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Inhibition of tumor cell proliferation by thieno[2,3-*d*]pyrimidin-4(1*H*)-one-based analogs

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Abstract—On the basis of a screening lead from an assay using a pair of p21 isogenic cell lines (p21-proficient cells and p21-deficient cells) to identify chemoselective agents, a series of novel thieno[2,3-*d*]pyrimidin-4(1*H*)-one-based analogs was prepared. Some analogs inhibited the growth of human colon tumor cells.

Progression from one phase of the cell cycle to the next is controlled by a series of sensors and arresting mechanisms called cell cycle checkpoints.[1,2] Through regulation of the cyclin-dependent kinases (CDKs), these checkpoints ensure that each step in the cell cycle has been successfully completed before the onset of the next phase.[3] Loss of checkpoint control is a hallmark of tumor cells, as it leads to an increase in mutation rate and allows a more rapid progression to the tumorigenic state.[1] The failure of cell cycle arrest responses in malignant cells can be exploited therapeutically in a screening approach: compounds that selectively kill checkpoint-deficient cells compared with checkpoint-proficient cells can be expected to target tumor cells preferentially, while sparing normal cells.[1,4–6] Anti-tumor agents identified by these screening methods are likely to be safer and more effective than current cancer therapies.

The protein $p21^{Wafl/Cipl/Sdi1}$ (hereafter referred to as p21), a downstream effector of the major tumor suppressor p53, blocks progression of the cell cycle in response to DNA damage or abnormal DNA content by suppressing the activity of CDKs.[7,8] A p21-deficient (p21−/−) cell line was generated from HCT116, a human colorectal cancer cell line with an intact p53/p21 checkpoint function (p21+/+), by targeted gene deletion.[9] The p21−/− cells showed increased sensitivity, compared to the isogenic p21+/+ cells, to several clinically used anti-tumor drugs, validating the role of cell cycle checkpoints in determining chemosensitivity.[4,5] In an effort to identify structurally novel agents for cancer treatment, we used this isogenic pair of cell lines, in parallel, to screen compound libraries for therapeutic molecules that preferentially inhibit the p21−/− cells.

We recently reported the identification of pyrazolo[1,5-*a*]pyrimidines **1** (Fig. 1) as selective inhibitors of p21−/− cells relative to p21+/+ cells using this isogenic pair of cell lines.[10] Compound **1** also showed inhibitory activity against a panel of human colon tumor cell lines. We now report the identification of **2**, a tricyclic 5,6,7,8-tetrahydrobenzothieno[2,3-*d*]pyrimidin-4(1*H*)-one with a 3,4,5-trimethoxyphenyl group at C-2, using the same screening approach (Fig. 1) and describe the synthesis,

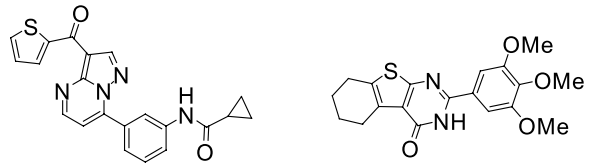

Figure 1. Leads from a high throughput screen for identification of p21 chemoselective agents.

Keywords: Anti-proliferative; p21 isogenic cell lines; Benzo[4,5]thieno[2,3-*d*]pyrimidin-4(1*H*)-ones.
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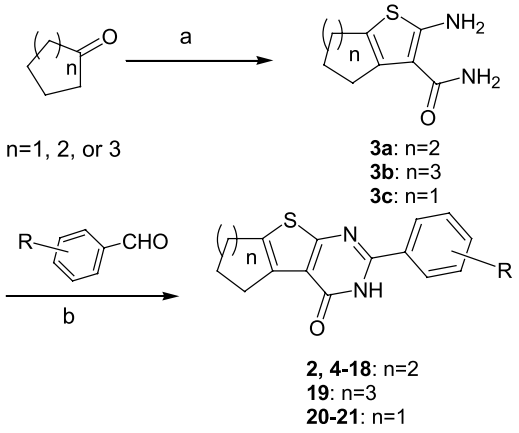
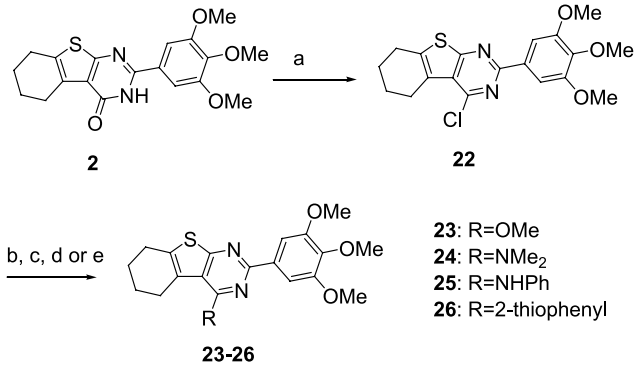


Scheme 1. Reagents and conditions: (a) cyanoacetamide/S_8/morpholine/EtOH and (b) HCl/MeOH, reflux.

