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Discovery of novel thieno[2,3-*d*]pyrimidin-4-yl hydrazone-based inhibitors of Cyclin D1-CDK4: Synthesis, biological evaluation, and structure–activity relationships

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ABSTRACT

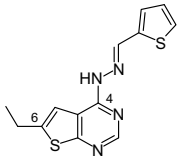
The synthesis and evaluation of new analogues of thieno[2,3-*d*]pyrimidin-4-yl hydrazones are described. 2-Pyrdinecarboxaldehyde [6-(*tert*-butyl)thieno[2,3-*d*]pyrimidine-4-yl]hydrazone derivatives have been identified as cyclin-dependent kinase 4 (CDK4) inhibitors. The potency, selectivity profile, and structure–activity relationship of this series of compounds are discussed.

The cell cycle of eukaryotic cells is regulated by cyclin-dependent kinases (CDKs). CDKs are a family of serine/threonine kinases which play a key role in the growth, development, proliferation, and death of eukaryotic cells.[1–3] CDKs, along with their regulatory subunit, the cyclins, are responsible for coordinating the events by which cells progress through the cell cycle and they become active at specific phases: G1, S, G2 and M.[4–6] In the G1 phase, as cells respond to the presence of mitogens, the cyclin D1 increases to trigger the activation of CDK4/6 in early G1 and then cyclin E/CDK2 activates.[7] These kinases phosphorylate the retinoblastoma tumor suppressor protein (Rb). The hyperphosphorylated Rb causes the release of members of the transcription factors, E2F proteins. E2F leads to transcriptional activation of gene expression that results in entry into the S phase of the cell cycle. It is noteworthy that CDK4 restricts the passage only through G1 phase, whereas CDK2 controls the passage through not only G1 but also S phase with cyclin A.[7–9]

In normal cells, CDK4/6 activities are negatively regulated by the tumor suppressor p16, a cyclin-dependent kinase inhibitor of the INK4 family, and the activity of CDK2 is negatively regulated by CKIs of the Cip/Kip family.[10] But many tumors have been reported to contain mutations, deletions or silencing of the p16 or the Rb gene.[11,12] Moreover, mutations in CDKs and abnormal expressions of their regulators have been found in a large percentage of melanoma patients.[13] From these findings, the deregulation of the Rb pathway, and CDK4, is important in cancer progression. Recently, Wyeth Research group[14] demonstrated that inhibition of endogenous cyclin D1 or CDK4 expression results in hypophosphorylation of Rb and accumulation of cells in the G1 phase. In addition, Malumbres[15] has reported that knockdown of CDK4 in mammary tumor cells prevents tumor formation. These results suggest that selective inhibition of CDK4 may restore normal cell activity and could be a more valuable approach to cancer therapy than that of CDK2, especially for those who have lost the INK4 family, such as p16.

A number of groups have identified CDK inhibitors.[16–18] Flavopiridol, seliciclib and several small-molecule CDK inhibitors have been developed and advanced to clinical trial. But it is still the case that more CDK2 inhibitors have been reported than CDK4 inhibitors and PD0332991[19] is known to be a selective CDK4 inhibitor under active development.

We report herein the synthesis of novel analogues of thieno[2,3-*d*]pyrimidin-4-yl hydrazones and their inhibitory activities for CDK4 with selectivity against CDK2. We also describe their cytotoxic potential against human cancer cell lines to verify our hypothesis that selective CDK4 inhibitor will provide an effective treatment for the inhibition of tumor growth.



1a

Figure 1. A compound identified by in-house high-throughput screening.

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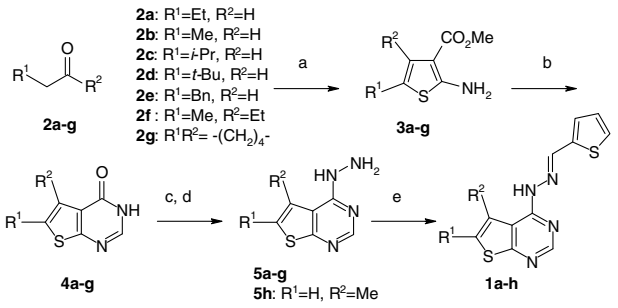
High-throughput screening efforts with the Daiichi Sankyo compound collection revealed that thieno[2,3-*d*] pyrimidin-4-yl hydrazone **1a** (Fig. 1) was CDK4 (IC_{50} = 0.75 μg/mL) inhibitor possessing modest selectivity against CDK2 (IC_{50} = 1.10 μg/mL) activity (Table 1). This finding prompted us to initiate our study to explore the activity of this class of compounds.

