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Synthesis and biological evaluation of 1-(2,4,5-trisubstituted phenyl)-3-(5-cyanopyrazin-2-yl)ureas as potent Chk1 kinase inhibitors

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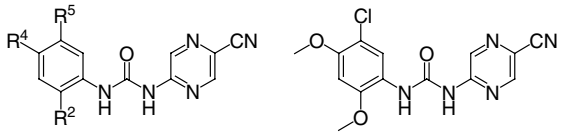
Abstract—Based on the X-ray crystallography of our lead compound 1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-cyanopyrazin-2-yl) urea in the checkpoint kinase 1 (Chk1) enzyme, we modified R^4, and to a lesser extent, R^2, and R^5 of the phenyl ring, and made a variety of *N*-aryl-*N*′-pyrazinylurea Chk1 inhibitors. Enzymatic activity less than 20 nM was observed in 15 of 41 compounds. Compound **8i** provided the best overall results in the cellular assays as it abrogated doxorubicin-induced cell cycle arrest (IC_{50} = 1.7 μM) and enhanced doxorubicin cytotoxicity (IC_{50} = 0.44 μM) while displaying no single agent activity.

When DNA is damaged by radiation or chemical reagents, normal cells arrest in the G1 phase, via the tumor suppressor protein *p*53, and attempt repair.[1,2] Tumor cells, however, often have mutated *p*53 and thus, must rely on the S and G2 checkpoints to repair their DNA.[1–3] Checkpoint kinase 1 (Chk1) is a human nuclear serine/threonine protein kinase. Upon DNA damage, Chk1 is activated and can phosphorylate and destabilize Cdc25A. This phosphorylation is necessary for S and G2 arrest.[4,5] Inhibition of Chk1 results in abrogation of arrest in the S and G2 phases, thereby allowing the DNA-damaged cells to progress prematurely into mitosis resulting in mitotic catastrophe or apoptosis.[6–8] Therefore, abrogation of the S and G2 checkpoints should lead to an increased and selective sensitivity of cancer cells to DNA damaging reagents in *p*53-deficient cells.[1–3,9] Thus, selective inhibitors of Chk1 may be of great therapeutic value in cancer treatment.

Several compounds such as UCN-01 have been reported as Chk1 inhibitors.[10,11] We[12,13] and others[14,15] recently discovered *N*-aryl-*N*′-pyrazinylureas to be a new class of Chk1 inhibitors. Our previous work focused on the modification of R^2 of the phenyl ring[12,13] (**1** wherein R^4 = H, Fig. 1). We also synthesized 1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-cyanopyrazin-2-yl)urea (**2**, Fig. 1) as a potent Chk1 kinase inhibitor (Chk1 $IC_{50} \sim 7$ nM).[12] Tolerance of the methoxy substituent at R^4 of the phenyl ring indicates that some amount of space exists in this region of the Chk1 protein and that other groups may be tolerated, as well. X-ray crystallography of compound **2** in the Chk1 enzyme[16] (Fig. 2) showed that R^2 points toward the ribose pocket of the Chk1 enzyme, while R^4 points toward the solvent front. Based on this, we decided to modify R^4 for possible improvement of physical properties such as polarity and solubility while keeping similar potency (**1**, Fig. 1). Initially, R^4 was altered, while R^2 and R^5 were held constant as the methoxy and chloro moieties,



1 **2**

Figure 1. Structure of compounds **1** and **2**.

