-MB-231 tumor cells treated with compound **2** at its IC_{80} (1.75 µM) were annexin V-positive after 48 h, with the larger proportion of these 7-AAD negative consistent with early apoptosis (Figure 7c, d). While compound **1** is clearly toxic to MDA-MB-231 cells at the tested concentration, only a modest (although statistically significant) increase in annexin V-positive cells was detected. There are several possibilities that could account for this, including: (i) that cell lysis occurs rapidly following induction of apoptosis by compound **1**, preventing significant accumulation of annexin V-positive bodies, or (ii) that a distinct (i.e., non-apoptotic/necrotic) cell death pathway is triggered by **1**. On the other hand, the robust accumulation of annexin V-positive cells observed following treatment with compound **2** is consistent with apoptotic cell death in a manner that is similar to the effects that were previously observed for the thieno[3,2-*d*]pyrimidine compounds shown in Figure 2a.[33]

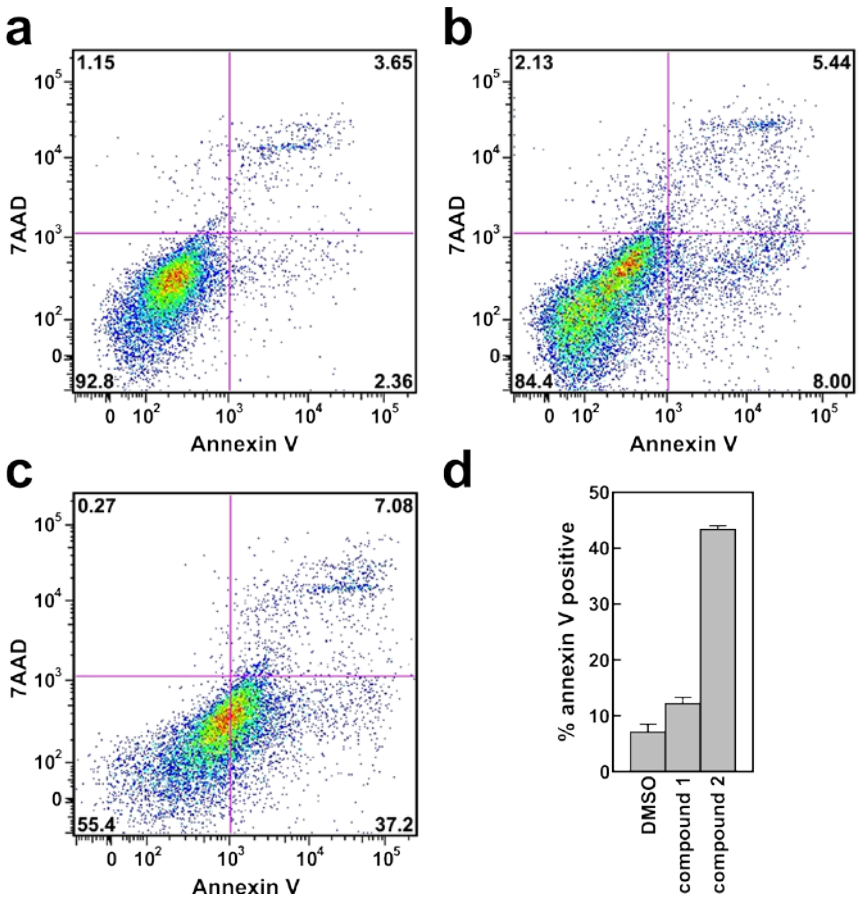


Figure 7. Apoptosis analyses of MDA-MB-231 cells following treatment with IC_{80} concentrations of compounds 1 and 2. Cells were treated with (a) an equal volume of vehicle (DMSO), (b) 15 µM compound 1, or (c) 1.75 µM compound 2. After 48 h, cells were stained using annexin V and 7-AAD and analyzed 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. (d) Total apoptotic (early + late) cell fractions detected in each treatment group. Bars show the mean ± SD values compiled across four independent experiments.