Our goal in this study was to improve the potency and clarify the SAR of HTS hit compounds. To get a starting exploration of structural requirements, substituted analogues of thieno[2,3-*d*]pyrimidin-4-yl hydrazones were prepared and tested in terms of CDK4 and CDK2 activity.

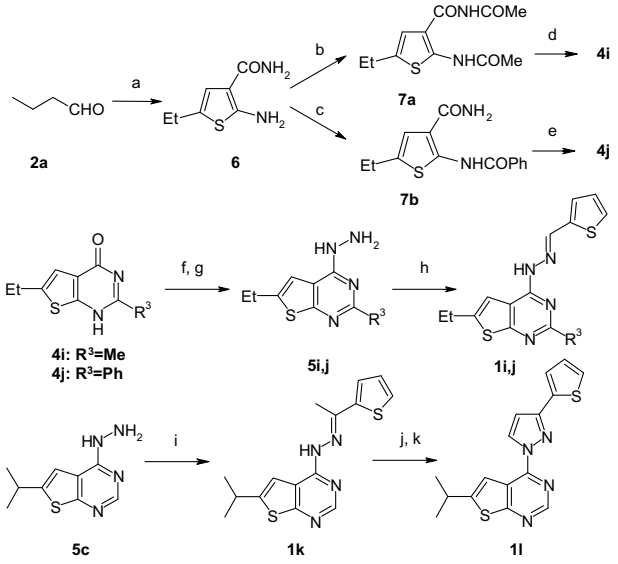
Preparation of thieno[2,3-*d*]pyrimidin-4-yl hydrazones **1a–h** was accomplished using a general synthetic route, as shown in Scheme 1. Thiophene intermediates **3a–g** were synthesized from commercially available aldehydes **2a–e** or ketones **2f,g** with methyl cyanoacetate in the presence of sulfur using the general method of Tinney et al.[20] Cyclization of **3a–g** with formamide obtained thieno[2,3-*d*]pyrimidin-4-ones **4a–g**. Hydrazines **5a–g** were prepared from chlorination of the carbonyl group at the C-4 position of **4a–g** with phosphorus oxychloride, followed by treatment with hydrazine monohydrate in ethanol under reflux.[21] Hydrazine **5h** is commercially available. Finally, hydrazones **1a–h** were produced by a classical condensation reaction with **5a–h** and 2-thiophenecarboxaldehyde in benzene.

The C-2 substituent compounds **1i,j** were prepared as shown in Scheme 2. Following the procedure reported by Gewald et al.,[22] intermediate **6** was prepared from *n*-butyraldehyde, 2-cyanoacetamide and sulfur in DMF. The resulting thiophene **6** was treated with acetic anhydride to obtain **7a**. Cyclization of **7a** with sodium ethoxide afforded thieno[2,3-*d*]pyrimidin-4-one **4i**. On the other hand, **6** was treated with benzoyl chloride to obtain **7b**, and **4j** was prepared by cyclization of **7b** with aqueous sodium hydroxide under reflux. The C-4 substituent compounds, ethylidene hydrazone derivative **1k** and ring-closed compound **1l**, were also synthesized from **5c**. Hydrazine **5c** was treated with 2-acetylthiophene to give **1k**. Then, following a procedure found in the literature,[23] **1k** was lithiated with *n*-butyl lithium, acylated and cyclized with ethyl formate, and finally treated with aqueous HCl to afford pyrazole **1l**.

Table 1 summarizes the CDK4/CDK2 inhibitory activity of thieno[2,3-*d*]pyrimidin-4-yl hydrazones containing HTS hit compound **1a**. Compounds **1c**,**d** with isopropyl or *tert*-butyl group at the C-6 (R^1) position had more potent CDK4 inhibitory activities

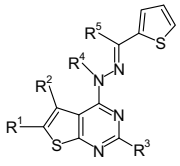


Scheme 1. Reagents and conditions: (a) methyl cyanoacetate, S_8, Et_3N, DMF; (b) $HCONH_2$, 210 °C, 7–84% from **2a–g**; (c) $POCl_3$, 110 °C; (d) $NH_2NH_2{\cdot}1H_2O$, EtOH, reflux, 33–57% from **4a–g**; (e) 2-thiophene-carboxaldehyde, benzene, reflux, 9–92%.



