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Parallel synthesis and biological evaluation of 5,6,7,8-tetrahydrobenzothieno[2,3-*d*]pyrimidin-4(3H)-one cytotoxic agents selective for p21-deficient cells

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Abstract—A novel series of inhibitors of cancer cell proliferation, selective against p21 cell cycle checkpoint-disrupted cells vs. cells with intact p21 checkpoint, were identified by high-throughput screening. Optimization of both ends of the lead molecule to improve potency, using parallel synthesis and iterative design, is described. The 2-(1,4-dibenzodioxane)-substituted derivative **14** was identified as a highly selective and potent agent displaying an IC_{50} of 91 nM in the p21-deficient cell line.

Cell cycle checkpoints are signal transduction pathways, which ensure that each step in the cell division cycle, has been successfully completed before the onset of the next phase.[1] Loss of checkpoint control is a hallmark of tumor cells, as it is thought to increase the mutation rate, allowing a more rapid progression of cells to the tumorigenic state.[1,2] However, inactivation of these checkpoints results in aberrant responses to cellular damage. This failure of checkpoint responses in malignant cells can be exploited in cancer drug discovery. Identification of compounds that selectively kill checkpoint-deficient cells can be expected to preferentially target tumor cells, while sparing normal cells.[3,4]

The p53 tumor suppressor gene is a major regulator of the G1/S-phase checkpoint and one of the most commonly mutated genes in human cancer.[5,6] p21, a downstream effector of p53, inhibits the cyclin-dependent kinases and arrests cell cycle progression in response to environmental insults causing DNA damage[7] or microtubule perturbation.[8] Disruption of this checkpoint by deletion of the p21 protein leads to failure of the cell to arrest, leading to endoreduplication, and finally, to apoptosis.[9,10] p21-deficient cells show increased sensitivity, compared to isogenic p21-proficient parental cells, to a variety of clinically used antineoplastic drugs, validating the role of checkpoints in chemosensitivity.

We have prepared p21-deficient cells from human colon carcinoma cell line HCT116 by targeted deletion of the p21 gene.[9] HCT116, the parental cell lines from which the p21−/− cells were derived, is one of the few colon cancer cell lines with an apparently intact p21 checkpoint. We anticipate that a search for compounds that preferentially kill the p21-deficient cells, compared to the p21-proficient cells, will enable us to identify compounds that are not simply toxic, but have selectivity toward cells defective in checkpoint control. Targeting these cells should improve the likelihood of obtaining lead molecules that preferentially inhibit the growth of tumor cells rather than normal cells, a requirement for an anticancer agent. Furthermore, since loss of p21 checkpoint is a hallmark of cancer (primarily through

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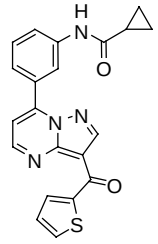
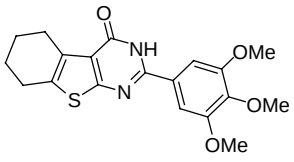
mutations in the upstream activator, p53), these compounds are widely used in treating a variety of them.

High-throughput screening using the p21-knockout isogenic cell screen described above led to the identification of two leads, pyrazolo[1,5,*a*]pyrimidin-7-yl phenyl amide **1**[11] and 2-(3,4,5-trimethoxyphenyl)-tetrahydrobenzothieno[2,3-*d*]pyrimidin-4(3H)-one **2**.[12] Preliminary SAR studies by others in our group[13] indicated that either the 4-methoxy or 3,4,5-trimethoxy substitution pattern on the phenyl ring of **2** was necessary for its activity. Replacement of the methoxy groups with chloro, methyl, or nitro, or deletion or rearrangement of the methoxy groups gave much less active compounds. The exocyclic oxygen was important to activity and conservative modifications to the tetrahydrobenzene ring were tolerated, giving slightly more active compounds. We presumed that a more thorough investigation of both the phenyl group and the tetrahydrobenzene ring SAR could be carried out using a parallel synthesis. Here, we report both the strategies adopted by us and the results of our effort.