Keywords: Urea; Chk1 inhibitor.
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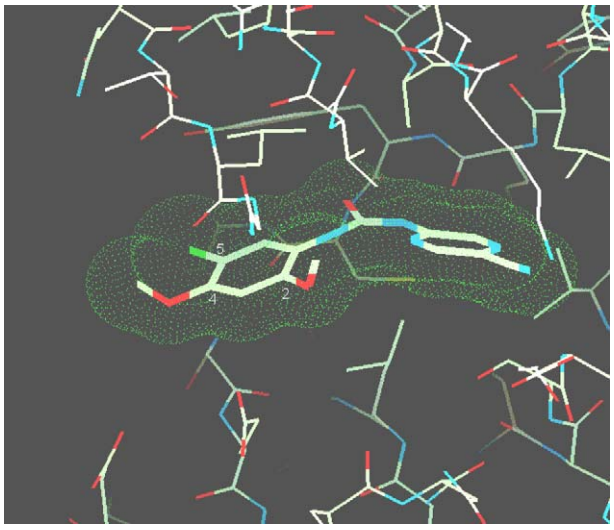


Figure 2. X-ray crystal structure of compound **2** in the Chk1 enzyme.

respectively (Table 1). Later, R^2 and R^4 were concomitantly altered, while R^5 was held constant as the chloro moiety or was removed completely (Table 2). The synthesis and biological results are presented.

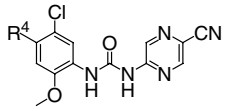
The synthesis of the urea compounds with various amide substituents at R^4 of the phenyl ring is shown in Scheme 1. Methylation of compound **4** provided compound **5**, which was refluxed with one equivalent of compound **3** in toluene[12,13] to give urea compound **6**. Reduction of the nitro group gave intermediate **7**, which was subsequently reacted with a variety of acid chlorides to provide compounds **8a–v**.

The preparation of compound **15** is shown in Scheme 2. Methylation of compound **9** was followed by nitro group reduction and protection of the resulting amino group with TFAA. The methyl group was then transformed through bromination to benzyl bromide **11**, subsequent hydrolysis and oxidation to the corresponding aldehyde **12**. Following removal of the trifluoroacetyl-protecting group, urea compound **14** was synthesized. Finally, reductive amination achieved compound **15**.

The preparation of compounds of structure **19** in Scheme 3 began with replacement of the 2-chloro substituent of 2,4,5-trichloronitrobenzene with one equivalent of pyrrolidine.[17] Diversity was introduced into the molecule in the next step by replacement of the 4-chloro moiety with a selection of alkyl alcohols. Subsequent reduction followed by urea formation provided compounds **19a–e**.

Scheme 4 illustrates the synthesis of compounds of structure **22.** Compound **20** was obtained via reflux of compound **16** and 10 equiv of NaOH in water for 3 days.[18] Aromatic nucleophilic substitution with 2° amines was followed by Mitsunobu reaction with a variety of alcohols and intermediate **21**. Nitro group reduction and urea formation completed the synthesis of compounds **22a–d**.

Table 1. IC_{50} of compounds **6**, **7**, **8a–v**, **14**, and **15**



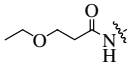
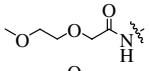
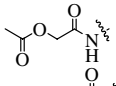
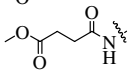
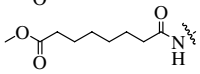
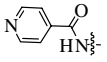
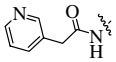
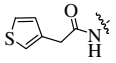
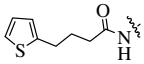
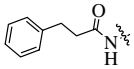
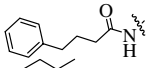
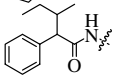
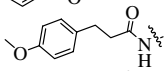
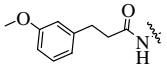
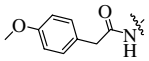
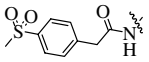
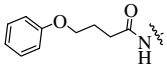
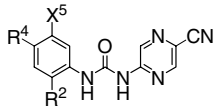
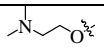
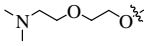
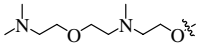
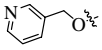
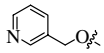
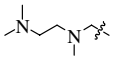
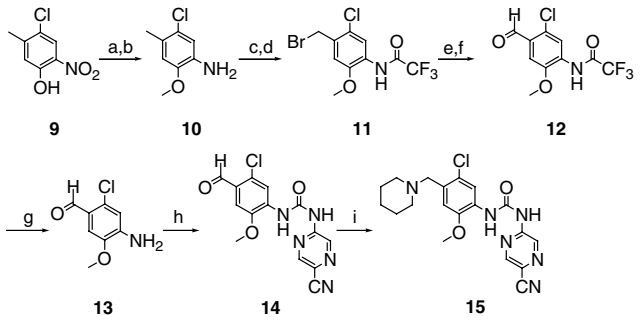
Compound	R^4–	Chk1 IC_{50} (nM)
6	$-NO_2$	42
7	$-NH_2$	5
8a		3
8b		22
8c		18
8d		9
8e		15
8f		23
8g		>10,000
8h		11
8i		8
8j		9
8k		111
8l		42
8m		58
8n		91
8o		68
8p		243
8q		328
8r		72
8s		51
8t		1365
8u		7
8v		228
14	–CHO	14
15		12