Discussion. Halogenated pyrrolo[3,2-*d*]pyrimidines have been commonly used as key intermediates to install a variety of functional groups on a pyrrolo[3,2-*d*]pyrimidine scaffold in order to evaluate the effect of substituents for a variety of medicinal properties. [26-27,39-40,42] However, the potential of halogenated pyrrolo[3,2-*d*]pyrimidines themselves as a therapeutically relevant scaffold has not previously been evaluated. Prompted by our earlier findings with the thieno[3,2-*d*]pyrimidines[33], 2,4-dichloropyrrolo[3,2-*d*]pyrimidines **1** and **2** were evaluated for their antiproliferative

properties against a number of cancer cell lines (Table 1 and Figure 2). Since, the importance of the C4 Cl group was already established[33], we studied the effect of pyrrole on the antiproliferative properties of an halogenated fused-pyrimidine scaffold. The activity of 2,4-dichloro pyrrolo[3,2-*d*]pyrimidine **1** is slightly lower than its thieno counterpart, however, the presence of 7-iodo enhanced the cytotoxicity to an extent that the activity was comparable to the halogenated thieno[3,2-*d*]pyrimidines shown in Figure 2. This notable increase in cytotoxic activity for **2** led to evaluation of bis-*O*-benzylated pyrrolo[3,2-*d*]pyrimidines **3** and **4** to assess the effect of C7 iodine independent of the C2 and C4 chlorines. The enhancement of cytotoxic activity for both **2** and **4** compared with the C7-unsubstituted parent compounds highlights the ability of the C7 iodine in imparting cytotoxic properties to the pyrrolo[3,2-*d*]pyrimidine scaffold independently warranted a detailed study of 2,4-substituted pyrrolo[3,2-*d*]pyrimidines possessing C7-iodine.

Our recent studies on 2,4-dichlorothieno[3,2-*d*]pyrimidines have shown that the presence of a bromine at C7 brought about complete alteration of the G2/M cell cycle arrest-induced tumor cell death to cell cycle-independent death in MDA-MB-231 cells.[64] Similarly, cell cycle distribution experiments conducted on pyrrolo[3,2-*d*]pyrimidines **1** and **2** indicated that both induce G2/M cell cycle arrest even though the presence of the C7 iodine marginally diminished the G2/M cell cycle arrest. Furthermore, apoptosis and necrosis studies revealed that compound **2** elicited a robust early stage apoptosis, but the same was not observed for compound **1**, which lacks the C7 iodine. Although intriguing, the difference in activity for compounds **1** and **2** follows a pattern that is similar to what

had been observed for the halogenated thieno[3,2-*d*]pyrimidines wherein the presence of an halogen at C7 alters the antiproliferative mechanism.

In summary, the antiproliferative activities of the halogenated-fused pyrimidines highlight the potential of reactive species as therapeutically useful scaffolds. Previously these types of covalent modifiers had been considered undesirable[65-72] due to their chemically labile nature, however the renewed interest in such compounds[73-75] have shown they may be exploited to treat cancer in a novel and effective manner.

Conclusions. We have identified interesting antiproliferative properties for several 2,4-dichloro pyrrolo[3,2-*d*]pyrimidines with emphasis on the ability of C7 iodine in markedly enhancing their cytotoxicity. These studies highlight their pharmacological relevance in addition to expanding the domain of the halogenated fused [3,2-*d*]pyrimidine scaffold. These halogenated compounds exhibited potent activity against triple negative breast cancer MDA-MB-231 cells by arresting the cell cycle in G2/M. We have previously established the importance of C4-Cl, however herein we have demonstrated the effect the halogen at C7 has on the potency and G2/M cell cycle-induced tumor cell death. Studies are underway to further expand the repertoire of these molecules with varied substituents on the scaffold to improve the antiproliferative activities of halogenated pyrrolo[3,2-*d*]pyrimidines. These results will help expand the chemical space of halogenated heterocyclic compounds with an ability to induce death in cancer cells.

Experimental. 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 NMRspectra 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.

Pyrrolo[3,2-*d*]pyrimidin-2,4-dione (5): To a stirred slurry of (E)-6-(2-(dimethylamino)vinyl)-5-nitropyrimidin-2,4-dione **4**[35,38] (1g, 4.4 mol) in fresh glacial AcOH (50 mL), Zinc dust (stabilized) (2 g) was added in two lots of 1g with an interval of 1 h. Upon overnight stirring the yellow slurry changed to a pale yellow to off-white slurry which was filtered and the filtrate concentrated *in vacuo* to obtain brown syrup. The product was precipitated from the brown syrup using ethanol to obtain **4** as white solid (0.6 g, 89.8%). The spectral data agrees with reported data.[38] ^1H NMR (400 MHz, DMSO-d_6): δ 5.82-5.83 (t, 1H, J=2.28 Hz), 7.12-7.13 (t, 1H, J=2.72 Hz, J=2.96 Hz),

10.57 (s, 1H), 10.74 (s, 1H), 11.82 (s, 1H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 96.5, 110.9, 127.4, 135.1, 152.0, 156.3.