Scheme 2. Reagents and conditions: (a) 2-cyanoacetamide, S_8, Et_3N, DMF, 62%; (b) Ac_2O, reflux, 24%; (c) BzCl, Et_3N, benzene, reflux, quant.; (d) NaOEt, EtOH, reflux, 57%; (e) NaOH aq., reflux, 25%; (f) $POCl_3$, 110 °C; (g) $NH_2NH_2{\cdot}1H_2O$, EtOH, reflux, 2 steps 97% (**5i**), 60% (**5j**); (h) 2-thiophene-carboxaldehyde, benzene, reflux, 65% (**1i**), 56% (**1j**); (i) 2-acetylthiophene, benzene, reflux, 34%; (j) *n*-BuLi, HCO_2Et; (k) HCl aq., reflux, 80% from **1k**.

Table 1
Enzyme inhibition of substituted thieno[2,3-*d*]pyrimidines (IC_{50} μg/mL) **1a–l** against CDK4 and CDK2



Compound	R^1	R^2	R^3	R^4	R^5	CDK4 IC_{50}[a] (μg/mL)	CDK2 IC_{50}[a] (μg/mL)
1a	Et	H	H	H	H	0.75	1.10
1b	Me	H	H	H	H	1.90	1.70
1c	*i*-Pr	H	H	H	H	0.05	0.35
1d	*t*-Bu	H	H	H	H	0.12	0.56
1e	Bn	H	H	H	H	>20.0	6.50
1f	Me	Et	H	H	H	16.00	12.00
1g	$-(CH_2)_4-$		H	H	H	>20.0	>20.0
1h	H	Me	H	H	H	>20.0	>20.0
1i	Et	H	Me	H	H	>20.0	>20.0
1j	Et	H	Ph	H	H	>20.0	>20.0
1k	*i*-Pr	H	H	H	Me	2.80	8.90
1l	*i*-Pr	H	H	–CH=CH–		>20.0	>20.0

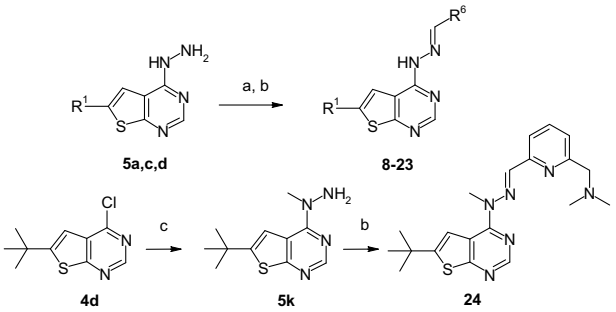
[a] Concentration (μg/mL) needed to inhibit Rb phosphorylation by 50%, as determined from the dose-response curve. Values are the means of at least two determinations.

(IC_{50} = 0.05 and 0.12 μg/mL, respectively) than **1a** with selectivity against CDK2 (5- to 7-fold). However, the benzyl compound **1e** showed no inhibitory activity up to 20 μg/mL. Compounds that alkyl groups were introduced to the C-5 (R^2) position, as exemplified in **1f** and **1h**, or fused to cyclohexane ring as in **1g**, showed only weak or no inhibitory activity. Substitution at the C-2 (R^3) position was found to be inactivated to CDK4 activity, as in **1i**,**j**. Introduction of methyl group into the R^5 position as in **1k** lead to significant decrease in potency. To elucidate the effect of the hydrazone part, pyrazole **1l** was synthesized as a fused ring compound. But no inhibitory activity up to 20 μg/mL was observed. In addition, compounds **1a–l** have extremely poor aqueous solubility, which is attributed to their highly lipophilic profiles. For example, the solubility of **1c** in water was below 0.1 μg/mL.

To further improve the CDK4 inhibitory activity and physical profile, our effort was focused on determining the effect of the thiophene ring at the C-4 (R^6) position. Alkyl or aryl groups were introduced instead of thiophene ring in combination with ethyl, isopropyl or *tert*-butyl group at the C-6 (R^1) position, as shown in Table 2.

