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Antiproliferative and apoptosis inducing properties of pyrano[3,2-*c*]pyridones accessible by a one-step multicomponent synthesis

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Abstract—4-Arylpyrano-[3,2-*c*]-pyridones have been prepared by a one-step cyclocondensation of 4-hydroxy-1,6-dimethylpyridin-2(1H)-one with various substituted benzaldehydes and malononitrile. These heterocycles exhibit micromolar and submicromolar antiproliferative activity in HeLa and induce apoptosis in Jurkat cell lines. Structure–activity studies performed on a small library of these compounds show a pronounced cytotoxicity enhancing effect of the bromo substituent at the meta position of the C4 aromatic moiety.
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2-Pyridone structural unit occurs in many natural and synthetic molecules exhibiting diverse biological activities.[1] A few selected examples include antitumor antibiotic diazaquinomycin A,[2] a non-nucleoside HIV reverse transcriptase inhibitor L-697,661[3], and a phosphodiesterase inhibitor milrinone,[4] used in the clinic for the treatment of heart failure (Fig. 1).

Many libraries of compounds containing a 2-pyridone moiety fused with another heterocyclic ring have been prepared and tested for various biological activities. Somewhat surprisingly, the biology of 4*H*-pyrano-[3,2-*c*]-pyridin-5(6*H*)-ones (**A**, Figure 2) has not been thoroughly investigated with the exception of antibacterial properties associated with some of these compounds.[5] For example, a literature search reveals that pyranopyridones **B**, whose preparation by way of cyclocondensation of arylidene malononitriles with 4-hydroxypyridine-2-ones has been reported on many occasions,[6] have not been evaluated for biological activities. In contrast, a number of recent publications and patents have described promising anticancer activity, associated with chromenes **C**.[7] These compounds,

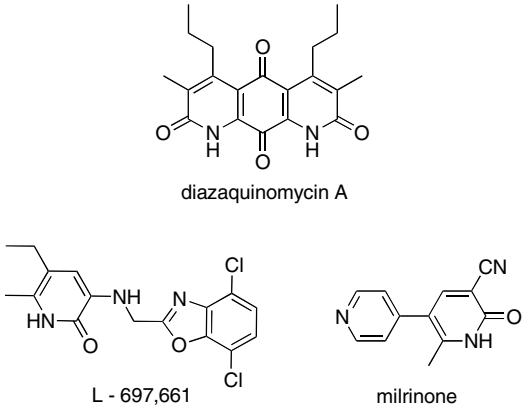

Figure 1. Structures of biologically active 2-pyridone-containing compounds.

Keywords: Multicomponent synthesis; Cytotoxicity; Apoptosis; Heterocycles.
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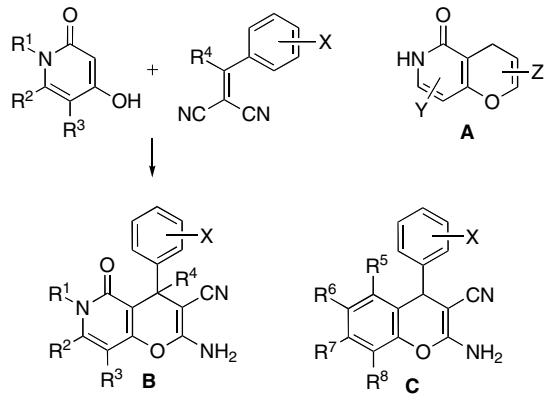


Figure 2. Structures of the scaffold **A**, pyranopyridone library **B**, and chromene library **C**.

shown to inhibit tubulin polymerization and induce apoptosis in cancer cells, exhibit high potency against taxol- and vinblastine-resistant, P-glycoprotein overexpressing cell types.[7d] Furthermore, chromenes **C** disrupt tumor vasculature in a number of human solid tumor xenografts and they are currently under development as anticancer agents.[7b,c]

Extensive SAR studies performed with chromenes **C** show that substituents on the benzene ring at positions 7 and 8 (R^7 and R^8) are well tolerated, while the introduction of substituents at positions 5 and 6 (R^5 and R^6) results in inactive compounds. Furthermore, replacement of the benzene ring of the chromene scaffold with a heterocyclic moiety has not been reported to the best of our knowledge. We reasoned that pyridones **B** would represent an interesting test because of the partial aromatic character of this ring brought about by the amide resonance conjugation. In addition, the amide group would be placed into the part of the structure that is most sensitive to alterations. Finally, amide resonance conjugation would make the compounds more polar addressing water solubility issues of the parent chromenes **C**.[8]

