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Synthesis and in-vitro biological activity of macrocyclic urea Chk1 inhibitors

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Abstract—A variety of macrocyclic urea compounds were prepared as potent Chk1 inhibitors by modifying the C5 position of the benzene ring of the macrocyclic urea with ether moieties, aliphatic carbon chains, amide and halides. Enzymatic activity less than 20 nM was observed in 29 of 40 compounds. Compounds **14**, **46d**, and **48j** provided the best overall results in the cellular assays as they abrogated doxorubicin-induced cell cycle arrest (IC_{50} = 3.31, 3.08, and 3.13 μM) and enhanced doxorubicin cytotoxicity (IC_{50} = 0.54, 1.27, and 0.96 μM) while displaying no single agent activity, respectively.

More than 50% of cancer cells have mutant *p*53 and are not capable of arresting in the G1 checkpoint.[1] As a result, cancer cells rely on Chk1-dependent S and G2 checkpoints to cope with DNA damages.[2–4] Inhibition of Chk1 abrogates the S and G2 checkpoints and leads to premature mitotic progression and mitotic catastrophe when combined with a DNA damaging agent.[5–7] In contrast, normal cells still arrest at G1, via *p*53, to repair their DNA damages. Therefore, Chk1 inhibitors should selectively sensitize cancer cells to DNA damaging reagents[2–4,8] and be of great therapeutic value in cancer treatment.

Several compounds are already known as Chk1 inhibitors.[9–12] UCN-01 is undergoing clinical trials as an anticancer agent.[13] Recently, we,[14–17] and others,[18–20] have disclosed *N*-aryl-*N*′-pyrazinyl ureas (**1**, Fig. 1) as a new class of Chk1 inhibitors. Much of our effort was focused on the modification of R^2, which points toward the ribose pocket of the Chk1 enzyme, and R^4, which points toward the solvent front. Hundreds of acyclic urea compounds we synthesized showed single-digit nM enzymatic activity and excellent selectivity.[14–16] Because diaryl ureas have been explored extensively in the patent literature,[14,18–20] we synthesized macrocyclic urea compounds[21,22] (**2**, Fig. 1). Since acyclic ureas with ethoxy (**3**, Fig. 1, IC_{50} = 31 nM) and amide (**4**, Fig. 1, IC_{50} = 22 nM) substitution at the R^5 position were active in the Chk1 enzymatic assay, we modified R^5 on the benzene ring of the macrocyclic urea. The chemistry and biological results of these modifications are presented here.

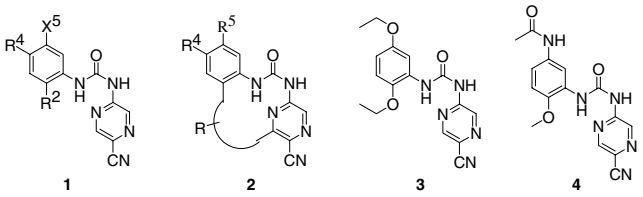

Figure 1. Structure of compounds **1**, **2**, **3**, and **4**.

Scheme 1 shows the initial synthesis of the macrocyclic ureas. After the protection of 2,4-dimethoxyaniline **7** with phthalic anhydride and demethylation with BBr_3, the C5-hydroxy of intermediate **8** was selectively protected with a TBDPS group, and subsequent allylation of the C2-hydroxy of intermediate **9** afforded compound **10**. Deprotection of compound **10** with hydrazine followed by coupling with intermediate **5** (Fig. 2) provided acyclic urea **12**. Grubbs cyclization afforded compound

