Structure-Based Design, Synthesis, and Biological Evaluation of Potent and Selective Macrocyclic Checkpoint Kinase 1 Inhibitors

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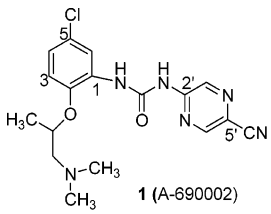
Based on the crystallographic analysis of a urea−checkpoint kinase 1 (Chk1) complex and molecular modeling, a class of macrocyclic Chk1 inhibitors were designed and their biological activities were evaluated. An efficient synthetic methodology for macrocyclic ureas was developed with Grubbs metathesis macrocyclization as the key step. The structure−activity relationship studies demonstrated that the macrocyclization retains full Chk1 inhibition activity and that the 4-position of the phenyl ring can tolerate a wide variety of substituents. These novel Chk1 inhibitors exhibit excellent selectivity over a panel of more than 70 kinases. Compounds **5b**, **5c**, **5f**, **15**, **16d**, **17g**, **17h**, **17k**, **18d**, and **22** were identified as ideal Chk1 inhibitors, which showed little or no single-agent activity but significantly potentiate the cytotoxicities of the DNA-damaging antitumor agents doxorubicin and camptothecin. These novel Chk1 inhibitors abrogate the doxorubicin-induced G2 and camptothecin-induced S checkpoint arrests, confirming that their potent biological activities are mechanism-based through Chk1 inhibition.

Introduction

DNA-damaging agents constitute a major class of anticancer therapeutics and have significantly contributed to the survival increases of cancer patients.[1] However, the clinical use of DNA-damaging anticancer agents is limited by their severe toxicity and by resistance from tumor cells, especially p53-deficient tumor cells. While there has been much interest in identifying more selective and effective agents that target DNA and its associated processes,[1] the development of adjuvant therapeutics that improve the efficacy and selectivity of DNA-damaging agents in the clinic has recently attracted particular attention. Such treatments may either sensitize tumor tissue or protect normal tissue from DNA damage. A promising approach to these treatments is the targeting of specific differential biological pathways in tumors such as DNA-damage-response pathways.[2−7]

With DNA damage, cells are arrested (G1, S, G2) to initiate the DNA repair process.[2−4] If cells progress into mitosis with damaged DNA, they will undergo mitotic catastrophe and eventually apoptosis. Most tumor cells distinguish themselves from normal cells by lacking the G1 checkpoint due to loss of p53, and therefore they are selectively arrested at the S or G2 checkpoint after DNA damage. If the S and G2 checkpoints are abrogated, G1-deficient cancer cells will not arrest to repair damaged DNA and will enter mitosis, which results in premature chromosome condensation and leads to cell death. In contrast, normal cells are still arrested in the G1 phase and are less affected by S and G2 checkpoint abrogation, suggesting that a favorable therapeutic window may be achieved for G2 and/or S abrogators.

Checkpoint kinase 1 (Chk 1)[a] is a serine/threonine protein kinase and a key mediator in the DNA damage-induced checkpoint network.[5−7] In response to DNA damage, ATM and ATR kinases activate Chk1 through phosphorylation at Ser317 and Ser345 in the SQ/TQ motif,[8,9] and Chk1 in turn inactivates Cdc2/cyclinB by downregulating the Cdc25 family of phosphatases (Cdc25A, B, and C) and upregulating Wee1, thereby inhibiting progression into mitosis.[10] The inhibition of Chk 1 abrogates the S and G2 checkpoints, thereby preferentially sensitizing tumor cells, especially p53-null cells, to various DNA damaging agents. Downregulation of Chk1 by siRNA or antisense abrogates DNA-damaging agent-induced G2/S arrest and sensitizes tumor cells to topoisomerase inhibitors, antimetabolite anticancer agents, and radiation.[10−14] Consequently, Chk1 has emerged as an attractive chemosensitization target, and the inhibition of Chk1 may significantly improve the efficacy and selectivity of DNA-damaging agents in the clinic.[3,15−18]



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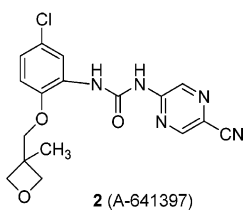

2 (A-641397)

An ideal Chk1 inhibitor should show no single-agent activity and should significantly potentiate DNA-damaging antitumor agents. To date, several classes of Chk1 inhibitors have been reported,[18] including indolocarbazoles such as 7-hydroxystaurosporine (UCN-01),[19] isogranulatimide,[20] debromohymenialdisine,[21] aminopyrimidine,[22] pyrrolopyridines,[23] indolinones,[24] and benzimidazole-quinolinones.[25] UCN-01 is a potent Chk1 inhibitor and has been extensively studied. It abrogates both the S and G2 checkpoints and sensitizes tumor cells to a wide

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[a] Abbreviations: APCI, atmospheric pressure chemical ionization; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3 related; BSA, bovine serum albumin; Chk1, checkpoint kinase 1; CPT, camptothecin; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; Dox, doxorubicin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FACS, fluorescence-activated cell sorting; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; SAR, structure−activity relationship; siRNA, small interfering ribonucleic acid; THF, tetrahydrofuran; UCN-01, 7-hydroxystaurosporine.

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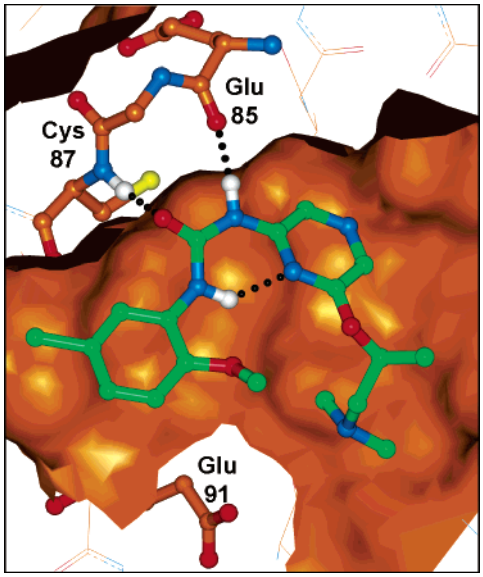


Figure 1. X-ray crystal structure of compound **4** (green carbon atoms) bound to Chk1 kinase (brown carbon atoms with brown protein surface). Hydrogen bonds are indicated with black dotted lines. Glu85 and Cys87 of the hinge and Glu91 of the ribose region are indicated. The side chain of hinge residue Tyr86 is not displayed.

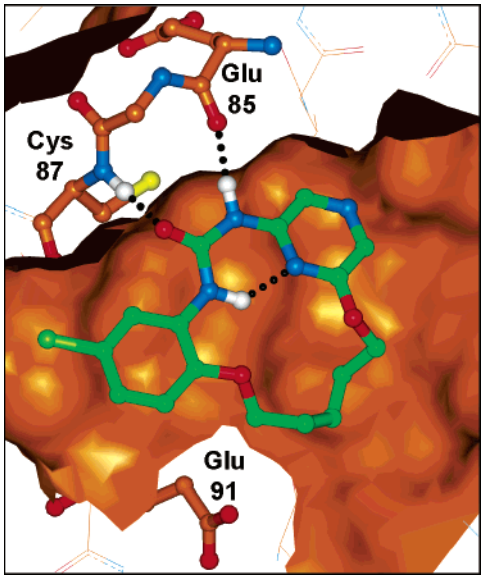


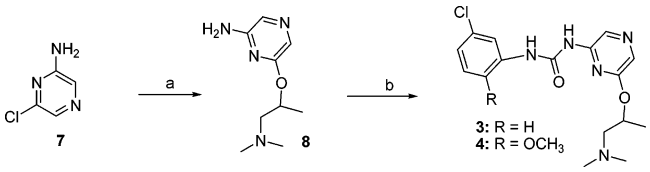
Figure 2. X-ray crystal structure of macrocyclic urea **5a** bound to Chk1 kinase. Hydrogen bonds are indicated with black dotted lines. Glu85 and Cys87 of the hinge and Glu91 of the ribose region are indicated. The side chain of hinge residue Tyr86 is not displayed.

spectrum of DNA-damaging agents.[26] However, UCN-01 also potently inhibits a number of other kinases and exhibits strong single-agent activity, and it has high human plasma protein binding affinity,[27] which could limit its use in the clinic. We recently reported two potent and selective urea-based Chk1 inhibitors, **1** and **2**.[28,29] Both compounds abrogate the cell cycle checkpoints and significantly potentiate the cytotoxicity of topoisomerase inhibitors. More importantly, these urea-based Chk1 inhibitors preferentially sensitize p53-deficient tumor cells to DNA-damaging agents.[28] The urea pharmacophore has been a very popular scaffold for kinase inhibitors[30] and has been well covered in the patent literature, and diaryl ureas have been specifically claimed as Chk1 inhibitors in several patents.[31−34] The work presented here further explores the urea class of Chk1 kinase inhibitors in the context of a macrocyclic ring system.[35,36]

Design

Previous SAR studies of urea-based Chk1 inhibitors disclosed that a variety of substituents are well tolerated at the C2-position of the phenyl ring.[37,38] X-ray crystallographic analysis and molecular modeling showed that both the 2-position of the phenyl ring and the C6′-position of the pyrazinyl ring point toward the vicinity of ribose pocket, indicating the C6′-position is an ideal site for further improvement.[38] This was confirmed by the synthesis of C6′-substituted ureas **3** and **4**. Both **3** (IC_{50} = 26 nM) and **4** (IC_{50} = 22 nM) possess potent inhibitory activity against Chk1. An X-ray cocrystal structure of a Chk1−**4** complex showed that the urea backbone forms complementary H-bonds with Cys87 and Glu85 in the hinge region (Figure 1; PDF ID: 2E9P). The substituents at the C6′- and C2-positions stretch into the ribose pocket, and their ends are essentially proximal. Importantly, the bound urea conformation showed that the urea amide bonds were in one cis and one trans orientation (Figure 1). This places vectors from positions 2 and 6′ pointing toward one another. We reasoned that potency might be improved by connecting these ribose pocket substituents in a macrocycle, as it has been well documented that restriction of conformation through macrocyclization can produce potent inhibitors.[39] Indeed, the Chk1 inhibition activity of macrocyclic urea **5a** (IC_{50} = 10 nM) was fully retained compared with **4** and dramatically improved, over 440-fold, compared with its synthetic acyclic urea precursor **6**. The X-ray cocrystal structure of **5a** (Figure 2) confirmed that the side chain linking the C2-position of phenyl ring to the C6′ substituent of the pyrazine ring lies in the vicinity of the ribose pocket as predicted from modeling studies.