2,4-Dichloropyrrolo[3,2-*d*]pyrimidine (1): To pyrrolo[3,2-*d*]pyrimidin-2,4-dione **5** (2.00 g, 13.2 mmol), 1N NaOH (15 mL), and 0.60 g NaOH in 15 mL H_2O was added and the mixture stirred at 40 °C until a clear solution was obtained. The solution was 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 1N 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 X 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 **1** 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.

7-iodo-2,4-dichloro pyrrolo[3,2-*d*]pyrimidine (2): To a solution of 2,4-dichloro pyrrolo[3,2-*d*]pyrimidine **1** (100 mg, 0.53 mmol) in anhydrous THF (5 mL), NIS (144

mg, 0.64 mmol) was added under N_2 atmosphere and stirred for 2 h after which TLC indicated consumption of **1**. The solvent was removed *in vacuo* and the residue dissolved in EtOAc. The organic phase was washed with aq. solution of $Na_2S_2O_3$ followed by water, brine and then dried over $MgSO_4$. The organic layer was concentrated *in vacuo* and loaded on silica. The product was purified using column chromatography eluting with 9:1 hexanes/EtOAc to obtain product as off-white solid (90 mg, 54%). R_f 0.55 in 3:1 hexanes/EtOAc. Mp decomposed from 160-230 °C. Spectral data agrees reported data.[40] ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.29 (s, 1H), 13.19 (s, 1H, *NH*). ^{13}C NMR (400 MHz, DMSO-d_6): δ 58.2, 124.4, 140.9, 143.5, 149.8, 153.5. ESI-MS *m/z* for calculated $[M+H]^+$ 313.8743, found 313.8740.

N-*tert*-butyloxycarbonyl-2,4-dichloro pyrrolo[3,2-*d*]pyrimidine (6): To a mixture of of 2,4-dichloro pyrrolo[3,2-*d*]pyrimidine **1** (50 mg, 0.26 mmol), di-*tert*-butyl carbonate (116 mg, 0.53 mmol) and DMAP (6.5 mg, 0.053 mmol), anhydrous THF (5 mL) was added under N_2 atmosphere and stirred overnight upon which TLC indicated consumption of **1**. The reaction mixture was concentrated and loaded on silica. The product was purified using column chromatography eluting with 49:1 hexanes/EtOAc to obtain product as white solid (50 mg, 65%). R_f 0.5 in 19:1 hexanes/EtOAc. Mp 101.0-103.2 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.66 (s, 9H), 6.71-6.72 (d, 1H, *J*=3.68 Hz), 8.01-8.02 (d, 1H, *J*=3.68 Hz). ^{13}C NMR (400 MHz, DMSO-d_6): δ 28.0, 87.2, 106.6, 123.4, 137.3, 146.5, 147.0, 153.1, 158.1. ESI-MS *m/z* for $C_{11}H_{11}C_{l2}N_3O_2$ calculated $[M+H]^+$ 288.0301, found 288.0301 ($2x^{35}Cl$), 289.0335 ($2x^{35}Cl$, ^{13}C), 290.0272 (^{35}Cl ^{37}Cl).

7-Iodo-pyrrolo[3,2-*d*]pyrimidine-2,4-dione (7): Pyrrolo[3,2-*d*]pyrimidine-2,4-dione **5** (500 mg, 3.31 mmol) was suspended in anhydrous DMF (10 mL) under N_2 atmosphere and cooled to -10 to -5 °C. To this slurry, N-iodosuccinimide (894 mg, 3.97 mmol) was added and the mixture stirred for 2 h at -10 to -5 °C, at which point the DMF was removed *in vacuo* to provide a brown sticky solid. The product was crystallized from the residue using 50% EtOH (6 mL) to give **7 (**800 mg, 87.2%) as a yellow solid. Mp >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 7.31 (s, 1H), 10.64 (br s, 1H), 10.76 (br s, 1H), 11.07 (br s, 1H). ^{13}C NMR: 93.5, 111.8, 131.7, 136.9, 151.8, 155.7. FAB-MS for $C_6H_4IN_3O_2$ calculated M^+ 276.9348, found 276.9345, calculated $[M+H]^+$ 277.9421, found 277.9419.