Keywords: Macrocyclic urea; Chk1 inhibitor.
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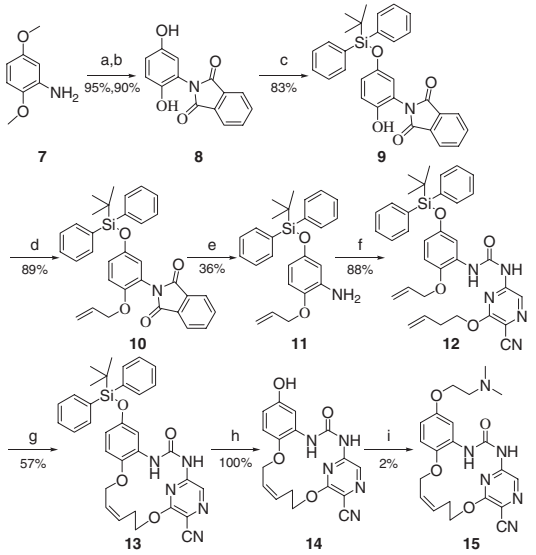


Scheme 1. Reagents and conditions: (a) phthalic anhydride, 180 °C, 15 min; (b) BBr_3, CH_2Cl_2, −78 to 0 °C, 2 h; (c) *tert*-butylchlorodiphenylsilane, imidazole, DMF, 50 °C, 72 h; (d) allyl bromide, K_2CO_3, acetone, reflux, 12 h; (e) H_2NNH_2, CH_3OH, rt, 2 h; (f) **5**, DMF, 70 °C, 12 h; (g) Grubbs catalyst, 2nd generation, CH_2Cl_2, 40 °C, 12 h; (h) TBAF, THF, rt, 3 h; (i) 2-dimethylamino-ethanol, di-*tert*-butylazodicarboxylate, PPh_3-polymer supported, THF, rt, 12 h.

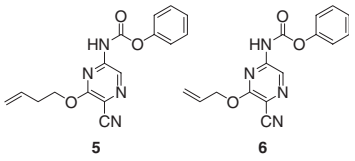
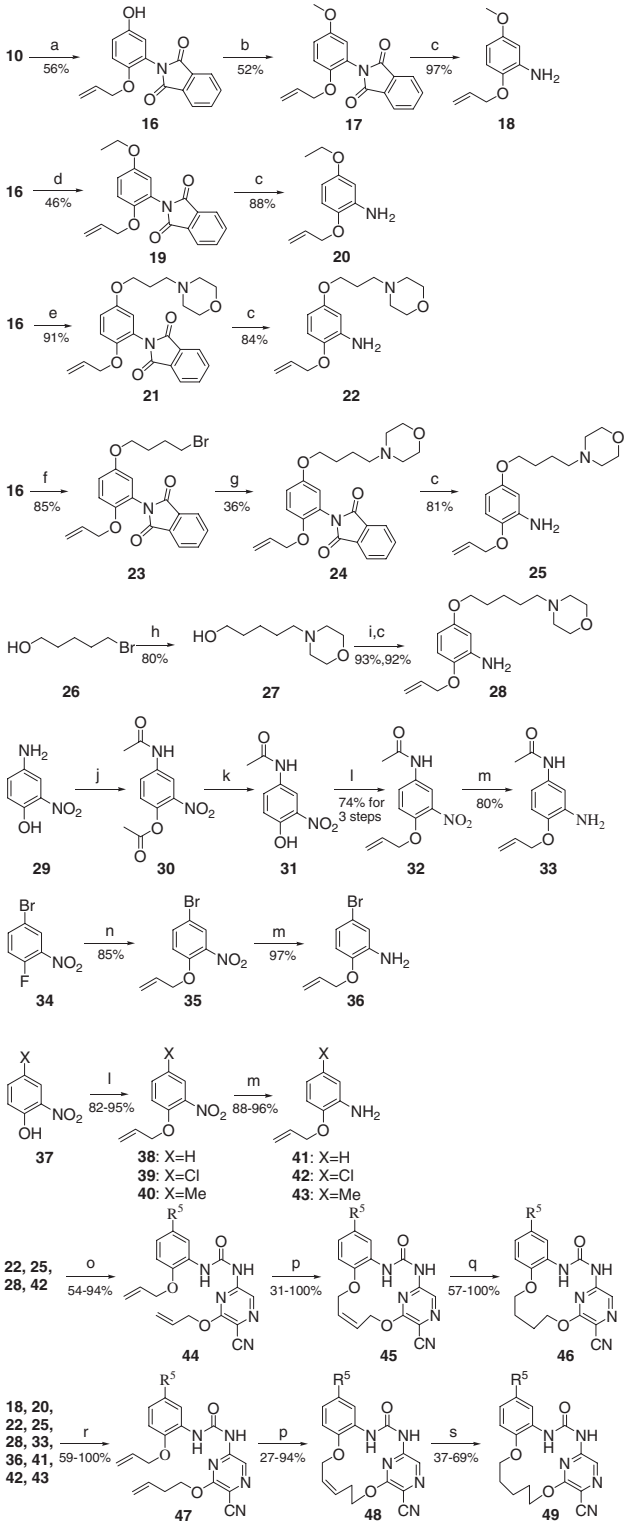