Scheme 1[a]



[a] Reaction conditions: (a) NaH, 1-dimethylaminopropan-2-ol, dioxane, 100 °C, overnight, 83%; (b) 1-chloro-3-isocyanatobenzenes, toluene, 120 °C, 50−70%.

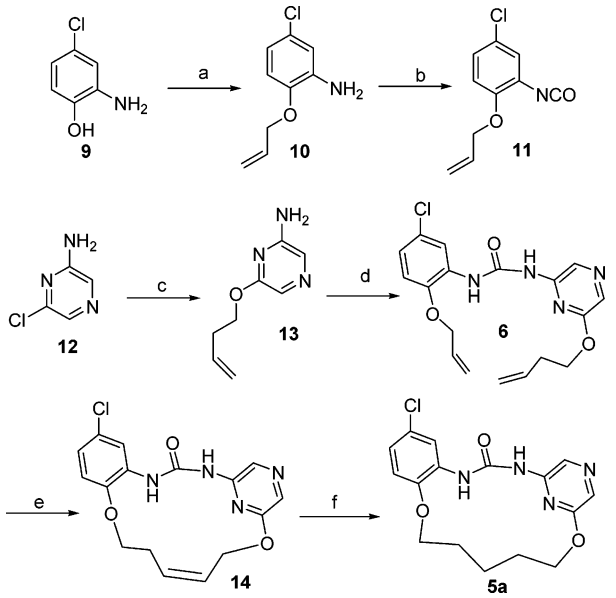
Chemistry

The synthesis of the acyclic ureas is outlined in Scheme 1. The nucleophilic displacement of chloride from **7** smoothly gave the aminopyrazinyl ether **8**, which was coupled with chlorophenyl isocyanates to give compounds **3** and **4** in moderate to good yield.

The synthesis of macrocycle **5a** is shown in Scheme 2. Alkylation of the commercially available aminophenol **9** by allyl bromide gave **10**, which was treated with phosgene in toluene to generate isocyanate **11** in very good yield. Aminopyrazinyl ether **13**, prepared through the nucleophilic displacement of chloride from **12** by 3-butenol, was coupled to **11** to give the key intermediate **6** in moderate yield. The macrocylization of **6** was achieved under a condition of Grubbs olefin metathesis ring-closure. Saturation of the cyclic olefin **14** provided **5a**. Macrocyclic ureas **5b**−**f** (Table 1) were prepared by synthetic procedures similar to that described for **5a**.

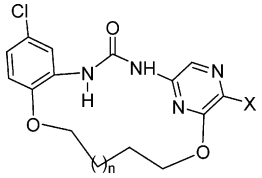
Scheme 3 outlines the synthesis of macrocyclic ureas in which a substituent is attached to the phenyl ring by an ether linkage. Removal of the SEM group from the 4-position of **5e** released the hydroxyl group in quantitative yield. Alkylation of the hydroxyl group by Mitsunobu reaction under various conditions

Scheme 2[a]



[a] Reaction conditions: (a) allyl bromide, K_2CO_3, acetone, rt, 65%; (b) phosgene, toluene, 110 °C, quantitative yield; (c) 3-buten-1-ol, NaH, dioxane, 100 °C, 31%; (d) **11**, toluene, 110 °C, 52%; (e) Grubbs' catalyst (second generation), CH_2Cl_2, reflux, 75%; (f) H_2, Pt/C (10%), MeOH−THF (3:1), 80%.

Table 1. Ring Size−Activity Relationship of Macrocyclic Ureas

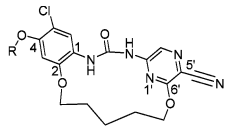


compd	ring size	X	Chk1 inhibition (IC_{50}, nM)	compd	ring size	X	Chk1 inhibition (IC_{50}, nM)
5a	$n = 2$ 15-member	H	10	**5c**	$n = 1$ 14-member	CN	6
5b	$n = 2$ 15-member	CN	7	**5d**	$n = 3$ 16-member	CN	28

gave only poor yields. Fortunately, direct alkylation of the phenol with organic bromides was achieved in the presence of Cs_2CO_3 in DMF in 20−90% yield without affecting the urea NH. This simple alkylation expedited the synthesis of ether analogues **16** (Table 2) and greatly facilitated SAR studies, showing the utility of the parallel synthesis.

Scheme 4 outlines the synthesis for macrocyclic ureas in which a substituent is linked to the phenyl ring by a nitrogen linker. Although alkylation of an inactive aniline through reductive amination has historically proven difficult, we were able to successfully perform the reaction by employing a modification of a recently published procedure.[40] Thus, various aldehydes were coupled to aniline **5f** in the presence of sulfuric

Table 2. Structure−Activity Relationship of Ether Analogues at the C4-Position

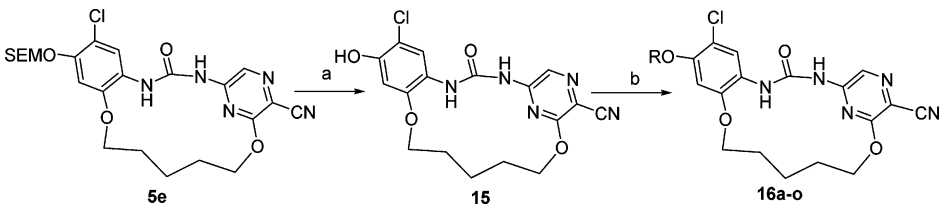


Compounds	R=	Chk1 Inhibition (IC_{50}, nM)	Compounds	R=	Chk1 Inhibition (IC_{50}, nM)
15	H	6	**16h**		1060
16a		2	**16i**		> 10,000
16b		1	**16j**	Et	6
16c		3	**16k**		13
16d		6	**16l**		12
16e		5	**16m**		9
16f		9	**16n**		63
16g		1	**16o**		3

acid, and the resulting imines were reduced to **17** (Table 3) in situ by $NaBH_4$ in an aqueous THF system. Additionally, acylation of **5f** with acyl chlorides in the presence of pyridine in methylene chloride afforded the amide analogues **18a**−**c**, and sulfonylation of **5f** with sulfonyl chloride at 0 °C in the presence of pyridine in methylene chloride afforded sulfonamide analogue **18d** (Table 3).

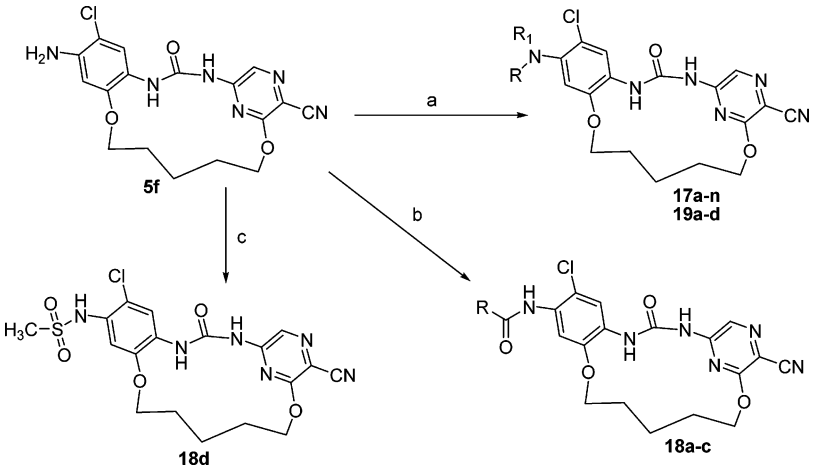
The synthesis of macrocyclic urea analogues bearing carbon-linked substituents on the phenyl ring is shown in Scheme 5. Macrocyclic urea **15** was converted to the triflate **20** in 82% yield. Various alkynes were coupled with **20** under Sonogashira reaction conditions to provide **21a**−**d**. It is worth noting that the coupling yield was significantly improved by addition of tetrabutylammonium iodide as reported in recent literature.[41] Complete saturation of the triple bond of **21e** followed by the removal of the THP group provided **22**. Coupling of the triflate **20** with tributylallyltin was effected in a system of $PdCl_2(PPh_3)_2$-Ph_3P-LiCl in DMF at 110 °C to provide **23** in 65% yield. Olefin **23** was oxidized by osmium tetraoxide under Upjohn conditions[42] to provide the diol **24** in good yield.

Scheme 3[a]



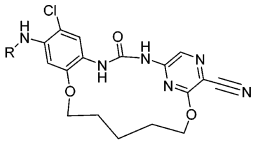
[a] Reaction conditions: (a) HCl, dioxane−CH_2Cl_2−EtOH, 96%; (b) RBr, Cs_2CO_3, DMF, 40 °C, 20−90%.