Table 2. IC_{50} of compounds **19a–e**, **22a–c**, **25**, **28**, and **29a–e**

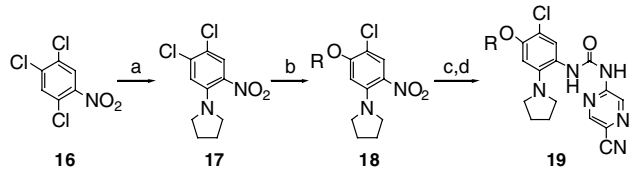


Compound	R^2–	R^4–	X^5–	Chk1 IC_{50} (nM)
19a			–Cl	10
19b			–Cl	25
19c			–Cl	64
19d			–Cl	38
19e			–Cl	15
22a			–Cl	66
22b			–Cl	38
22c			–Cl	43
25			–Cl	32
28	$-OCH_3$	–CHO	–H	45
29a	$-OCH_3$		–H	10
29b	$-OCH_3$		–H	96
29c	$-OCH_3$		–H	73
29d	$-OCH_3$		–H	13
29e	$-OCH_3$		–H	112

The synthesis of compound **25** is shown in Scheme 5. Replacement of the 2-chloro moiety was effected by reacting 2,4,5-trichloronitrobenzene with excess (3-methyl-oxetan-3-yl)-methanol and one equivalent of NaOH.[18] The 4-chloro moiety was then replaced using



Scheme 2. Reagents and conditions: (a) MeI, K_2CO_3, DMF, rt, 72 h; (b) $SnCl_2$, MeOH, 50 °C, 24 h; (c) TFAA, pyridine, CH_2Cl_2, 0 °C, 3 h; (d) NBS, AIBN, CCl_4, reflux, 5 h; (e) Celite®, dioxane, H_2O, reflux, 4 h; (f) Dess–Martin periodinane, CH_2Cl_2, rt, 5 min; (g) K_2CO_3, MeOH, H_2O, rt, 2 h; (h) **3**, toluene, 100 °C, 12 h; (i) piperidine, $NaBH_3CN$, CH_2Cl_2, MeOH, AcOH, 50 °C, 24 h.

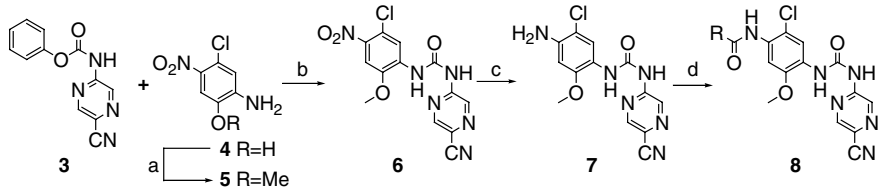


Scheme 3. Reagents and conditions: (a) pyrrolidine, CH_3CN, reflux, 3 hrs; (b) alcohols, NaOH, 70 °C, 12 h; (c) Raney Ni, H_2NNH_2, EtOH, rt, 8 h; (d) **3**, toluene, reflux, 3 h.