The synthesis of the pyridone **B** library is shown in Figure 3. Pyridone **1** was prepared by treating the corresponding commercially available pyrone with aqueous $MeNH_2$ following a literature procedure.[9] A three-component reaction of pyridone **1** with malononitrile and various aromatic aldehydes in a 1:1:1 ratio proceeds smoothly in refluxing ethanol containing a small quantity of Et_3N.[10] Pyranopyridones **2–12** precipitate directly from the refluxing reaction mixtures and require no further purification. The product yields are given in Table 1.[11,12]

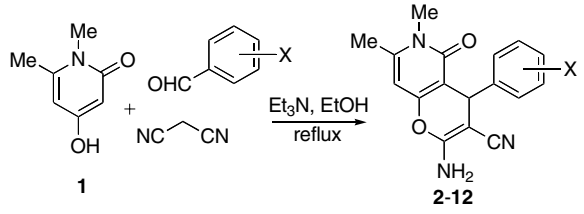


Figure 3. Three-component synthesis of pyrano-[3,2-*c*]-pyridones.

The analogue library was tested for antiproliferative activity using HeLa cell line as a model for human cervical adenocarcinoma. The cells were treated with respective compounds for 48 h and cell viability was assessed through measurements of mitochondrial dehydrogenase activity using MTT method (Table 1).[13] It is noteworthy that all potent analogues have a 3-bromo substituent on the aromatic ring at position C4 of the pyranopyridone skeleton (compounds **2–8**) and this preference is uniform irrespective of the substitution pattern of this aromatic moiety. The 3-chloro (**9**) and other variously substituted analogues (**10–12**) are significantly less potent or are totally inactive. Further, the substitution of the nitrogen in the pyridone ring by oxygen, as in pyranopyranone **13**, abolishes the activity as well. Moderate potency of the *N*-(β-arylethyl)pyridone **14**, synthesized in a manner analogous to the rest of the library, warrants further investigation of compounds having a bulky moiety on the pyridone nitrogen. Efforts to prepare a library of such compounds are underway in our laboratories.

Since many clinically used anticancer agents induce apoptosis in cancer cells, we tested the pyranopyridone analogues for their ability to induce apoptosis in Jurkat cells using a flow cytometric annexin-V/propidium iodide assay (Fig. 4). Compounds **2–8**, exhibiting submicromolar or low micromolar potencies for the inhibition of proliferation of HeLa cells, were found to be strong inducers of apoptosis in Jurkats at 5 μM concentrations. The magnitude of apoptosis induction (50–60% after 36 h treatment) is comparable to the known antimitotic agent colchicine used at the same concentration. In contrast, compounds **9**, **11**, **14**, which are much less potent or totally inactive in the HeLa MTT assay, show no apoptosis induction of Jurkats at this concentration.

For comparison we selected some of the most potent chromene **C** (Fig. 2) analogues on the basis of the literature data (e.g., R^5, R^6, $R^8 = H$; $R^7 = NMe_2$; X = 3,4,5-*tri*-OMe or X = 3-Br-4,5-*di*-OMe),[7] synthesized, and evaluated them in our assays. While the chromenes are significantly more cytotoxic to HeLa cells (IC_{50} = 1–10 nM), the magnitude of apoptosis induction in Jurkats is similar to compounds **2–8** (50–55% at 5 μM).

The images of Jurkat cells after 48-h treatment are shown in Figure 5. Cells treated with an inactive compound **11** (Fig. 5B) look similar to the DMSO treated counterparts (Fig. 5A). In contrast, extensive deformation and fragmentation are observed with cells treated with a potent analogue **3** (Fig. 5C).

Lastly, the flow cytometric cell cycle analysis, performed with pyranopyridones **2** and **3** using Jurkat cell line, shows pronounced cell cycle arrest in the G_2/M phase (Table 2). This effect is characteristic of antimitotic agents disrupting microtubule assembly, and is also observed with chromenes **C** that bind to or near the colchicine binding site on β-tubulin.[7d] This observation is

Table 1. Synthetic yields and antiproliferative activity of pyrano-[3,2-*c*]-pyridones