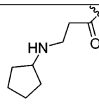
Scheme 4[a]



[a] Reaction conditions: (a) RCHO, H_2SO_4, THF, then $NaBH_4$, 0 °C; (b) RCOCl, pyridine, CH_2Cl_2, rt; (c) CH_3SO_2Cl, pyridine, CH_2Cl_2, 0 °C.

Table 3. Structure−Activity Relationship of Amino Analogues at the C4-Position



Compound	R=	Chk1 Inhibition (IC_{50}, nM)	Compound	R=	Chk1 Inhibition (IC_{50}, nM)
5f	H	3	**17j**		231
17a	CH_3	2	**17k**		6
17b	CH_3CH_2	4	**17l**		7
17c	n-Bu	15	**17m**		162
17d		2	**17n**		2
17e		11	**18a**		7
17f		17	**18b**		1
17g		12	**18c**		2
17h		13	**18d**		15
17i		266			

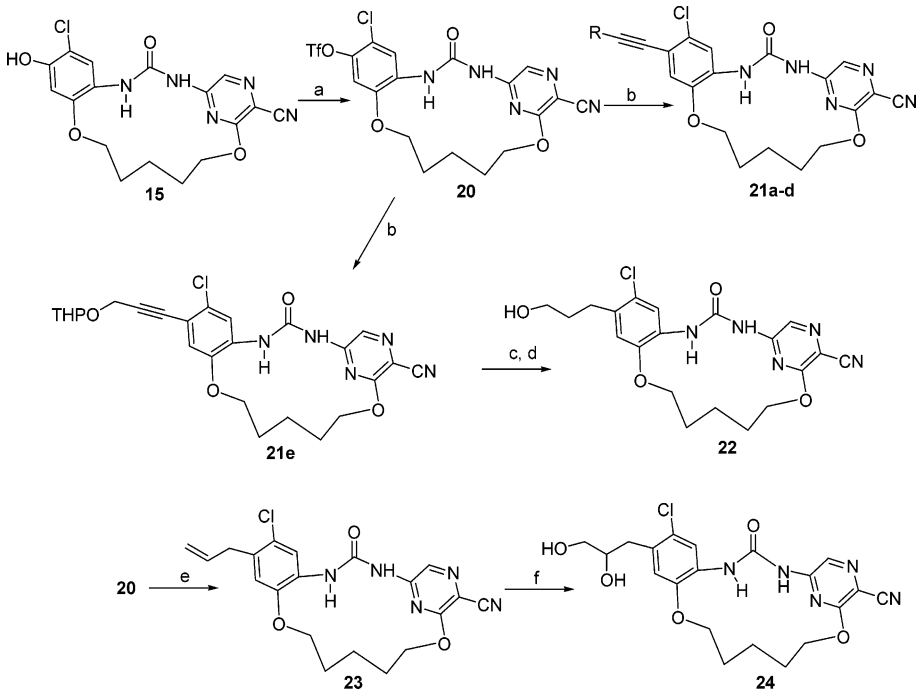
Results and Discussion

Ring Size−Activity Relationship. Molecular modeling studies suggested that a linker length of 6−8 atoms between ring positions 2 and 6′ would be sufficient to span the required distance, yet not induce strain from too small a ring or clash with Chk1 protein from too large a ring. Thus, 14−16-member macrocyclic ureas were prepared and tested against Chk1. As shown in Table 1, the 15-member macrocyclic ureas **5a** and **5b** both exhibit potent activity for the inhibition of Chk1 in vitro. Although the enzymatic inhibition potencies of these two compounds are comparable, **5b** showed superior cellular activity to **5a** (*vide infra*), indicating an important role for the CN group at the 5′-position of the pyrazinyl ring of **5b**. For this reason, we focused on CN analogues in the following SAR studies. The 15-member macrocycle **5b** and 14-member **5c** are both very potent, with the same IC_{50} (6 and 7 nM, respectively). In contrast, the 16-member macrocycle **5d** is less potent against Chk1, but its Chk1 inhibition activity is still fully retained compared with the acyclic analogue **4**. The reduced activity of **5d** may be due to the additional conformational flexibility resulting from the presence of more rotatable bonds in the carbon chain linker. This is consistent with the idea that the restriction of conformation produces more potent inhibitors.[39]

Structure−Activity Relationship on the C4-Position of the Phenyl Ring. Examination of the X-ray cocrystal structure of **5a**−Chk1 complex (Figure 2; PDB ID: 2E9U) shows that the substituent (R_4) at the 4-position of the phenyl ring is pointing into in a solvent-accessible region, which should be able to accommodate a variety of groups. To further improve the potency and physicochemical properties of the macrocyclic Chk1 inhibitors, we performed extensive SAR studies on the 4-position based on the 15-member-ring macrocyclic urea **5b**. Table 2 shows the SAR results of ether analogues. As predicted, most of the analogues exhibited potent activity for Chk1 inhibition. The introduction of a polar group such as an amino group at the terminal position of various long chain substituents resulted in a substantial increase in potency (e.g., **16g** vs **16h**). This may be attributed to the hydration of the polar group in the solvent-exposed region. Conversely, the introduction of a hydrophobic group results in lessened activity, and the activity decreases the further the hydrophobic group extends into the solvent-exposed region (**16k** vs **16h** vs **16i**). Furthermore, the nitrogen position in the pyridyl group of compounds **16l**−**n** has a remarkable impact on the potency. The 4″-pyridyl (**16l)** and 3″-pyridyl (**16m)** analogues possess much better activity than the 2″-pyridyl analogue **16n**.

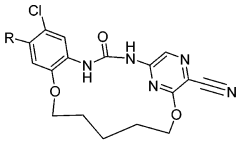
Tables 3 and 4 show the SAR results of analogues having substituents connected to the C4-position by a nitrogen atom.

Scheme 5[a]



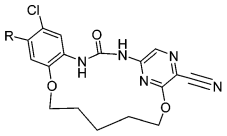
[a] Reaction conditions: (a) trifluoromethanesulfonyl chloride, TEA, DMF, 0 °C, 82%; (b) alkynes, TRA, $(PPh_3)_4Pd$, CuI, *n*-Bu_4NI, DMF, 70 °C; (c) Pt/C (5%), H_2, THF, 80%; (d) HOAc, THF, H_2O, 45 °C, 85%; (e) $PdCl_2(PPh_3)_2$, Ph_3P, LiCl, tributylallyltin, DMF, 110 °C, 65%; (f) *N*-methylmorpholine *N*-oxide, 2.5% (wt %) OsO_4 in 2-methyl-2-propanol, THF, H_2O, rt, 85%.

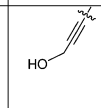
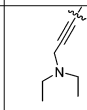
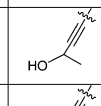
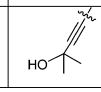
Table 4. Structure−Activity Relationship of Dialkylamino Analogues at the C4-Position



Compound	R=	Chk1 Inhibition (IC_{50}, nM)	Compound	R=	Chk1 Inhibition (IC_{50}, nM)
19a		7	**19c**		4
19b		248	**19d**		139

Table 5. Structure−Activity Relationship at the C4-Position with Carbon Linkage



Compound	R=	Chk1 Inhibition (IC_{50}, nM)	Compound	R=	Chk1 Inhibition (IC_{50}, nM)
21a		10	**21d**		2
21b		26	**22**		5
21c		109	**24**		5

The amine analogues in Table 3 showed SAR similar to the ether analogues in Table 2. Again, the 3- and 4-pyridyl amine analogues **17g** and **17h** showed potent activity, and removal of the nitrogen atom (i.e., benzyl analogue **17i**) caused a considerable reduction in potency. The importance of 3′-endocyclic nitrogen for potency was also observed for the five-membered ring heteroaromatic analogues. For example, **17k** and **17l** are much more potent than the thiophene analogue **17m**. It is also interesting to note that methylation of **17j** results in a >100-fold increase in potency (**17n**), a result not well understood at this stage. The amide (**18a**) and the corresponding primary amine-substituted analogues (**17b**) show comparable potency, but the sulfonamide analogue (**18d**) is slightly less potent. Table 4 shows that analogues with dialkylamino substituents at the C4-position are less active than their monoalkyl amino counterparts (**19b** vs **17b**).

Analogues with carbon-connected substituents at the C4-position are depicted in Table 5. These results further confirm that the connecting atom at the 4-position has little impact on potency (**16d** vs **17d** vs **22**). The rigid alkynyl spacer showed no negative impact on potency (e.g., **21d**), and the terminal group on the spacer is again the determinant for potency.