Figure 2. Structure of intermediates **5** and **6**, synthesized according to Ref. 21.

13. TBAF deprotection provided compound **14**. Mitsunobu reaction gave compound **15**.

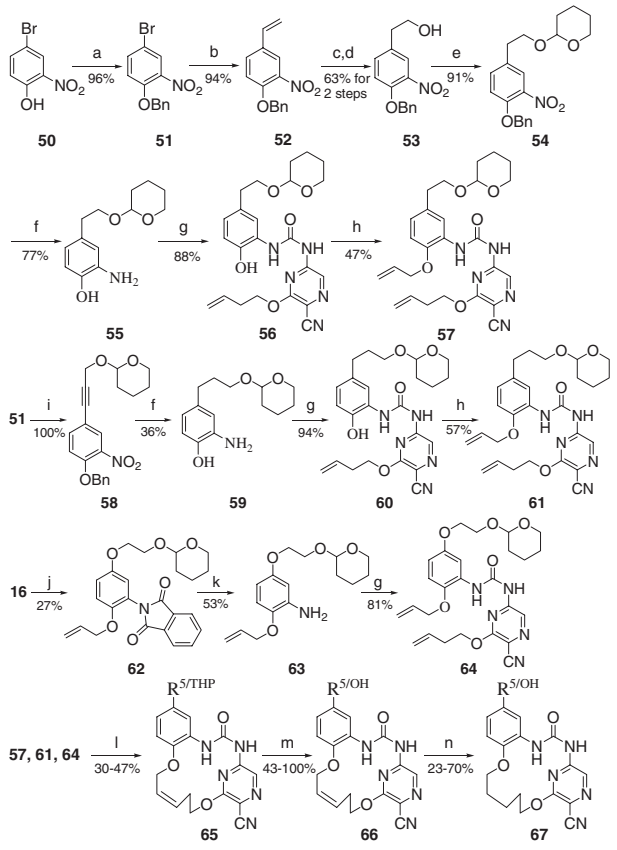
Since the Mitsunobu reaction in Scheme 1 did not work well, we used an alternate strategy in Scheme 2. Compound **10** was deprotected with TBAF to give compound **16**, which was then alkylated, and deprotected with hydrazine to provide amino intermediates **18** and **20**. The Mitsunobu reactions of compound **16** with 3-morpholinopropanol and 5-morpholinopentanol (made from the reaction of compound **26** with morpholine via step **h**) and subsequent deprotection with hydrazine afforded amino intermediates **22** and **28**. The Mitsunobu reaction of compound **16** with 4-bromo-1-butanol followed by substitution and deprotection produced amino intermediate **25**. Compound **29** was converted to the amino intermediate **33** in four steps via global acetylation of compound **29**; selective removal of the acetyl group from the resulting phenolic ester; allylation of