As shown in Scheme 3, C-4 substituent analogues **8–23**, focused on the aryl moiety at the hydrazone, were synthesized using selected hydrazines and appropriate aldehydes instead of 2-thiophenecarboxaldehyde. In addition, methylhydrazone **24** was prepared, from the intermediate chloride **4d** and methyl hydrazine.

Alkyl compounds **8** and **9** were less active than the parent compound **1a**. Heteroaryl compounds **10–16** had moderate CDK4 inhibitory activity, but most of them lost their selectivity against CDK2 and only 2-pyridinyl derivatives **10** and **15** showed some selectivity (3.5- and 3.1-fold, respectively). When substituent groups were introduced on the pyridine ring, better selectivity was observed with 5- and 6-methyl-2-pyridine derivatives **17** and **18** (13.6- and 13.3-fold, respectively). Our interest was focused on introducing substituents on the pyridine ring to increase the aqueous solubility. As a result, we found that the series of 6-aminomethyl derivatives **19–22** relatively maintained their inhibitory activity, and **20–22** had good selectivity (28.4-, 25.0-, and 32.4-fold, respectively). In particular, the 6-[(dimethylamino)methyl]pyridine compound **21** was significantly improved in its CDK4 inhibitory activity (IC_{50} = 0.056 μg/mL) and also improved in its solubility in water to 44 μg/mL. In contrast, the 6-hydroxymethyl compound **23** was less potent than **21**. Moreover, when the NH proton of the hydrazones was converted to a methyl group as compound **24** (Scheme 3), the resulting analogue did not show any inhibitory activity. This result suggests that the NH proton of the hydrazone part is essential for CDK4 inhibitory activity.

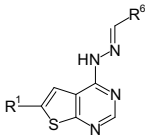


Scheme 3. Reagents and conditions: (a) aldehyde, benzene or toluene, reflux; (b) deprotection if necessary, 41–95% from **5a,c,d**; (c) $MeNHNH_2$, EtOH, reflux, 79%; (d) 6-[(dimethylamino)methyl]-pyridine-2-carbaldehyde, EtOH, reflux, 73%.

The derivatives were also tested for their antiproliferative activities in tumor cell lines. Compounds **10**, **15**, **17**, **19** and **23** had potent antiproliferative activities in human colon carcinoma (HCT116) and human lung carcinoma (PC6) cell lines with IC_{50}s ranging from 0.003 to 0.059 μg/mL, whereas compound **21** with the strongest CDK4 inhibitory activity showed less potent antiproliferative activity.

Further investigations of the antiproliferative effects were carried with **1a**, **15**, **20** and **21** against various tumor cell lines. Compound **15** had very potent antiproliferative activity in all the cell lines, as shown in Table 3.

Table 2
Enzymatic and cellular activity for substituted thieno[2,3-*d*]pyrimidines



Compound	R^1	R^6	CDK4 IC_{50}[a] (μg/mL)	CDK2 IC_{50}[a] (μg/mL)	HCT116 IC_{50}[b] (μg/mL)	PC6 IC_{50}[b] (μg/mL)
1a	Et	Thiophenyl	0.75	1.10	1.610	0.566
8	Et	*t*-Bu	4.40	3.80	1.660	1.550
9	Et	Cyclopropyl	3.20	4.10	NT[c]	NT[c]
10	*i*-Pr	2-Pyridinyl	0.88	3.10	0.010	0.003
11	*i*-Pr	3-Pyridinyl	0.55	0.70	NT[c]	0.889
12	*i*-Pr	4-Pyridinyl	0.24	0.33	NT[c]	>10.0
13	*i*-Pr	Phenyl	0.47	0.88	NT[c]	0.651
14	*i*-Pr	2-Franyl	0.39	0.55	NT[c]	3.780
15	*t*-Bu	2-Pyridinyl	0.90	2.80	0.014	0.009
16	*t*-Bu	3-Pyridinyl	0.50	0.56	NT[c]	1.450
17	*t*-Bu	5-Methyl-2-pyridinyl	0.88	12.00	0.020	0.009
18	*t*-Bu	6-Methyl-2-pyridinyl	0.52	6.90	0.268	0.258
19	*t*-Bu	6-Aminomethyl-2-pyridinyl	0.59	3.60	0.059	0.036
20	*t*-Bu	6-[(Methylamino)methyl]-2-pyridinyl	0.25	7.10	0.185	0.189
21	*t*-Bu	6-[(Dimethylamino)methyl]-2-pyridinyl	0.056	1.40	0.429	0.381
22	*t*-Bu	6-(Morpholin-4-ylmethyl)-2-pyridinyl	0.17	5.50	0.363	0.261
23	*t*-Bu	6-Hydroxymethyl-2-pyridinyl	0.35	2.10	0.039	0.034