X-ray Crystallographic Analysis of Macrocyclic Urea−Chk1 Complex. To better understand the SAR results and the binding mode of macrocylic Chk1 inhibitors, we performed an X-ray crystallographic analysis of **17h**−Chk1 complex at a resolution of 2.0 Å (Figure 3; PDB ID: 2E9V). Compound **17h** binds to the ATP-binding pocket of Chk1 in the same mode as compound **5a**. The urea amino group at the 2′-position of the pyrazinyl ring forms a hydrogen bond (3.1 Å) to the backbone carbonyl oxygen of Glu85, while the urea carbonyl oxygen accepts a hydrogen bond (2.7 Å) from the amide nitrogen of Cys87 in the hinge region. This complementary hydrogen-bonding pattern is the key interaction for most potent ATP-

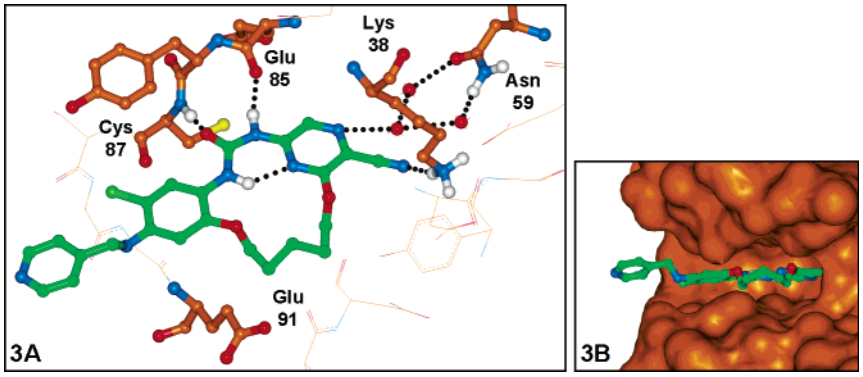


Figure 3. (A) X-ray crystal structure of **17h** bound to Chk1 kinase. Hydrogen bonds are indicated as in Figure 2 with the additional H-bond shown between the inhibitor nitrile group and Lys38 (N···N distance = 2.8 Å, C−N···N bond angle = 140°). The three water molecules of the "water pocket"[18a,46] are shown with oxygen atoms as red balls. (B) Surface of entire active site, showing 4-pyridyl unit of **17h** exposed to solvent.

competitive kinase inhibitors. One face of the diaryl urea core makes many favorable van der Waals contacts with the backbone and/or side chains of residues from the N-terminal domain, including Leu15, Val23, Ala36, and Leu84. The other face of the core makes van der Waals interactions with the side chains of Val68 and Leu137. The carbon chain connecting the two aryl rings sits in the ribose pocket and makes favorable van der Waals contacts with Glu91, Asn135, Leu137, Glu134, and Ser147. The 5′-CN is situated in the polar region surrounded by Ser147, Lys38, and Asp148 and forms a hydrogen bond with the side chain of Lys38 (3.2 Å). The 4′-N of the pyrazinyl ring points to the water pocket lined by Asn59, Leu84, and Phe149. The water pocket, occupied by three water molecules, is unique for Chk1 kinase. It has been suggested that this pocket is an attractive target for gaining both potency and selectivity for Chk1 since it might be entropically favorable to displace the pocket's water molecules into the bulk solvent.[18a,43] A related water pocket has been reported for Wee1 kinase.[44] In this kinase, two specific waters were observed that correspond to the two waters in Figure 3 that are closest to Lys38. A His residue in Wee1 replaces Asn 59 of Chk1, and its His NE2 atom corresponds to the third specific water reported here for Chk1. The water structure in these two kinases is potentially an area of interest in future inhibitor design. Other aspects of the crystal structure of **17h** include (a) the extension of its 4-pyridinylmethylamino group of the phenyl ring into the solvent-exposed region (Figure 3b) and (b) an intramolecular hydrogen bond (2.9 Å) between a urea amino group and the 1′-pyrazinyl nitrogen.

Kinase Selectivity Profiles of Macrocyclic Urea Chk1 Inhibitors. The selectivity profiles of three macrocyclic urea Chk1 inhibitors (**16a**, **18d**, and **22**) against other kinases were tested (Table 6). Under our assay conditions, remarkable selectivity for all three compounds over a panel of more than 70 kinases was observed. Especially, **18d** exhibited no inhibitory activity toward other kinases in this panel at the highest concentration tested except Plk1, but with a 170-fold selectivity window.

Potentiation of Cytotoxicity of DNA-Damaging Agents by Chk1 Inhibitors. Compounds identified as potent (IC_{50} < 20 nM) in an enzymatic assay were routinely screened for antiproliferative activity in HeLa cells, a p53-null human cervical cancer cell line. The EC_{50} values for the compounds were determined either alone or in the presence of 150 nM doxorubicin (Dox), a clinical topoisomerase II inhibitor known to arrest the G2/M checkpoint at this concentration in HeLa cells. The EC_{50} values for Chk1 inhibitors in combination with Dox were calculated from the percentage of inhibition by Chk1 compounds at various concentrations above the background inhibition by 150 nM Dox. The ability of Chk1 inhibitors to potentiate Dox is represented by the ratio of the EC_{50} values of the inhibitor alone and the inhibitor with Dox. As shown in Table 7, compounds **5b**, **5c**, **5f**, **15**, **16d**, **17g**, **17h**, **17k**, **18d**, and **22** stood out as ideal Chk1 inhibitors, showing little or no antiproliferative activity alone but significant activity in the presence of Dox. It is noteworthy that the CN group at the C5′-position of the pyrazynyl ring improved the cellular potency (**5a** vs **5b**). In addition, the substituents at the C4-position have significant impact on the cellular activity, and amino analogues are usually more potent in the cellular assays than the carbon-

Table 6. Kinase Selectivity of Macrocyclic Chk1 Inhibitors

	K_i,[a] μM				K_i,[a] μM		
kinase	**18d**	**16a**	**22**	kinase	**18d**	**16a**	**22**
Chk1	0.0137	0.0047	0.0043	InsR	>10	>10	>10
Abl	>10	>10	>10	IRAK4	>10	>10	>10
Akt1	>10	1.07	3.15	JAK2	>10	>10	>10
Akt2	>10	1.12	3.97	JAK3	>10	>10	>10
Akt3	>10	4.89	>10	Jnk1a1	>10	>10	>10
Aurora1	>10	>10	>10	Jnk2a2	>10	>10	>10
Aurora2	>10	>10	>10	KDR	>8.21	>8.21	>8.21
Blk	>10	>10	>10	LCK	>10	>10	>10
Cdc2	>10	>10	>10	Limk1	>10	>10	>10
Cdc42BPA	>10	6.63	4.44	Lyn	>10	>10	>10
Cdk2	>10	>10	5.76	Mk2	>10	0.88	1.57
Cdk5/p25	>10	>10	>10	MK3	>10	>10	>10
Chk2	>10	5.67	>10	Nek2	>10	>10	>10
Ck2	>10	>10	>10	p38d	>10	>10	>10
Camk4	>10	>10	>10	p38g	>10	>10	>10
Ck1delt	>10	>10	>10	PAK1	>10	>10	>10
cKit	>10	>10	>10	PAK4	>10	>10	>10
cMet	>10	>10	>10	PDK1	>10	2.07	0.74
Dyrk1A	>10	>10	>10	PKA	>10	>10	>10
EGFR	>10	>10	>10	PKCd	>10	>10	>10
EMK	>10	>10	7.35	PKCg	>10	>10	>10
Erk2	>10	8.21	>10	PKCz	>8	>8	>8
EphA2	>10	>10	>10	Pim1	>10	1.10	0.28
ErbB2	>10	>10	>10	Pim2	>10	>10	>10
ErbB4	>10	>10	>10	Pkd2	>10	>10	>10
FGFR1	>10	>10	>10	Plk1	2.33	1.28	0.94
FGFR3	>10	>10	>10	Plk3	>10	0.82	0.56
Flt1	>10	>10	>10	Plk4	>10	>10	>10
Flt3	>10	>10	>10	Rock1	>10	>10	>10
Flt4	>10	>10	>10	Rock2	>10	>10	>10
Fyn	>10	>10	>10	Sgk	>10	>10	>10
GSK3a	>10	>10	>10	Src	>10	>10	>10
IGF1R	>10	>10	>10	TrkA	>10	>10	>10
ITK	>10	>10	>10	TrkB	>10	>10	>10
IKKa	>10	>10	>10	Tyk2	>10	>10	>10
IKKb	>10	0.34	0.38	Zipk	>10	>10	>10

[a] Inhibition constant (K_i) values are calculated from the Cheng−Prusoff equation, $K_i = IC_{50}/(1 + ([ATP]/K_m)$.

Table 7. Cellular Activities of Macrocyclic Chk1 Inhibitors

compd	MTS EC_{50}, μM: compd + Dox	MTS EC_{50}, μM: compd alone	MTS potentiation of CPT	FACS EC_{50}, μM: compd + Dox	FACS EC_{50}, μM: compd alone
5a	37.8	>59	NA[a]	7.5	>10
5b	3.04	57.6	NA	2.7	>10
5c	3.08	57.6	NA	1.3	>10
5f	0.43	>59	NA	0.24	7.6
15	1.9	>59	NA	NA	NA
16d	3.01	>59	6-fold	0.7	>10
17g	1.6	>59	8-fold	0.33	>10
17h	1.4	>59	20-fold	1.2	>10
17k	1.2	>59	NA	0.11	>10
18d	2.7	>59	5-fold	1.6	>10
22	0.86	28.7	NA	0.19	>10

[a] NA, data not available.

connected and ether analogues. The lack of a perfect correlation between Chk1 enzymatic inhibition potency and cellular antiproliferative activity may be due to other physicochemical properties such as variation in cellular penetration, as has been well documented in the literature.[45]

The ability of selected compounds to sensitize tumor cells was further evaluated by measuring the antiproliferative EC_{50} of camptothecin (CPT) in the presence of a fixed concentration of Chk 1 inhibitors in a MTS assay. CPT is a major anticancer agent in clinical use. It damages DNA through the inhibition of topoisomerase I and is known to induce S-phase arrest.[46] Thus, SW620 cells (a p53-deficient colon carcinoma cell line) were treated with increasing doses of CPT in the presence and absence of Chk1 inhibitors. The ratio of EC_{50} value of CPT alone and EC_{50} value of CPT in combination with Chk1 inhibitor was defined as the potentiation ratio. As shown in Table 7, Chk1 compounds potentiate the cytotoxity of CPT 5−20-fold at a concentration of 10 μM. Reduced but still significant potentiation was also achieved at a Chk1 inhibitor concentration of 3 μM, indicating that the potentiation was dose-dependent on the Chk1 inhibitors (data not shown).