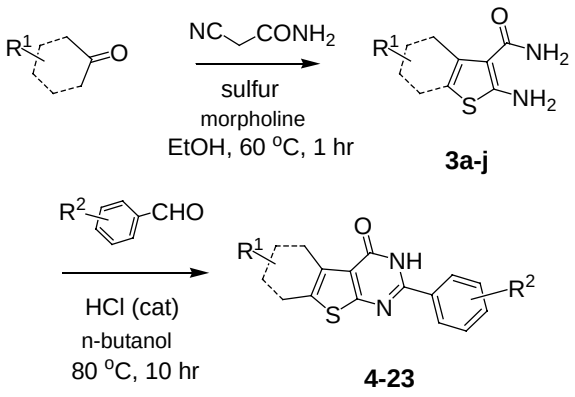


1



2

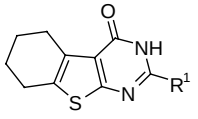
The compounds in this study were all made following the published procedure for the preparation of thienopyrimidinones.[12] 2-Amino-thiophene-3-carboxamides were prepared from cyclic ketones via the Gewald thiophene synthesis[14] and the fused ring thiophene intermediates were, in turn, condensed with aldehydes in parallel to give the desired thienopyrimidinones (Scheme 1). As the capacity of the team to assay new compounds was limited to a certain extent, we rejected the idea of making a full combinatorial library using large sets of ketone and aldehyde building blocks. Instead, we first investigated the effect of variation of the aromatic substituent (right-hand side) on activity, while keeping the left-hand side of the molecule constant.



Scheme 1. General route for the preparation of thienopyrimidinones.

The collection of aldehydes available to us for use in synthesis was so large that a physical property-based design approach to select building blocks from this large set (i.e., limiting the number of building blocks) had to be adopted.[15] First, physical properties [MW, hydrogen bond donor and acceptor counts, flexible bond counts, clogP, CMR, PSA, and MW of substituents in the *ortho*, *meta*, and *para* positions (where applicable)] of the 133 available benzaldehydes and heteroaryl aldehydes were calculated. Second, three principal components (vectors of property space) of this resulting data set were identified via multivariate analysis using SIMPCA-P.[16,17] Two selections of aldehyde building block sets were made using this information: (1) a 'diversity' set of 12 aldehydes was selected by D-optimal selection using the principal components and (2) a 'similarity' set of 13 aldehydes was selected on the basis of closest similarity to 4-methoxybenzaldehyde. Additionally, a third set was selected by hand from a set of commercially available heterocyclic aldehydes. In total, 47 aldehydes were selected. Products of the reaction of 2-amino-4,5,6,7-tetrahydrobenzo[*b*]-thiophene-3-carboxamide (**3a**) with the selected aldehydes were prepared[18] and tested for both activity and selectivity for the p21−/− cells over the p21+/+ cells (Table 1). Alone out of the entire compound set, the new thienopyrimidinone **4**, having the 2-(1,4-dibenzodioxane) substituent, evoked cell death in the p21−/− cell line at a submicromolar dose (IC_{50} p21−/− cells, 0.42 μM, $n = 2$), with excellent selectivity for p21−/− cells over the p21 proficient cells. This discovery represented a substantial improvement over the lead.

Table 1. Inhibition of p21+/+ and p21−/− cell proliferation by 5,6,7,8-tetrahydrobenzothieno[2,3-*d*]pyrimidin-4(3H)-ones[19,21]

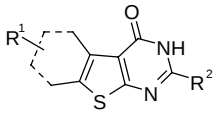


Compound	R^1	p21+/+ IC_{50} (μM)	p21−/− IC_{50} (μM)	Ratio
2	3,4,5-Trimethoxyphenyl	>20	2.8	>9
4	2,3-Dihydro-benzo[1,4]dioxin-6-yl	>20	0.42	>47
5	4-Hydroxy-naphthalen-1-yl	8.4	1.7	5
6	[2,2′]Bithiophenyl-5-yl	>20	9.3	>2
7	2-Bromo-3,4-dimethoxyphenyl	>20	9.3	>2
8	3-Acetyloxyphenyl	>20	12	>2
9	3-Chloro-4-hydroxy-5-methoxyphenyl	>20	12.5	>2
10	4-Isopropenyl-cyclohex-1-enyl	>20	12.8	>2