7-Iodo-1,3,5-tris-methoxymethyl-1,5-dihydropyrrolo[3,2-*d*]pyrimidine-2,4-dione (8): A solution of **5** (200 mg, 0.72 mmol) in anhydrous DMF (5 mL) was cooled on ice under N_2 atmosphere to which 60% suspension of NaH in oil (130 mg, 3.25 mmol) was added and stirred for 15 min. The ice was removed and the mixture stirred at room temperature for an additional 15 min at which point CH_3OCH_2Cl (0.19 mL, 2.53 mmol) was added dropwise over 15 min. The reaction mixture was stirred for 30 min on ice after which water (5 mL) was added followed by extraction with CH_2Cl_2. The organic layer was washed with brine and dried over $MgSO_4$. The organic layer was concentrated *in vacuo* and loaded on silica. The product was purified using column chromatography eluting with 9:1 and 4:1 hexanes/EtOAc to obtain product **8** as off-white solid (178 mg, 60%). R_f 0.5 in 3:1 hexanes/EtOAc. Mp 143.5-148 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 3.22 (s, 3H), 3.30 (s, 3H), 3.34 (s, 3H), 5.32 (s, 2H), 5.53 (s, 2H), 5.55 (s, 2H), 7.10 (s, 1H).^{13}C NMR: 28.2, 45.2, 56.5, 56.7, 58.0, 72.5, 78.4, 111.6, 134.6, 136.9, 152.2, 154.4. FAB-MS for

$C_{12}H_{16}IN_3O_5$ calculated M^+ 409.0134, found 409.0127, calculated $[M+H]^+$ 410.0207 found 410.0201.

2,4-Bis-benzyloxy-5-nitro-6-dimethylaminovinyl pyrimidine (10): To a solution of 2,4-Bis-O-Benzyl-6-methyl-5-nitro pyrimidine **9**[37] (2.3 g, 6.5 mmol) in DMF (20 mL), DMF-dimethyl acetal (1.74 mL, 13 mmol) was added at room temperature under N_2 atmosphere. The reaction was lowered in a preheated oil bath at 60-65 ° C and stirred overnight upon which TLC indicated absence of starting material. The solvents were removed and the residue loaded on silica. The product was purified using column chromatography eluting with 9:1 hexanes/EtOAc to obtain product **10** as orange-yellow solid (2 g, 75%). R_f 0.5 in 3:1 hexanes/EtOAc. Spectral data agrees with reported data.[37] ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.87-2.94 (br d, 6H), 5.33-5.36 (d, 1H, *J*=12.36 Hz), 5.38 (s, 2H), 5.44 (s, 2H), 7.31-7.41 (m, 10H), 7.98-8.01 (d, 1H, *J*=12.36 Hz). ^{13}C NMR (400 MHz, $CDCl_3$): δ 68.9, 69.5, 87.9, 127.5, 128.0, 128.1, 128.3 128.5, 135.8, 136.6, 151.8, 160.7, 161.6, 163.5. FAB-MS for $C_{22}H_{22}N_4O_4$ calculated $[M+H]^+$ 407.1714, found 407.1717.

2,4-Bis-benzyloxy-5*H*-pyrrolo[3,2-*d*]pyrimidine[34] **(3):** To a suspension of 2,4-Bis-O-Benzyl-5-nitro-6-β-dimethylaminovinyl pyrimidine **10** (2 g, 4.9 mmol) in AcOH (40 ml), Zn (4 g) was added in lot of 2g with an interval of 4hrs. The reaction mixture was stirred overnight at room temperature during which a dark yellow suspension became pale yellow suspension. The reaction mixture was filtered and the filtrate concentrated *in vacuo* to obtain syrup which was dissolved in CH_2Cl_2 then washed with saturated aq. $NaHCO_3$ followed by brine. The organic phase was dried over $MgSO_4$ and loaded on silica. The product was purified using column chromatography eluting with 4:1 and 1:1

hexanes/EtOAc to obtain product as pale-yellow solid (1.45 g, 90%). R_f 0.3 in 1:3 hexanes/EtOAc. Spectral data agrees with reported data.[37] ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.47 (s, 2H), 5.54 (s, 1H), 6.50-6.51 (dd, 1H, *J*=1.84, 2.28), 7.29-7.38 (m, 7H), 7.43-7.45 (m, 2H), 7.52-7.53 (m, 2H), 8.41 (br s, 1H, *NH*). ^{13}C NMR (400 MHz, $CDCl_3$): δ 68.3, 69.0, 102.6, 111.9, 127.8, 128.3, 128.4, 128.5, 128.6, 128.7, 128.8, 136.1, 137.3, 151.8, 156.79, 159.7. FAB-MS for $C_{20}H_{17}N_3O_2$ calculated $[M+H]^+$ 332.1394, found 332.1398.