FACS Analysis of Cell Cycle Profiles Treated with Chk1 Inhibitors. To establish that the macrocyclic Chk1 inhibitors are truly abrogating the checkpoints induced by DNA-damaging agents and not simply altering cell populations by affecting cell cycle progression, a detailed cell cycle kinetic analysis was carried out by use of fluorescence-activated cell sorting (FACS). The analysis was undertaken with the Chk1 inhibitors in H1299 cells. As expected, Dox alone conferred a prominent G2/M-phase arrest. Table 7 shows the EC_{50} values for abrogating G2/M caused by Dox in the presence of various concentrations of Chk1 inhibitors. The regular cell cycle profile was not altered by Chk1 inhibitors even at the highest concentration (10 μM) since the FACS profile was indistinguishable from that of the DMSO control (data not shown). This observation is consistent with the fact that Chk1 knockout through Chk1 siRNA did not change the cell-cycle distributions.[47] Chk1 inhibitors abrogated the Dox-induced-G2/M checkpoint with EC_{50} values of submicromolar to low (single−digit) micromolar. An increasing amount of cells accumulated in the sub-G0/G1 phase was noticed, indicating that the checkpoint abrogation results in apoptosis, again demonstrating the potentiation effect. In addition, we confirmed that the macrocyclic Chk1 inhibitors efficiently abrogated CPT-induced S arrest. As shown in Figure 4, both **17h** and **18d** abrogated CPT-induced S arrest at 3 and 10 μM.

Conclusions

We have discovered macrocyclic ureas as a novel class of Chk1 inhibitors. An efficient synthetic methodology for macrocyclic ureas was developed with Grubbs metathesis macrocyclization as the key step. Structure−activity relationship studies demonstrated that macrocyclic ring size had only modest impact on the Chk1 inhibition activity and that the 4-position of the phenyl ring can tolerate a wide variety of substituents. These novel Chk1 inhibitors exhibited remarkable selectivity over a panel of more than 70 kinases. Compounds **5b**, **5c**, **5f**, **15**, **16d**, **17g**, **17h**, **17k**, **18d**, and **22** were identified as ideal Chk1 inhibitors, which exhibited little or no antiproliferative activity alone but significantly potentiated the cytotoxicities of the DNA-damaging agents Dox and CPT. FACS analysis of cell cycle profiles confirmed that these compounds express their potent biological activities through the inhibition of Chk1. To our knowledge, these are the first examples of macrocyclic kinase inhibitors based on the urea scaffold. The efficient synthetic methodology developed here should facilitate the utilization of these macrocylic compounds as medically useful molecules, especially in the field of kinase inhibitors.

Experimental Section

General Information. All reactions were carried out under N_2 atmosphere unless otherwise specified. Reagents and solvents were obtained from commercial suppliers and were used without further purification. ^{1}H NMR spectra were obtained on a Varian Unity or Inova (500 MHz), Varian Unity (400 MHz), or Varian Unity plus or Mercury (300 MHz) instrument. Chemical shifts are reported as δ values (ppm) downfield relative to tetramethylsilane (TMS) as an internal standard, with multiplicities reported in the usual manner. Mass spectral analyses were performed on a Finnigan SSQ7000 GC/MS mass spectrometer using different techniques, including electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), as specified for individual compounds. Exact mass measurement was performed on a Finnigan FTMS Newstar T70 mass spectrometer. The compound is determined to be "consistent" with the chemical formula if the exact mass measurement is within 5.0 ppm relative mass error (RME) of the exact monoisotopic mass. Elemental analyses were performed by Quantitative Technologies, Inc., Whitehouse, NJ. Column chromatography was carried out on a Horizon Pioneer system (Biotage, Inc.).

Preparative reverse-phase HPLC was performed on an automated Gilson HPLC system, with a SymmetryPrep Shield RP18 prep cartridge, 250 mm × 21.20 mm i.d., 10 um, and a flow rate of 25 mL/min; λ = 214, 245 nm; mobile phase A, 0.1% trifluoroacetic acid (TFA) in H_2O; mobile phase B, CH_3CN; linear gradient 0−70% B in 40 min.

Analytical liquid chromatography/mass spectrometry (LC/MS) was performed on a Finnigan Navigator mass spectrometer and Agilent 1100 HPLC system running Xcalibur 1.2 and Open-Access 1.3 software. The mass spectrometer was operated under positive APCI ionization conditions. The HPLC system comprised an Agilent Quaternary pump, degasser, column compartment, autosampler, and diode-array detector, with a Sedere Sedex 75 evaporative light-scattering detector. The column used was a Phenomenex Luna Combi-HTS C8(2) 5 μm 100 Å (2.1 mm × 30 mm). TFA method (method A): A gradient of 10−100% acetonitrile (solvent 1) and 0.1% trifluoroacetic acid in water (solvent 2) was used, at a flow rate of 2 mL/min (0−0.1 min, 10% solvent 1; 0.1−2.6 min, 10−100% solvent 1; 2.6−2.9 min, 100−10% solvent 1; 2.9−3.0 min, 100−10% solvent 1). Ammonium method (method B): A gradient of 10−100% acetonitrile (solvent 1) and 10 mM NH_4OAc in water (solvent 2) was used, at a flow rate of 1.5 mL/min (0−0.1 min, 10% solvent 1; 0.1−3.1 min, 10−100% solvent 1; 3.1−3.9 min, 100−10% solvent 1; 3.9−4.0 min, 100−10% solvent 1).

6-[1-(Dimethylamino)propan-2-yloxy]pyrazin-2-amine (8). To a suspension of NaH (60%, 123.5 mg, 3.08 mmol) in dioxane was added dropwise dimethylaminopropanol (0.37 mL, 3.08 mmol). After the mixture was stirred for 30 min, 6-chloropyrazin-2-amine (200 mg, 1.54 mmol) was added. The resulting mixture was heated

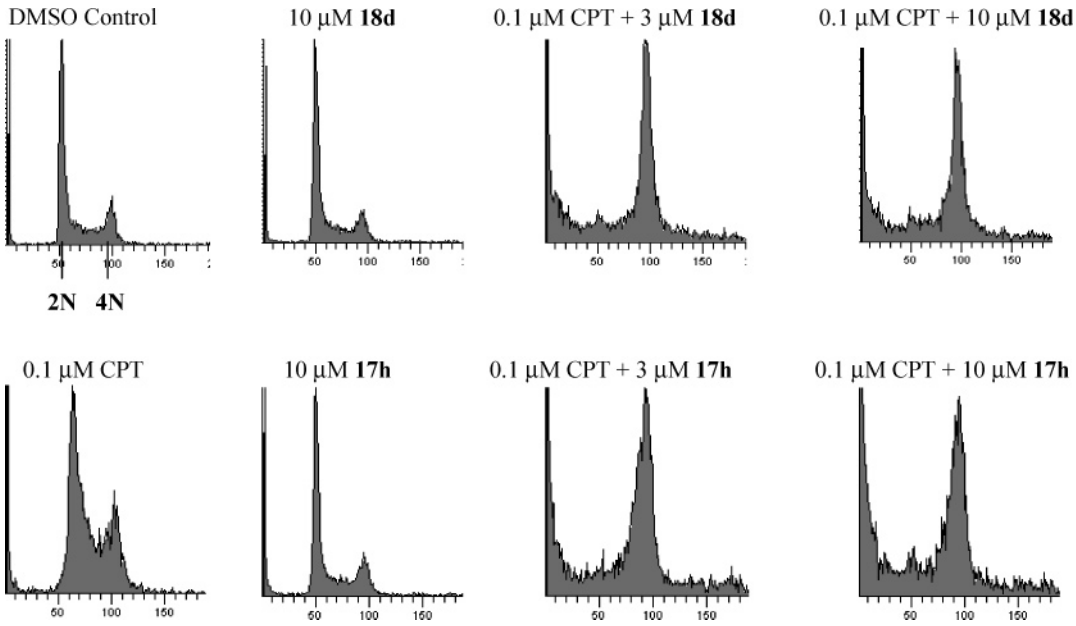


Figure 4. FACS profiles of SW620 cells treated with CPT in the presence and absence of macrocyclic Chk1 inhibitors **17h** and **18d**. The cells were stained with propidium iodide for DNA contents. G0/G1 cells contain 2*N* DNA while cells in G2/M phase have 4*N* DNA. S phase cells have DNA content between 2*N* and 4*N*. Apoptotic cells contain less than 2*N* DNA.

at 100 °C overnight and concentrated. The residue was purified by flash chromatography, eluted with a mixture of ethyl acetate/methanol/ammonium hydroxide (100:6:1), to provide 250 mg of **8** in 83% yield. MS (DCI/NH_3) *m*/*z* 197.11 $(M + H)^+$.

1-(3-Chlorophenyl)-3-[6-[1-(dimethylamino)propan-2-yloxy]-pyrazin-2-yl]urea (3). A mixture of **8** (50 mg, 0.25 mmol) and 1-chloro-3-isocyanatobenzene (0.03 mL, 0.25 mmol) in toluene was refluxed for 4 h and concentrated. The residue was triturated with a mixture of ethyl acetate and hexane to provide 61.2 mg of **3** in 70% yield. MS (DCI/NH_3) *m*/*z* 350.09 $(M + H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.29 (d, $J = 6.44$ Hz, 3H), 2.35−2.42 (m, 1H), 2.52−2.61 (m, 1H), 5.15−5.28 (m, $J = 11.70$, 6.61 Hz, 1H), 7.06−7.11 (m, 1H), 7.25−7.38 (m, 2H), 7.72 (t, $J = 1.86$ Hz, 1H), 7.85 (s, 1H), 8.67 (s, 1H), 9.23 (s, 1H), 9.35 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 18.1, 45.7, 63.6, 69.9, 116.9, 117.8, 122.2, 125.0, 127.4, 130.5, 133.2, 140.3, 146.1, 151.3, 157.5, 183.1. HRMS (ESI-TOF) calcd for $C_{16}H_{21}ClN_5O_2$ $(M + H)^+$ 350.1384; found 350.1391.