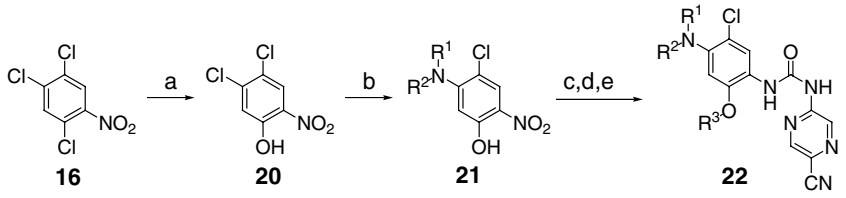
2-dimethylaminoethanol. Subsequent nitro reduction and urea formation provided compound **25**.

Preparation of compound **29**, as shown in Scheme 6, began by amidation of compound **26** with MeNHOMe. $LiAlH_4$ reduction resulted in intermediate **27**. Urea formation provided core compound **28**. Reductive amination using a variety of secondary amines afforded compounds **29a–e**.

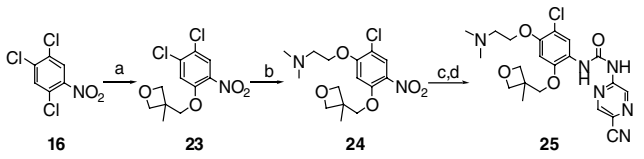
Results of the enzymatic assay[12,13] of compounds **8a–v** are listed in Table 1. Compounds **8a–f**, which contain flexible chains, provided excellent enzymatic activity ranging from 3 to 23 nM. Compound **8a** carries a tertiary aliphatic amine, which is very hydrophilic, and showed the best enzymatic activity (IC_{50} = 3 nM). Compounds **8b**, **8c**, and **8f** vary in chain length from 5 to 9 atoms, yet they showed little variance in enzymatic activity with IC_{50}'s of 22, 18, and 23 nM, respectively. Another subset of compounds we looked at were pyridine-containing compounds **8g–j**. Compounds **8h–j** showed excellent enzymatic activity



Scheme 1. Reagents and conditions: (a) MeI, K_2CO_3, DMF, rt, 72 h; (b) **3**, toluene, reflux, 2 h; (c) $SnCl_2$, MeOH, 50 °C, 12 h; (d) acid chlorides, pyridine, CH_2Cl_2, rt, 2 h.



Scheme 4. Reagents and conditions: (a) NaOH, H_2O, reflux, 72 h; (b) 2° amines, CH_3CN, reflux, 3 h; (c) alcohols, PPh_3-polymer supported, di-*tert*-butyl azodicarboxylate, THF, rt, 12 h; (d) Raney Ni, H_2NNH_2, EtOH, rt, 8 h; (e) **3**, toluene, reflux, 2 h.



Scheme 5. Reagents and conditions: (a) (3-methyl-oxetan-3-yl)-methanol, NaOH, 70 °C, 12 h; (b) 2-dimethylamino-ethanol, NaOH, 70 °C, 12 h; (c) Raney Ni, H_2NNH_2, EtOH, 0 °C, 0.5 h; (d) **3**, toluene, reflux, 2 h.