In the second phase of our optimization of **2**, we prepared a 9×6 combinatorial array comprised of the reaction of nine differently substituted 2-amino thiophene-3 carboxamides **3b**–**j**, prepared from nine commercially available cyclohexanones and piperidin-4-ones, with six aldehydes. Thirty-two out of 54 possible reaction products were successfully prepared and purified in parallel. Result from testing these products in our assay (Table 2) confirmed the desirability of the 2-(2,3-benzo[1,4]dioxinyl)-thienopyrimidin-4-one substituent group and revealed further novel compounds **11**–**23** useful for inducing apoptosis in p21−/− cells in preference to p21+/+ cells. Compound **11** (IC_{50} p21−/− cells, 0.091 μM, $n = 3$) represented a second significant advance in potency over the lead. Furthermore, enrichment of the second library in active compounds ($IC_{50} < 10$ μM), relative to the first library, was pronounced, a testimony to our success at identifying right-hand side substituents in the first iteration that improved activity.

The most potent compounds from the two libraries were selected for further characterization in a panel of human colon carcinoma cell lines (Table 3). Compounds displayed moderate to good potency across the panel. Potency seemed to track with potency of the lethal effect on p21−/− cells. To assess the therapeutic potential of the best compound from this series, **11** was tested in a human LoVo cell xenograft model in nude mice. Disappointingly however, **11** was not active at either 30 or

Table 2. Inhibition of p21+/+ and p21−/− cell proliferation by fused thieno[2,3-*d*]pyrimidin-4-ones[20,21]



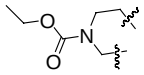
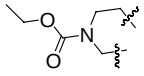
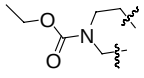
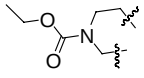
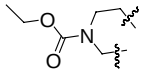
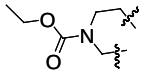
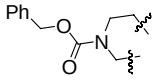
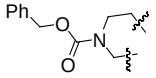
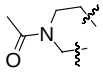
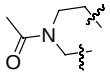
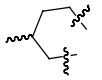
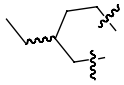
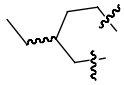
Compound	R^1	R^2	p21+/+ IC_{50} (μM)	p21−/− IC_{50} (μM)	Ratio
11		2,3-Dihydrobenzo[1,4]dioxin-6-yl	3.8	0.091	42
12		2-Benzo[1,3]dioxol-5-yl	6.8	0.68	10
13		4-Methoxyphenyl	>20	1.9	>10
14		4-Isopropenylcyclohex-1-enyl	>20	1.9	>10
15		3,4,5-Trimethoxyphenyl	>20	2	>10
16		4-(Methylthio)phenyl	>20	3.6	>6
17		4-Methoxyphenyl	11	0.8	14
18		3,4,5-Trimethoxyphenyl	>20	8.9	>2
19		2,3-Dihydrobenzo[1,4]dioxin-6-yl	20	1.8	11
20		4-Methoxyphenyl	>20	2.8	>7
21		3,4,5-Trimethoxyphenyl	>20	2.2	>9
22		3,4,5-Trimethoxyphenyl	>20	2.8	>7
23		2,3-Dihydrobenzo[1,4]dioxin-6-yl	>20	5.6	>4

Table 3. Activity comparison in the colon cell line panel

Compound	LoVo IC_{50} (μM)	SW620 IC_{50} (μM)	DLD1 IC_{50} (μM)	HT29 IC_{50} (μM)
2	0.14	0.19	0.29	0.24
4	0.08	0.12	0.15	0.18
11	0.02	0.03	0.04	0.04
12	0.05	0.07	0.09	0.12
13	0.11	0.14	0.14	0.15
15	0.18	0.24	0.29	0.36
17	0.17	0.17	0.18	0.15
19	0.19	0.42	0.41	0.42

Table 4. Binding of thienopyrimidinone cell proliferation inhibitors to tubulin in a fluorescence binding assay[22]

Compound	$K_{d(apparent)}$
11	1.8 μM ± 0.14
12	4.0 μM ± 0.4
16	1.6 μM ± 0.26

50 mg/kg (oral dosing, once-daily). Solubility of **11** was low (4 μg/mL at pH 7.4). The lack of in vivo efficacy may be on account of the low bioavailability caused by poor physical properties. However, a pharmacokinetic study on **11** was not done.