1-(5-Chloro-2-methoxyphenyl)-3-[6-[1-(dimethylamino)propan-2-yloxy]pyrazin-2-yl]urea (4). Compound **4** was synthesized by a synthetic procedure similar to that described for **3**, in 55% yield. MS (DCI/NH_3) *m*/*z* 380.10 $(M + H)^+$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.30 (s, 3H), 2.18 (s, 6H), 2.34−2.43 (m, 1H), 2.52−2.61 (m, 1H), 3.89 (s, 3H), 5.19−5.29 (m, 1H), 7.05 (s, 2H), 7.86 (s, 1H), 8.23 (s, 1H), 8.68 (s, 1H), 9.16 (s, 1H), 9.95 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 18.1, 45.7, 56.2, 63.7, 69.9, 112.1, 118.0, 121.7, 124.2, 125.1, 127.2, 129.2, 146.2, 146.8, 151.3, 157.4, 182.6. HRMS (ESI-TOF) calcd for $C_{17}H_{23}ClN_5O_3$ $(M + H)^+$ 380.1489; found 380.1497.

2-(Allyloxy)-5-chloroaniline (10). To a mixture of 2-amino-4-chlorophenol (10 g, 69.65 mmol) and K_2CO_3 (14.53 g, 105 mmol) in acetone (160 mL) was added allyl bromide (9.03 mL, 104 mmol). The reaction mixture was stirred at room temperature overnight. Inorganic salts were filtered off and washed with acetone. The combined filtrate was concentrated. The residue was purified by flash chromatography, eluted with hexane/EtOAc (10:1), to give 8.31 g (65%) of the desired compound as a light-brown oil. MS (DCI/NH_3) *m*/*z* 184.02 $(M + H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.51 (m, 2H), 5.02 (s, 2H), 5.24 (dd, $J = 10.53$, 1.68 Hz, 1H), 5.42 (m, 1H), 6.04 (m, 1H), 6.47 (dd, $J = 8.54$, 2.44 Hz, 1H), 6.66 (d, $J = 2.75$ Hz, 1H), 6.75 (d, $J = 8.54$ Hz, 1H).

1-(Allyloxy)-4-chloro-2-isocyanatobenzene (11). To a solution of 5 mL (47.3 mmol) of 20% phosgene in toluene (6 mL) was added slowly a solution of **10** (1 g, 5.44 mmol) in toluene (10 mL) with stirring under reflux. The resulting reaction mixture was refluxed at 110 °C for 20 h and then cooled. Evaporation of the solvent gave the desired product as an oil in quantitative yield. ^{1}H NMR (500 MHz, CD_2Cl_2) δ 4.62 (d, $J = 5.30$ Hz, 2H), 5.33 (dd, $J = 10.76$, 1.40 Hz, 1H), 5.46 (dd, $J = 17.31$, 1.40 Hz, 1H), 6.07 (m, 1H), 6.84 (d, $J = 8.73$ Hz, 1H), 6.99 (d, $J = 2.50$ Hz, 1H), 7.10 (dd, $J = 8.74$, 2.50 Hz, 1H).

6-(But-3-enyloxy)pyrazin-2-amine (13). To a suspension of NaH (60%, 618 mg, 15.45 mmol) in dioxane (30 mL) was injected 3-buten-1-ol (1.33 mL, 15.45 mmol) at 0 °C. After the reaction mixture was stirred for 2 h, 2-amino-6-chloropyrazine (1 g, 7.72 mmol) was added. The reaction mixture was further stirred at 100 °C for 2.5 days, cooled, and diluted with ethyl acetate. The resulting mixture was washed with water and dried over $MgSO_4$. Evaporation of ethyl acetate gave a residue, which was purified by flash chromatography, eluted with hexane/ethyl acetate (2:1). The title compound (390 mg, 31%) was obtained. MS (DCI/NH_3) *m*/*z* 166.12 $(M + H)^+$. ^{1}H NMR (500 MHz, benzene-d_6) δ 2.66 (m, 2H), 4.42 (t, $J = 6.87$ Hz, 2H), 5.24 (dd, $J = 10.22$, 1.98 Hz, 1H), 5.30 (m, 1H), 6.04 (m, 1H), 7.64 (s, 1H), 7.65 (s, 1H).

1-[2-(Allyloxy)-5-chlorophenyl]-3-[6-(but-3-enyloxy)pyrazin-2-yl]urea (6). A mixture of **11** (201 mg, 0.96 mmol) and **13** (158 mg, 0.96 mmol) in toluene (15 mL) was stirred at 110 °C for 15 h. Evaporation of solvent gave a residue, which was purified by flash chromatography, eluted with hexane/ethyl acetate (1:1). The title compound (185 mg, 52%) was obtained. MS (DCI/NH_3) *m*/*z* 375.12$(M + H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.52 (m, 2H), 4.33 (t, $J = 6.55$ Hz, 2H), 4.70 (d, $J = 5.30$ Hz, 2H), 5.09 (dd, $J = 10.29$, 1.25 Hz, 1H), 5.16 (dd, $J = 17.31$, 1.40 Hz, 1H), 5.31 (d, $J = 10.61$ Hz, 1H), 5.43 (dd, $J = 17.16$, 1.25 Hz, 1H), 5.88 (m, 1H), 6.08 (m, 1H), 7.02 (d, $J = 8.75$ Hz, 1H), 7.06 (dd, $J = 8.75$, 2.5 Hz, 1H), 7.89 (s, 1H), 8.22 (d, $J = 2.50$ Hz, 1H), 8.69 (s, 1H), 9.07 (s, 1H), 10.08 (s, 1H).

Macrocyclic Diarylurea 14. Compound **6** (60 mg, 0.16 mmol) in CH_2Cl_2 (66 mL) was treated with the second-generation Grubbs' catalyst (20 mg, 0.024 mmol). The reaction mixture was stirred at 50 °C overnight. Solvent was removed under reduced pressure. The residue was purified by flash chromatography, eluted with hexane/ethyl acetate (1:1). The title compound (22 mg, 75%) was obtained. MS (DCI/NH_3) *m*/*z* 347.11 $(M + H)^+$. ^{1}H NMR (500 MHz, CD_2Cl_2) δ 2.70 (d, $J = 7.25$ Hz, 2H), 4.63 (m, 4H), 6.05 (m, 2H), 6.90

(d, J = 8.73 Hz, 1H), 7.02 (dd, J = 8.73, 2.57 Hz, 1H), 7.15 (s, 1H), 7.72 (s, 1H), 7.84 (s, 1H), 8.25 (d, J = 2.57 Hz, 1H), 10.55 (s, 1H).

Macrocyclic Diarylurea 5a. To a suspension of platinum−carbon (10%, 2 mg) in a mixture of methanol−THF (3:1, 3 mL) was added **14** (17 mg, 0.049 mmol). The reaction mixture was bubbled with hydrogen for 10 min. Platinum−carbon was removed by filtration through Celite. The filtrate was concentrated and the residue was purified by recrystallization from ethyl acetate. The title compound (13.7 mg, 80%) was obtained. MS (DCI/NH_3) m/z 349.11 $(M + H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.64 (m, 2H), 1.84 (m, 2H), 1.89 (m, 2H), 4.14 (t, J = 5.16 Hz, 2H), 4.48 (t, J = 7.80 Hz, 2H), 7.08 (d, J = 2.50 Hz, 1H), 7.09 (s, 1H), 7.89 (s, 1H), 7.95 (s, 1H), 8.23 (d, J = 2.50 Hz, 1H), 10.26 (s, 1H), 10.32 (s, 1H). Anal. ($C_{16}H_{17}ClN_4O_3$) C, H, N.

Compounds **5b**−**f** were prepared by synthetic procedures similar to those described for **5a**.

Macrocyclic Diarylurea 5b. MS (DCI/NH_3) m/z 391.3 $(M + NH_4)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.61 (dd, J = 12.16, 6.24 Hz, 2H), 1.82 (m, 2H), 1.95 (m, 2H), 4.19 (t, J = 5.15 Hz, 2H), 4.62 (t, J = 8.45 Hz, 2H), 7.13 (m, 2H), 7.99 (s, 1H), 8.20 (d, J = 2.18 Hz, 1H), 9.95 (s, 1H), 10.94 (s, 1H). Anal. ($C_{17}H_{16}ClN_5O_3$) C, H, N.

Macrocyclic Diarylurea 5c. MS (ESI) m/z 357.91 $(M - H)^-$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.85−1.91 (m, 2H), 2.00−2.06 (m, 2H), 4.17 (t, J = 5.49 Hz, 2H), 4.75 (t, J = 7.32 Hz, 2H), 7.09 (dd, J = 8.85, 2.14 Hz, 1H), 7.18 (d, J = 8.54 Hz, 1H), 7.94 (s, 1H), 8.34 (s, 1H), 10.83 (s, 1H), 11.05 (s, 1H). Anal. ($C_{16}H_{14}ClN_5O_3$) C, H, N.

Macrocyclic Diarylurea 5d. MS (ESI) m/z 386.08 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.43−1.59 (m, 4H), 1.74−1.92 (m, 4H), 4.15 (s, 2H), 4.52 (t, J = 6.90 Hz, 2H), 7.15 (s, 2H), 7.96 (s, 1H), 8.00 (s, 1H), 9.61 (s, 1H), 10.91 (s, 1H). Anal. ($C_{18}H_{18}ClN_5O_3$) C, H, N.