7-iodo-2,4-bis-benzyloxy-5*H*-pyrrolo[3,2-*d*]pyrimidine[34] (**4**):To a stirred solution of 2,4-bis-O-benzyl-5*H*-pyrrolo[3,2-*d*]pyrimidine **3** (1.43 g, 4.3 mmol) in anhydrous CH_2Cl_2 (15 mL) under N_2, NIS (1.069 g, 4.7 mmol) was added at which point the reaction mixture turned from pink to orange. The mixture was stirred overnight until the TLC indicated the absence of starting material. The reaction mixture was washed with aqueous $Na_2S_2O_3$ (15 mL) followed by brine (15 mL). The organic layer was dried over $MgSO_4$, loaded onto silica and purified using column chromatography eluting with 4:1 then 1:1 hexanes/EtOAc to obtain **4** as a pale-yellow solid (1.77 g, 3.88 mmol, 90%).[34] R_f 0.4 in 1:3 hexanes/EtOAc. Mp 157.8-158.4 ˚C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.53 (s, 4H), 7.32 -7.41 (m, 9H), 7.55-7.57 (m, 2H), 8.71 (br s, 1H, *NH*). ^{13}C NMR: 57.3, 68.7, 69.3, 111.9, 127.9, 128.3, 128.6, 128.66, 128.7, 128.9, 132.4, 135.8, 137.2, 152.0, 156.9, 160.1. FAB-MS *m/z* for $C_{20}H_{16}IN_3O_2$ calculated $[M+H]^+$ 458.0360, found 458.0357.

2,4-Bis-benzyloxy-5-*N*-boc-7-iodopyrrolo[3,2-*d*]pyrimidine (11): To a solution of 7-iodo-2,4-bis-benzyloxy-5*H*-pyrrolo[3,2-*d*]pyrimidine **4** (100 mg, 0.218 mmol) in dry THF (5 mL), Boc_2O (96 mg, 0.437 mmol) and DMAP (145.33 mg, 0.0437 mmol) were added and the mixture stirred for 1 hr until TLC indicated the absence of starting material. The solvent was removed *in vacuo* and the residue purified by column

chromatography eluting with 49:1 hexanes/EtOAc to obtain **11** as a white solid (65.1 mg, 116 mmol, 53.4%).[34] R_f 0.5 in 19:1 hexanes/EtOAc. Mp 118.5-122.5 ˚C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.51 (d, 9H), 5.59 (s, 2H), 5.50 (s, 2H), 7.38-7.30 (m, 6H), 7.47 (d, 2H), 7.56 (d, 2H), 7.91 (s, 1H). ^{13}C NMR: δ 27.8, 64.3, 68.9, 69.5, 85.3, 110.9, 127.9, 128.4, 128.5, 128.8, 136.2, 136.5, 136.9, 147.2, 156.6, 157.9, 161.1. FAB-MS *m/z* for $C_{25}H_{24}IN_3O_4$ calculated $[M+H]^+$ 558.0884, found MH^+ 558.0885.

2-[(Dimethylamino)methyleneimino]-4-benzyloxy-5*H*-pyrrolo[3,2-*d*]pyrimidine (12): A slurry of 2-[(Dimethylamino)methyleneimino]-4-benzyloxy-5-nitro-6-β-dimethylaminovinyl pyrimidin-4-one[37] (3 g, 8.1 mmol) in EtOH (100 mL) and water (20 mL) was heated to 90-95 °C and $Na_2S_2O_4$ (8.5 g, 50.5 mmol) added in lots of 1.7 g after every 30-45 min. After 8.5 g, the slurry changed to pale yellow slurry and TLC indicated absence of starting material. The EtOH was removed *in vacuo* and the residual solids were extracted with CH_2Cl_2 (2X200 mL). The organic phase was washed with water, then dried over $MgSO_4$. The organic phase was removed in vacuo to obtain **12** as pale yellow solid (1.7 g, 71%). R_f 0.45 in CH_2Cl_2/MeOH. Spectral data agrees with reported data.[37] ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.99 (s, 3H), 3.04 (s, 3H), 6.22-6.24 (t, 1H, *J*=2.72 Hz, *J*=2.32 Hz), 7.06-7.07 (t, 1H, *J*=3.2 Hz, *J*=2.76 Hz), 7.11-7.25 (m, 3H), 7.31-7.33 (m, 2H), 9.68 (br s, 1H, *NH*). ^{13}C NMR (400 MHz, $CDCl_3$): δ 35.0, 40.9, 45.5, 102.1, 115.2, 126.8, 127.6, 128.0, 128.2, 138.9, 144.4, 154.1, 156.0, 156.4. FAB-MS *m/z* for $C_{16}H_{17}N_5O$ calculated M^+ 295.1433, found 295.14288. Calculated $[M+H]^+$ 296.1506, found 296.1508.