Scheme 2. Reagents and conditions: (a) TBAF, THF, rt, 3 h; (b) iodomethane, K_2CO_3, acetone, reflux, 3 h; (c) H_2NNH_2, CH_3OH, rt, 2 h; (d) iodoethane, K_2CO_3, acetone, reflux, 3 h; (e) 3-morpholinopropanol, di-*tert*-butylazodicarboxylate, PPh_3-polymer supported, THF, rt, 12 h; (f) 4-bromo-1-butanol, di-*tert*-butylazodicarboxylate, PPh_3-polymer supported, THF, rt, 12 h; (g) morpholine, triethylamine, CH_3CN, 60 °C, 12 h; (h) morpholine, CH_3CN, 80 °C, 12 h; (i) **16**, di-*tert*-butylazodicarboxylate, PPh_3-polymer supported, THF, rt, 12 h; (j) acetyl chloride, pyridine, rt, 12 h; (k) K_2CO_3, MeOH, rt, 72 h; (l) allyl bromide, K_2CO_3, acetone, reflux, 6 h; (m) Fe, NH_4Cl, EtOH, H_2O, 80 °C, 3 h; (n) allyl alcohol, NaH, THF, rt, 12 h; (o) **6**, DMF, 70 °C, 12 h; (p) Grubbs catalyst, 2nd generation, CH_2Cl_2, 40 °C, 12 h; (q) $Rh(I)(PPh_3)_3Cl$, 60 psi H_2, THF, rt, 72 h; (r) **5**, DMF, 70 °C, 12 h; (s) 5% Pt/C, THF, 50 psi H_2, rt, 3 h.

the phenol; and finally iron reduction of the nitro group. Amino intermediate **36** was synthesized by substitution and reduction of compound **34**. Amino intermediates **41**–**43** were made through allylation and reduction. Intermediates **22**, **25**, **28**, and **42** were coupled with intermediate **6** (Fig. 2) separately to afford the corresponding acyclic ureas. After Grubbs cyclization and hydrogenation, 14-membered ring macrocyclic urea compounds **45a**–**d** and **46a**–**d** were obtained. Moreover, all the amino intermediates in Scheme 2 were coupled with intermediate **5** separately to afford all the corresponding acyclic urea intermediates. Grubbs cyclization and hydrogenation provided the 15-membered ring macrocyclic urea compounds **48a**–**j** and **49a**–**j**.

As shown in Scheme 3, several alcohol groups were introduced at the C5 position. Starting material **50** was benzylated to give intermediate **51**. The bromo moiety was then replaced with vinyl via Stille coupling. After hydroboration and oxidation, a hydroxyethyl group was introduced at this position. THP protection afforded compound **54**. Hydrogenation followed by urea coupling and Mitsunobu reaction provided acyclic urea intermediate **57**. A triple bond was introduced on the bromo site of intermediate **51** via the Sonogashira reaction and intermediate **58** was made. After hydrogenation, urea coupling and Mitsunobu reaction, intermediate **61** was obtained. Alkylation of compound **16** followed by hydrazine deprotection and urea coupling afforded intermediate **64**. Intermediates **57**, **61**, and **64** were cyclized to produce compounds **65a**–**c**. THP deprotection provided compounds **66a**–**c**. Hydrogenation afforded compounds **67a**–**c**.



Scheme 3. Reagents and conditions: (a) benzylbromide, K_2CO_3, acetone, reflux, 3 h; (b) tributyl(vinyl)tin, $Pd(PPh_3)_4$, DMF, 80 °C, 12 h; (c) 9-BBN, THF, rt, 12 h; (d) NaOH, H_2O_2, rt, 6 h; (e) 3,4-dihydro-2*H*-pyran, *p*-TsOH·H_2O, CH_2Cl_2, rt, 12 h; (f) 20% $Pd(OH)_2$/C, 60 psi H_2, CH_3OH, 50 °C, 4 h; (g) **5**, DMF, 70 °C, 12 h; (h) allyl alcohol, di-*tert*-butylazodicarboxylate, PPh_3-polymer supported, THF, rt, 12 h; (i) tetrahydro-2-(2-propynyloxy)-2*H*-pyran, $Pd(PPh_3)_2Cl_2$, PPh_3, CuI, triethylamine, 120 °C, 25 min, microwave; (j) 2-(2-bromoethoxy)-tetrahydro-2*H*-pyran, K_2CO_3, acetone, reflux, 3 h; (k) H_2NNH_2, CH_3OH, rt, 2 h; (l) Grubbs catalyst, 2nd generation, CH_2Cl_2, 40 °C, 12 h; (m) CH_3CO_2H/THF/H_2O (4:2:1), 45 °C, 12 h; (n) 5% Pt/C, THF, 50 psi H_2, rt, 3 h.