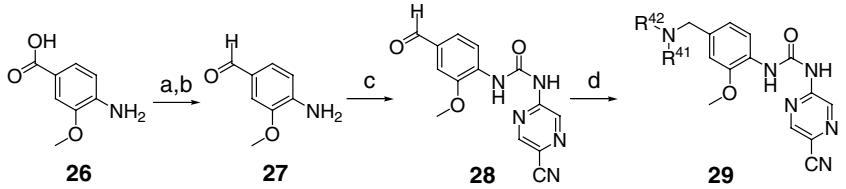
(IC_{50}'s = 8–11 nM). However, compound **8g** is inactive (IC_{50} > 10 μM). Compound **8g** differs from compounds **8h** and **8i** only by placement of the pyridine nitrogen atom. It may be, in this case, that the nitrogen atom of the pyridine ring is unable to make the critical interaction needed to impart activity. In compounds **8k–v,** a variety of furan, thiophene, substituted, and unsubstituted phenyl rings were introduced into the molecule. In general, the activities of these compounds are worse than those seen for the compounds that contain heteroaliphatic chains (**8a–f**) or pyridine rings (**8h–j**, **8g** is an exception). However, compound **8u**, which possesses the highly polar methylsulfonyl group, is an exception with an IC_{50} of 7 nM. Compound **8j** is 6.4-fold more active than compound **8m**. This indicates pyridine rings may provide better interaction with the enzyme than thiophene rings. Compounds **8o**, **8r**, and **8s**, all of which have a phenethyl group, showed similar potencies despite addition or placement of a methoxy substituent with IC_{50}'s of 68, 72, and 51 nM, respectively. Compounds **8t** and **8u** form an interesting comparison. Compound **8u** is 195-fold more active than compound **8t**. This difference in activity stems from a single structural change, compound **8u** has a methylsulfonyl group at the para position of the benzyl ring, while compound **8t** has a methoxy group.

The enzymatic activity of compounds **6**, **7**, **14**, and **15** is also shown in Table 1. Reduction of the nitro moiety of compound **6** to the amino group of compound **7** imparted an 8-fold increase in activity. Alternatively, the increased bulk of compound **15** from the reductive amination of compound **14** does not impart a significant change in activity. Compounds **15** and **14** showed very similar potency, 12 and 14 nM, respectively.

The SAR of compounds **19a–e**, **22a–c**, and **25** are listed in Table 2. Overall, these compounds showed very good to excellent enzymatic activity with IC_{50}'s ranging from 10 to 66 nM. The heteroaliphatic-substituted compounds **19a–c** showed decreased activity with increased chain length. Their IC_{50}'s are 10, 25, and 64 nM, respectively. In compounds **22a–c,** R^4 was held constant as the methylpiperidinyl moiety, while R^2 was modified. Although the R^2 modifications ranged in size, polarity, and flexibility, compounds **22a–c** do not differ significantly in activity (IC_{50} = 38–66 nM).

The SAR of compounds **28** and **29a–e** are also shown in Table 2. Removal of the chloro group from R^5 was not detrimental to the ability of these compounds to inhibit Chk1. Direct comparison of compounds **28** and **29a** with their chloro-containing counterparts, compounds **14** and **15**, respectively, showed a 3-fold decrease for one compound and a slight increase in activity for the other compound. Among compounds **29a–e**, **29a**, and **29d** gave the best enzymatic activity, 10 and 13 nM, respectively. Compounds **29a–c** all have six-membered heteroaromatic rings, however, compound **29b** has a morpholine ring and is 9.6-fold less active than compound **29a**. Compound **29c** has a methylpiperidine ring and is 7.3-fold less active than compound **29a**.

A selection of compounds possessing the best IC_{50} values was also tested in one or more cellular assays, including a MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) cell proliferation assay and FACS (fluorescence-activated cell sorting).[12,13] The MTS assay measures the amount of surviving cells as an assessment



Scheme 6. Reagents and conditions: (a) MeNHOMe·HCl, EDC, DMF/Et_3N, rt, 12 h; (b) $LiAlH_4$, THF, 0 °C, 1 h; (c) **3**, toluene, reflux, 2 h; (d) 2° amines, $NaBH_3CN$, CH_2Cl_2, MeOH, AcOH, 50 °C, 24 h.