9-deaza-2'-deoxyxanthosine (13): White solid Mp 185.3-186.8 ˚C. ^{1}H NMR (400 MHz, DMSO *d6*): δ 1.84-1.95 (m, 2H), 3.49-3.57 (m, 2H), 3.75 (br s, 1H), 4.19 (br s, 1H), 4.96

(d, 1H, *J*=3.24 Hz), 5.05 (dd, 1H, *J*=5.92 Hz, *J*=10.08 Hz), 5.62 (br s, 1H), 10.56 (br s, 1H, *NH*), 10.58 (br s, 1H, *NH*), 11.67 (br s, 1H, *NH*). ^{13}C NMR (400 MHz, DMSO *d6*): δ 43.0, 62.7, 73.6, 73.7, 87.7, 111.2, 111.5, 125.2, 132.7, 151.8, 156.3. FAB-MS for $C_{11}H_{13}N_3O_5$ calculated M^+ 267.0855, found 267.0853; calculated [$M+H^+$] 268.0928 found 268.0930.

9-deaza-2'-deoxyguanosine (14): White solid Mp >300 ˚C. R_f 0.5 in 4:1 CH_2Cl_2: MeOH. ^{1}H NMR (400 MHz, DMSO *d6*): δ 1.86 (dd, 1H, *J*=5.26 Hz, *J*=12.62 Hz), 2.13 (td, 1H, *J*=5.04 Hz, *J*=11.69 Hz), 3.37 (ddd, 2H, *J*=4.12 Hz, *J*=11.66 Hz, *J*=11.44 Hz), 3.69 (t, 1H, *J*=4.12 Hz), 4.15 (br s, 1H), 4.85 (d, 1H, *J*=3.64 Hz), 5.05 (dd, 1H, *J*=5.28 Hz, *J*=10.76 Hz), 5.66 (br s, 2H, *NH*$_2$), 5.81 (br s, 1H), 7.09 (d, 1H, *J*=3.2 Hz), 10.41 (br s, 1H, *NH*), 11.33 (br s, 1H, *NH*). ^{13}C NMR (400 MHz, DMSO *d6*): δ 42.6, 63.8, 73.6, 73.9, 88.3, 113.8, 115.7, 126.1, 151.3, 155.4, 164.0. ESI-MS *m/z* for $C_{11}H_{14}N_4O_4$ calculated [$M+H^+$] 267.1087, found 267.1089.

Cell proliferation assays: All assays were performed in 96-well microtiter plates. To each well were added (5−7.5) × 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%. MDA-MB-

231 cells (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal bovine serum (FBS). Cells were seeded in 96 well plates at 5×10^3 cells per well and treated with compounds **1** and **2** across a range of concentrations. After 48 h, relative numbers of viable cells were quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Assay kit (ATCC) according to the manufacturer's instructions. The fraction of viable cells relative to wells treated with vehicle alone was analyzed as a function of drug concentration using a sigmoidal dose response model with PRISM v3.03 software (GraphPad) to resolve IC_{50}.

Cell cycle distribution and apoptosis assays: The cell cycle distributions of MDA-MB-231 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_{80} values determined by cell viability assays. 48 h following treatment the cells were collected, fixed, and stained with propidium iodide (Sigma Aldrich) as described[76] immediately before analysis by flow cytometry. To analyze apoptotic cell death, MDA-MB-231 cells were seeded as described above but then treated with vehicle alone or compounds **1** or **2** at their IC80 concentrations of 15 μM and 1.75 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-amino-actinomycin D (7-AAD) using the BD Pharmingen PE Annexin V Apoptosis Detection Kit I (BD Pharmingen) as described.[77] 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.

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