NMR analysis confirmed that the Grubbs cyclization here provided the *cis* conformation. The enzymatic activities[14–16,21] of the macrocyclic urea compounds are listed in Table 1. Compound **13** showed no enzymatic activity because the TBDPS group prevented binding to the enzyme.

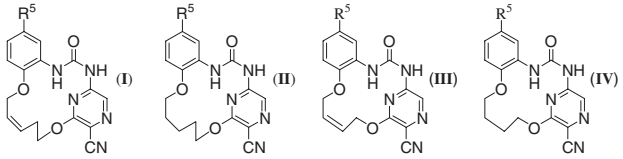
In most cases, the enzymatic activities of the macrocyclic ureas before and after the hydrogenation of the cyclization chain are comparable. For example, compounds **45b** and **46b** both possess IC_{50} = 4 nM. The other corresponding pairs generally follow the same trend. This indicates that the hydrogenation of the cyclization chain did not have a significant effect on the enzymatic activity. Also, the enzymatic activities of the 15-membered ring macrocyclic ureas and 14-membered ring macrocyclic ureas with the same side group R^5 are essentially equivalent. For example, the corresponding 14-membered ring versus 15-membered ring pairs of compounds **48c** and **45a**, **49d** and **46b**, **49e** and **46c** have similar IC_{50}'s. We did not make 16-membered ring macrocyclic urea compounds here because they are 4- to 5-fold less than active the 14- and 15-membered ring macrocyclic urea compounds.[22]

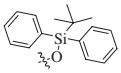
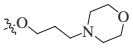
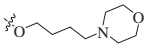
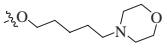
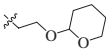
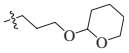
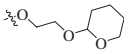
Comparison of compounds **65a** and **66a**, compounds **65b** and **66b**, and finally compounds **65c** and **66c** shows that the enzymatic activity does not change significantly upon THP deprotection. Also, increasing the side chain by one methylene group does not cause appreciable change in the enzymatic activity. Compounds **45a** and **45b** and the other analogous pairs have similar IC_{50}'s. This indicates that R_5 may be near to or occupy a position in free space because the enzymatic activity was not sensitive to the length of the side chain. This is consistent with the X-ray crystallography data reported in Ref. 16.

Comparison of the enzymatic activity of compound **65b**–**65c**, compound **66b**–**66c**, and finally compound **67b**–**67c** shows that in each case the latter of the pair is more active than the former. The only difference between these two types of compounds is that compounds **65b**, **66b**, and **67b** have a methylene group directly attached to the C5 position, while compounds **65c**, **66c**, and **67c** have an oxygen atom at this position. Obviously, oxygen provides a better interaction between the small molecule and the enzyme.

Comparing compounds **48c**–**e** with compound **14**, one can calculate that compounds **48c**–**e** are 6.80-, 5.67-,

Table 1. Chk1 IC_{50}'s of 15-membered ring and 14-membered ring macrocyclic urea compounds before and after the hydrogenation of the cyclization chain