Macrocyclic Diarylurea 5e. MS (DCI/NH_3) m/z 537.20 $(M + NH_4)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.00 (s, 9H), 0.90−0.94 (m, 2H), 1.59−1.65 (m, 2H), 1.83−1.87 (m, 2H), 1.94−2.01 (m, 2H), 3.77−3.80 (m, 2H), 4.19−4.21 (m, 2H), 4.60−4.63 (m, 2H), 5.35 (s, 2H), 7.05 (s, 1H), 8.00 (s, 1H), 8.17 (s, 1H), 9.84 (s, 1H), 10.90 (s, 1H).

Macrocyclic Diarylurea 5f. MS (DCI/NH_3) m/z 406.11 $(M + NH_4)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.54−1.59 (m, 2H), 1.76−1.81 (m, 2H), 1.90−1.96 (m, 2H), 4.05 (d, J = 5.49 Hz, 2H), 4.55 (t, J = 8.24 Hz, 2H), 5.19 (s, 2H), 6.56 (s, 1H), 7.87 (s, 1H), 7.95 (s, 1H), 9.64 (s, 1H), 10.76 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 22.7, 25.6, 26.8, 68.3, 69.0, 99.0, 106.5, 107.5, 115.5, 116.1, 122.2, 128.1, 141.4, 148.2, 149.4, 150.5, 160.3. Anal. ($C_{17}H_{17}ClN_6O_3$) C, H, N.

Macrocyclic Diarylurea 15. To a solution of **5e** (1.8 g, 3.46mmol) in a mixture of dichloromethane (100 mL) and ethanol (300 mL) was added 9 mL of 4 N HCl in 1,4-dioxane dropwise. The reaction mixture was stirred overnight, and the white precipitate was collected by filtration and dried. The desired product (1.3 g, 96%) was obtained as a white solid. MS (DCI/NH_3) m/z 407.08 $(M + NH_4)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.60−1.65 (m, 2H), 1.81−1.85 (m, 2H), 1.94−2.00 (m, 2H), 4.11 (t, J = 5.15 Hz, 2H), 4.59 (t, J = 8.11 Hz, 2H), 6.72 (s, 1H), 7.99 (s, 1H), 8.06 (s, 1H), 9.75 (s, 1H), 10.78 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 22.8, 25.6, 26.8, 68.4, 69.5, 100.8, 106.8, 109.7, 115.4, 118.7, 122.4, 128.1, 147.9, 149.4, 149.4, 150.6, 160.3. HRMS (ESI-TOF) calcd for $C_{17}H_{17}ClN_5O_4$ $(M + H)^+$ 390.0964; found 390.0960.

Macrocyclic Diarylurea 16a. A mixture of **15** (20 mg, 0.051 mmol), 3-bromopropanol (11 mg, 0.077 mmol), and Cs_2CO_3 (33.4 mg, 0.102 mmol) in DMF (2 mL) was heated at 40 °C overnight. Inorganic salts were filtered off, and the filtrate was concentrated. The residue was purified by HPLC, eluted with a gradient of 0−70% acetonitrile in 0.1% TFA aqueous solution. The desired product (8.2 mg, 36%) was obtained. MS (ESI) m/z 445.98 $(M - H)^-$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.57−1.62 (m, 2H), 1.80−1.84 (m, 2H), 1.85−1.90 (m, 2H), 1.93−1.99 (m, 2H), 3.58 (q, J = 5.90 Hz, 2H), 4.15 (t, J = 6.26 Hz, 2H), 4.24 (t, J = 5.03 Hz, 2H), 4.56−4.61 (m, 3H), 6.92 (s, 1H), 7.99 (s, 1H), 8.11 (s, 1H), 9.79 (s, 1H), 10.88 (s, 1H). Anal. ($C_{20}H_{22}ClN_5O_5 \cdot 0.4H_2O$) C, H, N.

Macrocyclic Diarylurea 16b. A mixture of **15** (25 mg, 0.064 mmol), 2-(3-bromopropyl)isoindoline-1,3-dione (25.7 mg, 0.096 mmol), and Cs_2CO_3 (41.8 mg, 0.128 mmol) in DMF (2 mL) was heated at 40 °C overnight. Inorganic salts were filtered off, and the filtrate was concentrated. The residue was treated with hydrazine monohydrate (0.016 mL, 0.32 mmol) in a mixture of THF (2 mL) and methanol (0.2 mL). The reaction mixture was stirred for 4 h and concentrated. The residue was purified by HPLC to provide **16b** as the TFA salt in 55% yield (for two steps). MS (APCI) m/z 447.36 $(M + H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.55−1.65 (m, 2H), 1.79−1.87 (m, 2H), 1.92−2.07 (m, 4H), 2.94−3.05 (m, 2H), 4.18 (t, J = 5.95 Hz, 2H), 4.24 (t, J = 5.03 Hz, 2H), 4.52−4.65 (m, 2H), 6.93 (s, 1H), 7.73 (s, 3H), 8.00 (s, 1H), 8.13 (s, 1H), 9.81 (s, 1H), 10.90 (s, 1H). Anal. ($C_{20}H_{23}ClN_6O_4 \cdot 0.2H_2O \cdot$TFA) C, H, N.

Macrocyclic Diarylurea 16c. A mixture of **15** (52 mg, 0.13 mmol), allyl bromide (0.0138 mL, 0.16 mmol), and K_2CO_3 (36.8 mg, 0.267 mmol) in DMF (5 mL) was stirred at room temperature overnight. Inorganic salts were filtered off, and the filtrate was concentrated. The residue was dissolved in a mixture of THF (20 mL) and water (2 mL), and the solution was treated with *N*-methylmorpholine *N*-oxide (36 mg, 0.31 mmol), followed by the addition of 2.5% (wt %) OsO_4 in 2-methyl-2-propanol (0.19 mL) at 0 °C. The reaction mixture was stirred overnight and concentrated. The residue was purified by HPLC, eluted with a gradient of 0−70% acetonitrile in 0.1% TFA aqueous solution. The desired product (22 mg, 36%) was obtained as a white solid. Analytical LC/MS with two different solvent systems indicated >95% purity. MS (ESI) m/z 462.20 $(M - H)^-$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.57−1.62 (m, 2H), 1.80−1.85 (m, 2H), 1.92−1.99 (m, 2H), 3.44−3.52 (m, 2H), 3.82 (m, 1H), 4.00 (m, 1H), 4.08 (m, 1H), 4.23 (t, J = 5.19 Hz, 2H), 4.58 (t, J = 8.24 Hz, 2H), 4.69 (t, J = 5.65 Hz, 1H), 4.98 (d, J = 4.88 Hz, 1H), 6.93 (s, 1H), 7.98 (s, 1H), 8.12 (s, 1H), 9.79 (s, 1H), 10.87 (s, 1H).

Macrocyclic Diarylurea 16d. A mixture of **15** (30 mg, 0.077 mmol), 2-(2-bromoethoxy)tetrahydropyran (32 mg, 0.154 mmol), and Cs_2CO_3 (33.4 mg, 0.102 mmol) in DMF (2 mL) was heated at 40 °C overnight. Inorganic salts were filtered off, and the filtrate was concentrated. The residue was treated with HOAc/THF/H_2O (4:2:1, 8 mL) at 45 °C overnight. The solvent was removed and the residue was purified by HPLC, eluted with a gradient of 0−70% acetonitrile in 0.1% TFA aqueous solution. The desired product (25 mg, 76%) was obtained. MS (ESI) m/z 432.04 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.57−1.63 (m, 2H), 1.80−1.85 (m, 2H), 1.92−2.00 (m, 2H), 3.74 (q, J = 5.22 Hz, 2H), 4.11 (t, J = 5.06 Hz, 2H), 4.23 (t, J = 5.06 Hz, 2H), 4.59 (t, J = 8.29 Hz, 2H), 4.88 (t, J = 5.37 Hz, 1H), 6.94 (s, 1H), 7.99 (s, 1H), 8.12 (s, 1H), 9.80 (s, 1H), 10.86 (s, 1H). Anal. ($C_{19}H_{20}ClN_5O_5$) C, H, N.

Compounds **16e**−**o** were prepared by synthetic procedures similar to that described for **16a**, substituting 3-bromopropanol with corresponding organic chlorides or bromides.

Macrocyclic Diarylurea 16e. MS (ESI) 446.01 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.57−1.63 (m, 2H), 1.80−1.85 (m, 2H), 1.92−1.99 (m, 2H), 3.34 (s, 3H), 3.68−3.70 (m, 2H), 4.20−4.25 (m, 4H), 4.59 (d, J = 7.98 Hz, 2H), 6.93 (s, 1H), 7.99 (s, 1H), 8.13 (s, 1H), 9.80 (s, 1H), 10.86 (s, 1H). Anal. ($C_{20}H_{22}ClN_5O_5 \cdot 0.1H_2O$) C, H, N.

Macrocyclic Diarylurea 16f. MS (ESI) 490.07 $(M - H)^-$, 492.07 $(M + H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.57−1.63 (m, 2H), 1.80−1.85 (m, 2H), 1.92−1.99 (m, 2H), 3.25 (s, 3H), 3.45−3.48 (m, 2H), 3.61−3.63 (m, 2H), 3.76 (d, J = 4.60 Hz, 2H), 4.21−4.25 (m, 4H), 4.60 (t, J = 8.29 Hz, 2H), 6.95 (s, 1H), 7.99 (s, 1H), 8.13 (s, 1H), 9.80 (s, 1H), 10.86 (s, 1H). Anal. ($C_{22}H_{26}ClN_5O_6 \cdot 0.5H_2O \cdot 0.1$TFA) C, H, N.