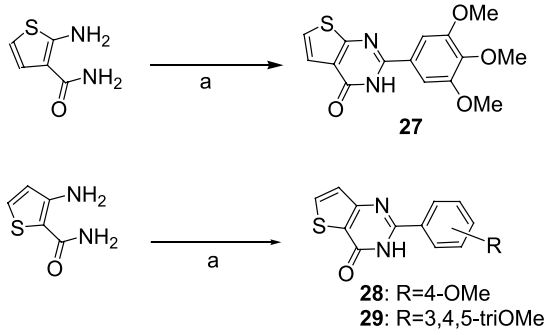
anti-proliferative activity, and the SAR of these compounds.

The tricyclic benzo[4,5]thieno[2,3-*d*]pyrimidin-4(1*H*)-ones with substituted phenyl groups at C-2 (compounds **2** and **4**–**18**) were prepared as shown in Scheme 1. Treatment of cyclohexanone with cyanoacetamide in the presence of sulfur and morpholine resulted in bicyclic 2-amino-4,5,6,7-tetrahydrobenzothiophene-3-carboxamide **3a**.[11] Cyclocondensation of **3a** with various substituted benzaldehydes under a modified literature procedure[12] provided the desired thieno[2,3-*d*]pyrimidin-4(1*H*)-ones, **2** and **4**–**18**, which all have saturated 6-membered cycloalkyl ring fused to the thiophene. In order to explore how the size of the cycloalkyl group affects the biological activity, compounds with 7- and 5-membered cycloalkyl rings fused to the thiophene (**19**–**21**) were also prepared similarly from cycloheptanone and cyclopentanone via intermediates **3b** and **3c**, respectively.

To investigate the SAR of the pyrimidone ring, compounds **23**–**26** with different substituents at the C-4 position were prepared (Scheme 2). Thus, chlorination of **2** with $POCl_3$ provided chloride **22**. Nucleophilic substitution of **22** with MeOH gave methyl ether derivative **23**.



Scheme 2. Reagents and conditions: (a) $POCl_3$, reflux; (b) Na, MeOH, reflux; (c) DMF, reflux; (d) aniline/pyridine–HCl/2-ethoxyethanol, 100 °C; (e) 2-thiopheneboronic acid/$Pd(PPh_3)_4$/aq $NaHCO_3$/DME, reflux.



Scheme 3. Reagents and conditions: (a) HCl/MeOH/3,4,5-trimethoxybenzaldehyde or 4-methoxybenzaldehyde, reflux.

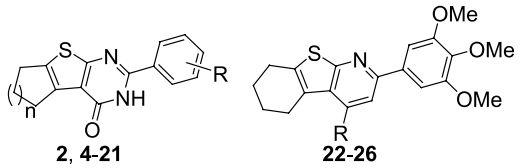
The dimethylamino derivative **24** was prepared by treatment of **22** with DMF under thermal conditions. Chloride **22** reacted with aniline in the presence of pyridine–HCl to provide **25** and Pd-mediated Suzuki coupling of **22** with 2-thiopheneboronic acid afforded **26**.

To probe whether the cycloalkyl ring is part of the essential pharmacophore, compounds **27**–**29** were prepared as shown in Scheme 3. A bicyclic thieno[2,3-*d*]pyrimidin-4(1*H*)-one without the cycloalkyl ring, **27**, was prepared via a cyclocondensation reaction of 2-amino-3-thiophenecarboxamide with 3,4,5-trimethoxy-benzaldehyde. The corresponding analogs of **27** with a reversed ring fusion, thieno[3,2-*d*]pyrimidin-4(1*H*)-ones (**28** and **29**), were prepared from 3-amino-2-thiophenecarboxamide and benzaldehydes.

The tricyclic 5,6,7,8-tetrahydrobenzo[4,5]-thieno[2,3-*d*]pyrimidin-4(1*H*)-ones and their structurally modified analogues were evaluated for their inhibitory activity in the isogenic cell lines.[13] Their activities and the selectivity ratios between the two cell lines are reported in Table 1. The HTS hit, **2**, showed inhibitory activity in p21−/− cells with an IC_{50} of 2.3 μM and a selectivity ratio of >8.7-fold against p21+/+ cells. Structural changes at the C-2 phenyl group appear to have a considerable effect on the biological activity. Compounds lacking only one of the methoxy groups of **2** resulted in total loss of activity (**4** and **5**). However, removal of both the 3- and 5-methoxy groups (compound **6**) caused only a slight change to its activity. As seen from the data in Table 1, replacement of the 4-methoxy group of **6** with a methylthio group was well tolerated. However, only small alkoxy groups were tolerated at the 4-position of the phenyl group. When the 4-methoxy group was replaced with a bulkier alkoxy group (compounds **7**–**10**) or a bulkier dimethylamino group (compound **12**), the activity of these analogs decreased dramatically. This may indicate a stringent spatial constraint for this region of the molecule.

It was also observed that the replacement of the 4-methoxy group with electron withdrawing groups (compounds **13** and **14**) or weak electron donating groups (compounds **15**–**17**) all resulted in loss of activity. Introducing a fluoro group at the *ortho* position (compound **18**) increased the cell activity in the p21−/− cells threefold, with **18** having an IC_{50} of 0.68 μM.