Table 3. Results of the MTS and FACS cellular assays (μM)

Compound	IC_{50} (nM)	MTS[a] (μM)			FACS[b] (μM)		
		Cpd alone	Cpd/Dox[c]	Ratio	EC_{50}	Cpd/Dox[d]	Ratio
7	5	>58.9	15.0	>3.9			
8a	3	8.65	0.89	9.7			
8b	22	>59.3	24.0	>2.5			
8f	23	>59.3	>59.3		>10	>10	
8i	8	>59.3	0.44	>136	>10	1.71	>5.9
8j	9	>58.9	12.2	>4.8			
8u	7	7.86	0.41	19.1			
15	12	1.54	0.76	2.0	>10	0.14	>71
19a	10	0.83	0.22	3.8	3.66	0.58	6.3
29a	10	14.5	4.66	3.1	>10	1.45	>6.9
29d	13	16.4	6.55	2.5			

[a] Tested using HeLa cells.
[b] Tested using H1299 cells.
[c] The Dox concentration was 100 nM.
[d] The Dox concentration was 500 nM.

for cytotoxicity. FACS analysis measures abrogation of the G2 checkpoint as an indicator of Chk1-based cellular mechanism for the compounds. These data, along with the enzymatic assay data (IC_{50}'s), are presented in Table 3 as IC_{50} values at which the compound reduces cell growth or decreases G2 cells by half. We have defined an ideal result as a compound possessing little or no antiproliferative activity when dosed alone (>59.3 μM in the MTS assay and >10 μM in the FACS assay), but possessing high antiproliferative activity in the presence of doxorubicin (Dox) (ideally $\leqslant$1 μM), thus providing the highest ratio.

From the data in Table 3, it is seen that good activity in the enzymatic assay does not always translate into good cellular activity. Factors such as cell permeability can contribute to these differences. Compounds **7**, **8b**, **8f**, and **8j** all have potent IC_{50}'s ranging from 5 to 23 nM, however, compounds **7**, **8b**, and **8j** have only very weak combination cellular activity: 15, 24.0, and 12.2 μM, respectively. Compound **8f** did not show any combination or single agent cellular activity in either the MTS or FACS assay. Overall, the two cellular assays corroborate well with each other. Compound **8i** had no single compound activity and showed strong combination activity in the MTS assay. FACS analysis also showed little single compound activity and had some combination activity. An apparent discrepancy between the MTS and FACS assays was observed for compounds **15**, **19a**, and **29a**. However, the different cell lines and compound concentrations used in the two assays could explain the limited variations. The MTS assay uses HeLa cells that are more sensitive than the H1299 cells used in the FACS assay. For example, a single compound EC_{50} value of 0.83 μM for compound **19a** in the MTS assay became 3.66 μM in the FACS assay. Moreover, the MTS assay measures proliferation, while FACS analysis evaluates Chk1-based cellular mechanism. The two parameters may vary in the cell lines. The single compound activities for compounds **15** and **29a** in the MTS assay were not detected in the FACS assay because the maximum concentration for FACS was only 10 μM. For combination treatments, all three of the compounds showed activity in both assays. Of the compounds tested, **8i** showed the best overall results among all three assays possessing an IC_{50} of 8 nM, MTS single agent activity for compound alone of >59.3 μM and combination activity with doxorubicin of 0.44 μM, and a ratio of single agent to combination activity of >10.0 μM/1.7 μM in the FACS assay. In addition, compounds **8a** and **8u** also showed moderate to good activity having 9.7- and 19.1-fold MTS single/combination ratios, respectively.

In summary, we have modified R^4 of the urea phenyl ring with a variety of chemical structures, and R^2 and R^5 to some degree. Several compounds showed very promising results as potent and selective Chk1 inhibitors. In particular, compound **8i** showed very good results throughout the enzymatic and cellular assays. Although we have progressed toward our goal of finding a potent and selective Chk1 inhibitor, additional work is needed to obtain greater insight into the SAR of these compounds.

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