R^5	I		II		III		IV	
	Compound	Chk1 IC_{50}[a] (nM)	Compound	Chk1 IC_{50}[a] (nM)	Compound	Chk1 IC_{50}[a] (nM)	Compound	Chk1 IC_{50}[a] (nM)
	**13**	>10,000						
–OH	**14**	34						
	**15**	15						
	**48a**	37	**49a**	25				
	**48b**	19	**49b**	55				
	**48c**	5	**49c**	7	**45a**	**4**	**46a**	2
	**48d**	6	**49d**	3	**45b**	**4**	**46b**	4
	**48e**	5	**49e**	3	**45c**	**2**	**46c**	3
	**48f**	16	**49f**	8				
–Br	**48g**	12	**49g**	12				
–H	**48h**	12	**49h**	16				
–Cl[b]	**48i**	10	**49i**	11	**45d**	**5**	**46d**	6
–Me	**48j**	15	**49j**	8				
	**65a**	108						
	**66a**	97	**67a**	39				
	**65b**	91						
	**66b**	69	**67b**	24				
	**65c**	19						
	**66c**	13	**67c**	11				

[a] Radiometric assay in the presence of 5 μM of ATP.
[b] See Ref. 22.

and 6.80-fold more active than compound **14** in the enzymatic assay. However, comparing compounds **15**, **48a**, **48b**, **65c**, and **66c** with compound **14**, one can see that the enzymatic activities did not change significantly. These results indicate that the terminal morpholine group on the side chain interacted more effectively with the solvent front[16] than the other side chains we introduced. Compounds **45a**–**c** and **46a**–**c** were prepared to determine if this trend would continue with the analogous 14-membered ring macrocyclic ureas. The excellent enzymatic assay results of compounds **45a**–**c** and **46a**–**c** confirmed this hypothesis.

The enzymatic activities of compounds **48f**, **48g**, **48i**, and **48j** are better than that of compound **14**. However, the acetamidyl, bromo, chloro, and methyl groups are larger than the hydroxyl group. It may be interesting to prepare more macrocyclic urea compounds with larger groups at this position to more fully develop and understand the SAR of these compounds.

A total of 29 compounds were further evaluated in the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt) cell proliferation assay and FACS (fluorescence-activated

cell sorting) analysis.[6,14–17,21] The MTS assay measures the amount of surviving cells as an assessment of cytotoxicity. FACS analysis measures abrogation of the G2 checkpoint as an indicator of Chk1-based cellular mechanism for the compounds. A Chk1 mechanism-based compound is expected to have little single agent activity when administered alone in MTS and FACS. Preferably, the IC_{50} values should be higher than the maximum concentration of 59.29 and 10.00 μM used for MTS and FACS analysis, respectively. In combination with DNA damaging agents such as doxorubicin (Dox), the Chk1 inhibitor should significantly enhance cytotoxicity.

Table 2 shows the results of the above two cellular assays, along with the enzymatic assay data (all presented as IC_{50} values). Because of compound-to-compound variabilities concerning such factors as cell permeability, compounds with good enzymatic activity do not always have good cellular activity. In Table 2, all 29 compounds have very good enzymatic activity, however, 12 compounds showed no combination activity in the MTS cell proliferation assay (>5.93 μM) and 7 compounds showed no combination activity in the FACS assay (>10.00 μM).

In Table 2, 12 compounds showed single agent activity in the MTS assay (<59.29 μM); while no compound showed single agent activity in the FACS assay (all >10.00 μM). Since MTS measures cell survival, compounds that had single agent activity could inhibit cell growth without alteration of the cell cycle profiles. The fact that most of the compounds were weak in MTS and inactive in FACS indicated that they lacked non-mechanism-based cellular profiles, which was consistent with the Chk1 siRNA data.[6]

Generally speaking, the two cellular assays correlate well with each other. Among all 29 compounds, six compounds (**48a**, **49a**, **49b**, **49g**, **49h**, and **67c**) did not show