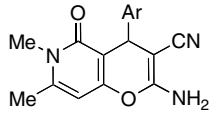
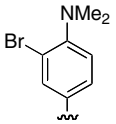
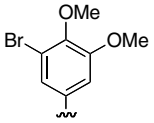
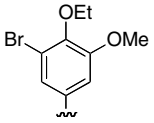
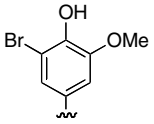
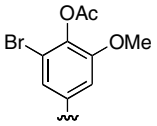
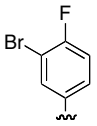
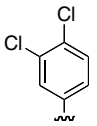
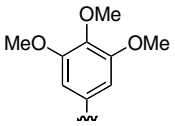
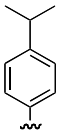
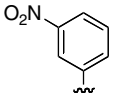
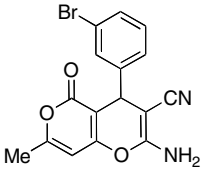
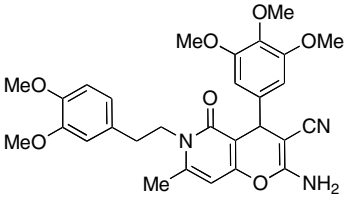
		Synthesis	Compound concentration required to reduce HeLa cell viability by 50% after 48-h treatment relative to 100% DMSO control as assessed with MTT assay in three independent experiments				
Analogue	Ar	% Yield	$IC_{50}(1)$, μM	$IC_{50}(2)$, μM	$IC_{50}(3)$, μM	IC_{50}, μM	SD, μM
2		83	0.3	0.3	0.4	0.33	0.06
3		87	0.75	0.5	0.5	0.58	0.14
4		88	2	0.75	0.5	1.08	0.8
5		75	2	2	4	2.67	1.1
6		83	4	2	4.5	3.5	1.3
7		84	7	7	5	6.33	1.1
8		97	7.5	7	5	6.5	1.3
9		98	20	15	20	18.3	2.9
10		97	40	50	40	43.3	5.1
11		81	>100	>100	>100	>100	n/a

Table 1 (*continued*)

Analogue	Ar	Synthesis % Yield	Compound concentration required to reduce HeLa cell viability by 50% after 48-h treatment relative to 100% DMSO control as assessed with MTT assay in three independent experiments				
			$IC_{50}(1)$, μM	$IC_{50}(2)$, μM	$IC_{50}(3)$, μM	IC_{50}, μM	SD, μM
12		97	20	25	60	35.0	21.8
13		87	>100	>100	>100	>100	n/a
14		80	30	18	20	22.7	6.4

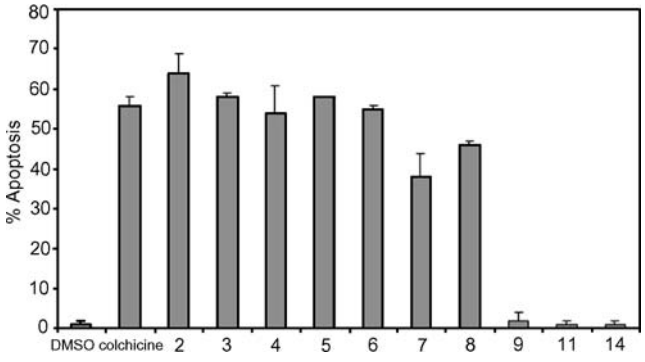


Figure 4. Induction of apoptosis in Jurkat cells treated for 36 h with DMSO control, colchicine (5 μM), and selected pyridone library analogues (5 μM) in flow cytometric annexin-V/propidium iodide assay. Error bars represent data from two experiments.

Table 2. Flow cytometric cell cycle analysis of Jurkat cells

Compound	% Relative DNA content ±SD after 15-h treatment as assessed with Vybrant Orange staining		
	G_0/G_1	S	G_2/M
DMSO	49.4 ± 2.7	26.6 ± 0.1	23.9 ± 2.4
2 (5 μM)	14.4 ± 1.7	18.1 ± 0.6	67.5 ± 2.3
3 (5 μM)	12.0 ± 3.5	19.0 ± 2.0	68.5 ± 4.7
Colchicine (25 μM)	19.0 ± 0.2	23.1 ± 0.1	57.9 ± 0.1

indicative of the antitubulin mechanism for pyranopyridones **B** similar to the one established for chromenes **C**.