The anti-tumor compound colchicine is known to work by inhibiting microtubule formation. Colchicine is also an inhibitor of p21−/− cell proliferation (IC_{50} p21−/− 0.02 μM) with approximately 10-fold selectivity for p21 deficient cells over p21 proficient cells. We were interested knowing whether or not the thienopyrimidinone series killed p21−/− cells by the same mechanism. Using a fluorescence binding assay,[22] **11** was found to bind saturably to the tubulin dimer with a one-to-one stoichiometry and showed a $K_{d(apparent)}$ of 1.8 μM. However, tubulin binding within the series (Table 4) was essentially invariant, while potency of induction of p21−/− cell apoptosis varied by orders of magnitude, indicating that there is no correlation between tubulin binding and induction of cell death in this series.

In summary, we have successfully used a parallel synthesis to rapidly improve the potency of an initial lead. A physical property-based approach was used to select the building blocks for synthesis. Although the preferred 2-(1,4-dibenzodioxane) substituent was selected on account of its similarity to the reference, 4-methoxybenzaldehyde, it was not clear from this study if a selection based on diversity or one based on similarity to a reference point was preferred. Probably, a combination approach, as practiced here, is the best. This effort has demonstrated the value of a parallel synthesis strategy for the optimization of leads which led to the identification of several analogs with submicromolar activity in a panel of colon cell lines.

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16. (a) Eriksson, L.; Johanasson, E.; Kettaneh-Wold, N.; Wold, S. *Introduction to Multi- and Megavariate Data Analysis Using Projection Methods (PCA % PLS)*; Umetrics AB: Umea, Sweden, 1999; (b) Calculations performed using software product SIMPCA-P (Umetrics AB, Umea, Sweden).
17. The most significant property contributors to the principal components were as follows: [t]1, correlation to *c*log*P* and reciprocal correlation to #HBD, #HBA, PSA, and $MW_{ortho\ substituent}$; [t]2, correlation to MW; [t]3, correlation to $MW_{para\ substituent}$ and reciprocal correlation to $MW_{meta\ substituent}$.
18. 2-Amino-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxamide (**3a**) (29 mg, 0.15 mmol) was suspended in 1 ml *n*-butanol. 1,4-Benzodioxane-6-carboxaldehyde (24.6 mg, 1 equiv) and 10 μL conc. HCl were added and the reaction mixture was heated to 80 °C for 10 h. The reaction product was collected by filtration, washed with water, and dried to give 30 mg **4** [LC/MS (Hewlett Packard 1100 MSD with ChemStation Software, Keystone Aquasil C_{18} 50 mm × 2 mm column, 5 μm particle size, at 40 °C, 10 mM NH_4OAc–acetonitrile solvent system at 0.8 mL/min flow rate, 254 nm DAD detection, API-ES scanning mode, fragmentor 70 mV): *m/z* 341 (M+H); retention time 2.59 min]. Compounds that were not judged to be sufficiently pure (>90%) by LC were purified by RP-HPLC with automatic fraction collection (Gilson Semi-Preparative HPLC system with Unipoint Software v. 1.71, Phenomenex C_{18} Luna column, 21.2 mm × 100 mm, 5 μm particle size, water–acetonitrile solvent system with added 0.05% NH_4OH buffer, at 22.5 mL/min).
19. Compounds not shown in the table had IC_{50} > 20 μM on 80S14, as well as HCT116 cell lines.
20. Compounds not shown on the table had IC_{50} > 10 μM on 80S14 as well as HCT116 cell lines.

21. p21+/+ and p21−/− isogenic cells and other colon carcinoma cell lines (LoVo, SW620, DLD1, and HT29) were plated in 96-well tissue culture plates. The following day, various dilutions of compounds were added and cells were cultured for an additional 4 days (colon cell panel) or 5 days (isogenic cell lines). Cell survival was determined using sulforhodamine B, a protein binding dye. The concentration of compound that inhibits cell growth by 50% (IC_{50}) was estimated from cytotoxicity curves. When multiple independent experiments were performed, IC_{50}s shown represent the mean value.
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