Table 2. Results of the MTS cell proliferation and FACS analysis

Compound	Chk1 IC_{50} (nM)	MTS EC_{50}[a] (μM)		FACS EC_{50}[b] (μM)	
		Compound alone	Compound/Dox[c]	Compound alone	Compound/Dox[d]
14	34	>59.29	3.31	>10.00	0.54
15	15	30.38	NV	>10.00	6.06
45b	4	7.56	0.50	ND[e]	ND[e]
45d[f]	5	>59.29	>5.93	>10.00	2.19
46d[f]	6	>59.29	3.08	>10.00	1.27
48a	37	>59.29	>5.93	>10.00	>10.00
48b	19	>59.29	NV	>10.00	2.71
48c	5	>59.29	4.25	>10.00	1.18
48e	5	5.27	3.04	>10.00	1.14
48f	16	39.91	5.92	>10.00	3.43
48g	12	29.37	2.86	>10.00	1.05
48h	12	39.95	3.91	>10.00	1.93
48i[f]	10	11.90	2.60	>10.00	4.53
48j	15	>59.29	3.13	>10.00	0.96
49a	25	>59.29	>5.93	>10.00	>10.00
49b	55	>59.29	>5.93	>10.00	>10.00
49c	7	4.03	3.99	>10.00	>10.00
49e	3	8.87	0.28	>10.00	1.23
49g	12	52.77	>5.93	>10.00	>10.00
49h	16	>59.29	>5.93	>10.00	>10.00
49i[f]	11	57.57	3.04	>10.00	2.65
65a	97	NV	>5.93	>10.00	3.12
65b	91	>59.29	3.13	>10.00	3.12
65c	19	30.86	5.54	>10.00	1.84
66b	69	>59.29	>5.93	>10.00	1.01
66c	13	>59.29	>5.53	>10.00	1.93
67a	39	>59.29	>5.93	>10.00	4.48
67b	24	>59.29	5.37	>10.00	2.29
67c	11	>59.29	>5.93	>10.00	>10.00

[a] Tested using HeLa cells, a p53-deficient human cervical cancer cell line.

[b] Tested using NCI-H1299 cells, a human lung cancer cell line.

[c] Calculated from the percentages of inhibition by varying concentration of the test compounds above the background inhibition by 100 nM of doxorubicin, a topoisomerase II inhibitor in clinical use known to confer G2/M checkpoint arrest at this concentration in HeLa cells. The ability of the compound to sensitize HeLa cells is represented by the ratio of the EC_{50} values of the compound alone and the compound with 100 nM doxorubicin.

[d] Compounds alone (10 μM) caused no alternation in cell cycle distribution in comparison to the DMSO control. Doxorubicin at 500 nM led to a dramatic shift of cell distribution in comparison with the DMSO control, with most of the cell population in the G2/M phase and a very low population of apoptotic cells. Increasing the concentration of the compounds such as **14**, **46d**, and **48j** significantly reduced the G2/M population and simultaneously increased apoptotic cells, which is consistent with the abrogation of the G2 checkpoint arrest and induction of apoptosis.

[e] Not determined.

[f] See Ref. 22.

any activity in either of the two cellular assays. Fourteen compounds (**14**, **46d**, **48c**, **48e**, **48f**, **48g**, **48h**, **48i**, **48j**, **49i**, **65b**, **65c**, and **67b**) showed activity in both cellular assays. Among these 14 compounds, compounds **14**, **46d**, **48c**, **48j**, **65b**, and **67b** showed the greatest inhibitory activity. The most active compounds in the cellular assays were compounds **14**, **46d**, and **48j**.

In summary, we have modified successfully the C5 position of the benzene ring of the macrocyclic urea core. Forty new macrocyclic urea compounds were prepared and 29 of these compounds showed excellent Chk1 enzymatic activity (<20 nM). Compounds **14**, **46d**, and **48j** showed the best overall results in all three assays with IC_{50}'s of 34, 6, and 15 nM; ratios of single agent to combination activity of >59.29 μM/3.31 μM, >59.29 μM/3.08 μM, and >59.29 μM/3.13 μM in the MTS assay; and ratios of single agent to combination activity of >10.00 μM/0.54 μM, >10.00 μM/1.27 μM, and >10.00 μM/0.96 μM in the FACS assay, respectively. Further investigation is needed to optimize the design of these Chk1 inhibitors.

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