[a] Concentration (μg/mL) needed to inhibit Rb phosphorylation by 50%, as determined from the dose–response curve. Values are the means of at least two determinations.
[b] Dose–response curves were determined at ten concentrations. The IC_{50} values are the concentrations needed to inhibit cell growth by 50%, as determined from these curves.
[c] NT = not tested.

Table 3
Antiproliferative effects of **1a**, **15**, **20** and **21** (IC_{50}: ng/mL)[a] against various tumor cell lines

Compound	WiDr[b]	DLD-1[b]	HCT116[b]/ Tere-1[c]	HCT116[b]/ SN2-3[d]	MKN28[e]	MDA-MB-231[f]	MDA-MB-468[f]	BL-6[g]	P388[h]
1a	1360	1120	1210	1340	2860	1870	48	2440	1010
15	19	37	7	5	49	19	8	45	6
20	263	221	290	269	686	279	159	379	131
21	889	506	973	755	1390	919	315	1090	370

[a] The IC_{50} values are the concentrations needed to inhibit cell growth by 50% by MTT assay.
[b] Human colon cancer.
[c] Docetaxal-resistant cell established in-house.
[d] SN-38-resistant cell established in-house.
[e] Human gastric cancer.
[f] Human breast cancer.
[g] Murine melanoma.
[h] Murine leukemia.

Table 4
Antitumor effects of **15** against HCT116 solid tumors

Route	dose (mg/kg)	Administration schedule	IRTVmax[a] (%)	BWLmax[b] (%)	D/N[c]
i.v.	200.0	qdx4	67.9	22.6	0/5
p.o.	200.0	qdx4	54.1	4.6	0/5

[a] Maximum Inhibition Rate of Tumor volume.
[b] Maximum Body Weight Loss.
[c] Number of mice dead from toxicity/Number of mice used.

In the G1-S transition of the cell cycle, inhibition of cellular CDK4 activity will result in cell cycle arrest at the G1 phase. To investigate whether these compounds will induce a G1 cell cycle arrest, we evaluated the effects of selected compounds **15** and **21** by cell cycle analysis in tumor cells. A cell cycle distribution study was performed by treating HCT116 cells with various concentrations of compound **15**. Cells were collected after 16 hr treatment of the compounds and the DNA content of the cells was assessed by flow cytometry analysis.[24] After treating the tumor cell with **15**, a significant accumulation of the G1 population (64%) was observed and there were decreases in the S and G2/M populations at a concentration of 500 ng/mL. Compound **21** caused an increase in the G1 population in a dose-dependent manner, too. This observation of G1 cell cycle arrest is consistent with its cyclin D1/CDK4 inhibitory activity.

Finally, compound **15** caused tumor growth delay in the xenograft model of human colon carcinoma HCT116 cell. Taking into account the poor solubility of **15**, hydrochloride salt dissolved with 40% Captisol[25] was used. A reduction in colon tumor growth of 67.9% was observed using i.v. administration of **15** when given at a dose of 200 mg/kg once a day for 4 days continuously. The antitumor effect was also found with a 54.1% reduction after oral administration at a similar dose without any serious toxicity, as shown in Table 4.

In conclusion, a series of thieno[2,3-*d*]pyrimidin-4-yl hydrazones was synthesized and evaluated, and several 2-pyrdinecarboxaldehyde [6-(*tert*-butyl)thieno [2,3-*d*]pyrimidine-4-yl]-hydrazones were shown to be potent, selective inhibitors of CDK4 with improved physical profiles. In addition, these compounds have antiproliferative activities and act as cytotoxic agents with the ability to prevent cell progression. Moreover, compound **15** is efficacious in xenograft models of human colon carcinoma. These results provide valuable information for the design of CDK4 inhibitor. Further work will be reported on the improvement of CDK4 selectivity and the physical profile in the near future.

Acknowledgments

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