Table 1. Inhibition of p21−/− and p21+/+ cell proliferation by thieno[2,3-*d*] pyrimidin-4(1*H*)-one-based analogs



2, 4-21 **22-26**

Compound	*n*	R	IC_{50} p21−/− (μM)[a]	IC_{50} p21+/+ (μM)[a]	Ratio
1	—	—	0.40	12	30
2	2	3,4,5-TriOMe	2.3	>20	>8.7
4	2	3,5-DiOMe	>20	>20	NA
5	2	3,4-DiOMe	>20	>20	NA
6	2	4-OMe	2.4	>20	>8.5
7	2	4-OEt	8.4	>20	>2.4
8	2	4-OPr-i	>20	>20	NA
9	2	4-OBu-n	>20	>20	NA
10	2	4-OPh	>20	>20	NA
11	2	4-SMe	4.1	>20	>4.9
12	2	4-NEt_2	>20	>20	NA
13	2	4-NO_2	>20	>20	NA
14	2	4-CO_2Me	>20	>20	NA
15	2	4-Ph	>20	>20	NA
16	2	4-Me	>20	>20	NA
17	2	4-Cl	>20	>20	NA
18	2	2-F-4-OMe	0.68	13	19
19	3	3,4,5-triOMe	0.75	6.6	8.7
20	1	3,4,5-triOMe	3.5	>20	>5.8
21	1	4-OMe	2.4	20	8.3
22	—	Cl	>20	>20	NA
23	—	OMe	>20	>20	NA
24	—	NMe_2	>20	>20	NA
25	—	NHPh	>20	>20	NA
26	—	2-Thiophene	>20	>20	NA
27	—	—	>20	>20	NA
28	—	—	>20	>20	NA
29	—	—	>20	>20	NA

[a] IC_{50} values reported for both p21−/− and p21+/+ cells represent the means of at least two separate determinations with typical variations of less than 40% between replicate values.

Changing the size of the cycloalkyl ring fused to the thiophene appears to have some effect on the cell activity. The compound with a 7-membered cycloalkyl ring, **19**, showed an improved IC_{50} in p21−/− cells by 3-fold compared to **2**, but the compounds with a 5-membered cycloalkyl ring, **20** and **21**, showed comparable activities to **2**, with IC_{50}s of 3.5 and 2.4 μM in the p21−/− cells, respectively.

Compounds **22**–**26**, which possessed substituents other than carbonyl at C-4 of the pyrimidine ring, were inactive against both the p21−/− and p21+/+ cells. Compounds with the cycloalkyl ring removed, **27**–**29**, were also inactive. This observation suggests that the cycloalkyl ring is a crucial component of the anti-proliferative pharmacophore.

A group of selected compounds, including HTS hit, **2**, were further evaluated against a panel of the human colon tumor cell lines (LoVo, SW620, DLD1, and HT29).[10] Their activities are shown in Table 2. Compounds that were active against p21−/− cells displayed moderate to good potency in the colon cell lines. The

Table 2. Activity in human colon tumor cell lines

Compound	IC_{50} (μM)[a]			
	LoVo	SW620	DLD1	HT29
2	0.14	0.19	0.28	0.24
6	0.15	0.23	0.29	0.33
7	2.6	5.0	4.6	5.0
11	0.43	0.80	0.89	1.0
18	0.46	0.42	0.22	0.42
19	0.17	0.22	0.35	0.45
20	0.13	0.19	0.26	0.30
21	0.15	0.23	0.29	0.33

[a] IC_{50} values reported for the above colon cell lines represent the means of at least two separate determinations with typical variations of less than 40% between replicate values.

HTS hit, **2**, had an IC_{50} range from 0.14 to 0.28 μM across the panel. The analog with a single methoxy group at the 4-position, **6**, showed similar activity with an IC_{50} of 0.16–0.33 μM. Compound **7**, the ethoxy analog of **6**, was much less active than **6**. Changing of the 4-methoxy group to methylthio rendered **11** less active across the panel. Although compounds **18** and **19** were somewhat more active in the p21−/− cells than **2**, they

showed analogous IC_{50}s in the colon cell lines to **2**. Likewise, compounds **20** and **21** showed indistinguishable activities compared to **2** in the colon cell lines.

In summary, we have identified tricyclic thieno[2,3-*d*]pyrimidin-4(1*H*)-one-based analogs as a novel class of anti-proliferative agents by an innovative cell-based screening method. We have described their anti-proliferative activity and the preliminary SAR study. The mechanism of action of these compounds at a molecular level will be discussed in future publications.

Acknowledgments

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13. Cytotoxicity assays. p21+/+ and p21−/− isogenic cells and other carcinoma cell lines (LoVo, SW620, DLD1, and HT2) were plated in 96-well tissue culture plates. The following day, dilutions of compounds were added and cells were cultured for 5 days (isogenic cell lines). Cell survival was determined using sulforhodamine B, a protein binding dye. The concentration of the compound that inhibits cell proliferation by 50% (IC_{50}) was estimated from inhibition curves.