Further optimization of the pyranopyridione library with the aim of identifying more potent analogues as

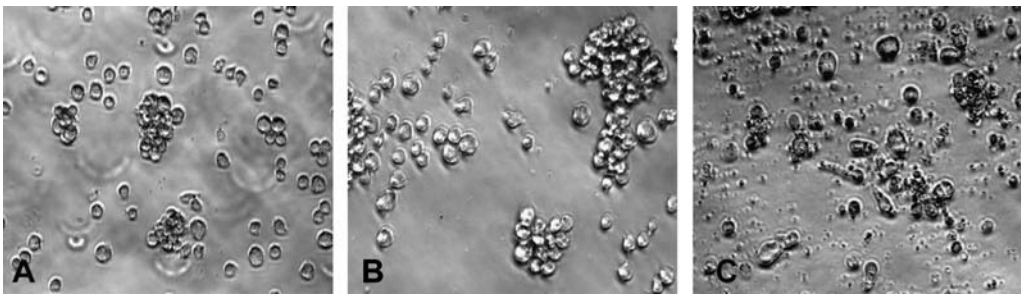


Figure 5. Jurkat cells after 48-h treatment with DMSO control (A), inactive compound **11** (B) and potent analogue **3** (C).

well as more detailed mechanistic studies are underway and will be reported in due course.

Acknowledgments

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8. The calculated log*P* values are 1.5–2 units lower for pyranopyridones **B** (log*P* ~ 2–2.5) compared with the previously reported ([7d]) 7-Me$_2$*N*-substituted (R^5, R^6, R^8 = H; R^7 = NMe$_2$) chromenes **C** (log*P* ~ 3.5–4.5).
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11. *General procedure for the synthesis of pyrano-[3,2-c]-pyridones* **2**–**12**: A mixture of 4-hydroxy-1,6-dimethylpyridin-2(1*H*)-one (0.8 mmol), malononitrile (0.8 mmol), triethylamine (0.05 mL), and a corresponding aldehyde (0.8 mmol) in EtOH (96% aqueous solution, 3 mL) was refluxed for 50 min. The reaction mixture was allowed to cool to room temperature, the precipitated product was collected by filtration and washed with EtOH (5 mL). In all cases the product was >98% pure as judged by ^{1}H NMR analysis.
12. *Selected characterization data*: Compound **2**: 83%; mp 248–250 °C (EtOH); ^{1}H NMR (DMSO-d_6) δ 7.27–7.08 (m, 5H), 6.06 (s, 1H), 4.28 (s, 1H), 3.33 (s, 3H), 2.66 (s, 6H), 2.33 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 161.7, 159.8, 155.4, 153.7, 148.8, 145.0, 143.1, 122.9, 112.7, 105.5, 97.4, 60.5, 56.6, 39.3, 37.2, 31.2, 20.8; HRMS *m*/*z* (ESI) calcd for $C_{19}H_{19}BrN_4O_2$ (M+Na$^+$) 437.0589, found 437.0580. Compound **4**: 88%; mp 258–260 °C (EtOH); ^{1}H NMR (DMSO-d_6) δ 7.09 (s, 2H), 6.90 (s, 1H), 6.79 (s, 1H), 6.07 (d, J = 2.7 Hz, 1H), 4.33 (d, J = 3.0 Hz, 1H), 3.92 (q, J = 3.0 Hz, 2H), 3.76 (s, 3H), 3.33 (s, 3H), 2.33 (s, 3H), 1.27 (t, J = 3.0 Hz. 3H); ^{13}C NMR (DMSO-d_6) δ 161.6, 159.8, 155.4, 153.8, 148.6, 144.3, 142.7, 122.9, 117.5, 112.6, 105.5, 97.42, 68.9, 57.8, 56.6, 37.1, 31.1, 20.8, 16.1; HRMS *m*/*z* (ESI) calcd for $C_{20}H_{20}BrN_3O_4$ (M+Na$^+$) 468.0535, found 468.0540. Compound **5**: 75%; mp 246–247 °C (EtOH); ^{1}H NMR (DMSO-d_6) δ 9.32 (s, 1H), 7.04 (s, 2H), 6.82–6.71 (m, 2H), 6.06 (s, 1H), 4.28 (s, 1H), 3.77 (s, 3H), 3.32 (s, 3H), 2.34 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 161.6, 159.6, 155.1, 148.5, 148.4, 143.0, 137.5, 123.1, 111.5, 109.6, 105.9, 97.4, 58.0, 56.7, 37.0, 31.1, 20.8; HRMS *m*/*z* (ESI) calcd for $C_{18}H_{16}BrN_3O_4$ (M+Na$^+$) 440.0222, found 440.0223.
13. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55.