Macrocyclic Diarylurea 16g. MS (ESI) 459.14 $(M - H)^-$, 461.07 $(M + H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.57−1.64 (m, 2H), 1.80−1.88 (m, 2H), 1.93−2.00 (m, 2H), 2.93 (s, 3H), 3.17 (s, 3H), 3.55−3.58 (m, 2H), 4.24−4.27 (m, 2H), 4.44 (t, J =

4.91 Hz, 2H), 4.60 (t, J = 8.29 Hz, 2H), 7.01 (s, 1H), 8.00 (s, 1H), 8.18 (s, 1H), 9.84 (s, 1H), 10.90 (s, 1H). Anal. ($C_{21}H_{25}ClN_6O_4$·0.1H_2O·TFA) C, H, N.

Macrocyclic Diarylurea 16h. MS (APCI) m/z 460.17 (M + H)$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.55−1.71 (m, 4H), 1.76−1.89 (m, 3H), 1.89−2.05 (m, 2H), 4.10 (t, J = 6.60 Hz, 2H), 4.23 (d, J = 4.60 Hz, 2H), 4.40−4.76 (m, 2H), 6.91 (s, 1H), 7.98 (s, 1H), 8.11 (s, 1H), 9.79 (s, 1H), 10.85 (s, 1H). Anal. ($C_{22}H_{26}ClN_5O_4$) C, H, N.

Macrocyclic Diarylurea 16i. MS (APCI) m/z 474.16 (M + H)$^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.91 (s, 3H), 0.93 (s, 3H), 1.31−1.39 (m, 2H), 1.57−1.67 (m, 3H), 1.71−1.79 (m, 2H), 1.80−1.89 (m, 2H), 1.93−2.03 (m, 2H), 4.08 (t, J = 6.41 Hz, 2H), 4.25 (t, J = 5.03 Hz, 2H), 4.49−4.74 (m, 2H), 6.91 (s, 1H), 8.00 (s, 1H), 8.14 (s, 1H), 9.80 (s, 1H), 10.90 (s, 1H). HRMS (ESI-TOF) calcd for $C_{23}H_{29}ClN_5O_4$ (M + H)$^+$ 474.1908; found 474.1908.

Macrocyclic Diarylurea 16j. MS (APCI) m/z 417.98 (M + H)$^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.35 (t, J = 7.02 Hz, 3H), 1.53−1.66 (m, 2H), 1.79−1.86 (m, 2H), 1.91−2.00 (m, 2H), 4.14 (q, J = 6.92 Hz, 2H), 4.23 (t, J = 5.03 Hz, 2H), 4.50−4.65 (m, 2H), 6.89 (s, 1H), 7.98 (s, 1H), 8.12 (s, 1H), 9.79 (s, 1H), 10.88 (s, 1H).

Macrocyclic Diarylurea 16k. MS (ESI) 429.97 (M − H)$^-$, 432.00 (M + H)$^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.28 (d, J = 6.10 Hz, 6H), 1.56−1.62 (m, 2H), 1.79−1.84 (m, 2H), 1.92−1.99 (m, 2H), 4.22 (t, J = 5.19 Hz, 2H), 4.59 (t, J = 8.24 Hz, 2H), 4.66−4.71 (m, 1H), 6.92 (s, 1H), 7.99 (s, 1H), 8.12 (s, 1H), 9.80 (s, 1H), 10.89 (s, 1H). Anal. ($C_{20}H_{22}ClN_5O_5$) C, H, N.

Macrocyclic Diarylurea 16l. MS (ESI) 479.02 (M − H)$^-$, 481.05 (M + H)$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.55−1.62 (m, 2H), 1.78−1.84 (m, 2H), 1.91−1.99 (m, 2H), 4.20−4.23 (m, 2H), 4.57−4.61 (m, 2H), 5.41 (s, 2H), 7.03 (s, 1H), 7.66 (d, J = 6.14 Hz, 2H), 7.99 (s, 1H), 8.18 (s, 1H), 8.72 (d, J = 6.44 Hz, 2H), 9.81 (s, 1H), 10.88 (s, 1H). Anal. ($C_{23}H_{21}ClN_6O_4$·0.6TFA) C, H, N.

Macrocyclic Diarylurea 16m. MS (ESI) 481.05 (M − H)$^-$, 479.07 (M + H)$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.56−1.63 (m, 2H), 1.79−1.85 (m, 2H), 1.91−2.00 (m, 2H), 4.24 (t, J = 5.06 Hz, 2H), 4.59 (t, J = 7.67 Hz, 2H), 5.29 (s, 2H), 7.08 (s, 1H), 7.45 (dd, J = 7.83, 4.14 Hz, 1H), 7.88 (m, 1H), 7.97 (s, 1H), 8.15 (s, 1H), 8.56 (dd, J = 4.76, 1.69 Hz, 1H), 8.69 (d, J = 2.15 Hz, 1H), 9.81 (s, 1H), 10.86 (s, 1H). Anal. ($C_{23}H_{21}ClN_6O_4$·0.7TFA) C, H, N.

Macrocyclic Diarylurea 16n. MS (ESI) 481.03 (M − H)$^-$, 479.03 (M + H)$^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.59−1.64 (m, 2H), 1.83−1.87 (m, 2H), 1.96−2.02 (m, 2H), 4.26 (t, J = 5.19 Hz, 2H), 4.63 (t, J = 8.24 Hz, 2H), 5.36 (s, 2H), 7.12 (s, 1H), 7.42 (dd, J = 7.17, 5.03 Hz, 1H), 7.64 (d, J = 7.63 Hz, 1H), 7.92−7.95 (m, 1H), 8.03 (s, 1H), 8.20 (s, 1H), 8.64 (d, J = 4.58 Hz, 1H), 9.85 (s, 1H), 10.94 (s, 1H). Anal. ($C_{23}H_{21}ClN_6O_4$·0.6TFA) C, H, N.

Macrocyclic Diarylurea 16o. MS (ESI) 482.03 (M − H)$^-$, 484.00 (M + H)$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.53−1.65 (m, 2H), 1.79−1.87 (m, 2H), 1.90−2.00 (m, 2H), 4.23 (t, J = 4.60 Hz, 2H), 4.47 (t, J = 5.06 Hz, 2H), 4.57−4.64 (m, 4H), 6.94 (s, 1H), 7.61 (s, 1H), 7.73 (s, 1H), 7.99 (s, 1H), 8.12 (s, 1H), 8.96 (s, 1H), 9.81 (s, 1H), 10.88 (s, 1H). Anal. ($C_{22}H_{22}ClN_7O_4$·0.1H_2O·0.8TFA) C, H, N.

Macrocyclic Diarylurea 17a. The desired product (7.7 mg, 25%) was prepared by a procedure similar to that described for **17g**. MS (APCI) m/z 403.24 (M + H)$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.54−1.62 (m, 2H), 1.78−1.85 (m, 2H), 1.92−2.00 (m, 2H), 2.78 (s, 3H), 4.21 (t, J = 5.22 Hz, 2H), 4.58 (t, J = 8.29 Hz, 2H), 5.33 (br s, 1H), 6.35 (s, 1H), 7.94 (s, 1H), 7.97 (s, 1H), 9.69 (s, 1H), 10.77 (s, 1H).

Macrocyclic Diarylurea 17b. The desired product (9.6 mg, 30%) was prepared by a procedure similar to that described for **17g** by replacing 3-pyridinecarboxaldehyde with acetylaldehyde. Analytical LC/MS with two different solvent systems indicated >95% purity. MS (APCI) m/z 417.29 (M + H)$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.17 (t, J = 7.06 Hz, 3H), 1.54−1.60 (m, 2H), 1.76−1.83 (m, 2H), 1.90−1.98 (m, 2H), 3.14−3.22 (m, 2H), 4.18 (t, J = 5.06 Hz, 2H), 4.56 (t, J = 8.29 Hz, 2H), 5.02 (t, J = 5.68 Hz, 1H), 6.39 (s, 1H), 7.93 (s, 1H), 7.96 (s, 1H), 9.66 (s, 1H), 10.74 (s, 1H). HRMS (ESI-TOF) calcd for $C_{19}H_{22}ClN_6O_3$ (M + H)$^+$ 417.1442; found 417.1441.

Macrocyclic Diarylurea 17c. The desired product was prepared by a procedure similar to that described for **17g** by replacing 3-pyridinecarboxaldehyde with butyraldehyde. Analytical LC/MS with two different solvent systems indicated > 95% purity. MS (APCI) m/z 445.64 (M + H)$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.92 (t, J = 7.36 Hz, 3H), 1.31−1.42 (m, 2H), 1.51−1.63 (m, 4H), 1.78−1.84 (m, 2H), 1.92−2.00 (m, 2H), 3.15 (q, J = 6.44 Hz, 2H), 4.19 (t, J = 5.22 Hz, 2H), 4.58 (t, J = 7.67 Hz, 2H), 5.01 (t, J = 5.83 Hz, 1H), 6.40 (s, 1H), 7.94 (s, 1H), 7.97 (s, 1H), 9.68 (s, 1H), 10.77 (s, 1H). HRMS (ESI-TOF) calcd for $C_{21}H_{26}ClN_6O_3$ (M + H)$^+$ 445.1755; found 445.1753.

Macrocyclic Diarylurea 17d. The desired product was prepared by a procedure similar to that described for **17g**. Analytical LC/MS with two different solvent systems indicated >95% purity. MS (APCI) m/z 433.34 (M + H)$^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.52−1.64 (m, 2H), 1.76−1.85 (m, 2H), 1.90−2.01 (m, 2H), 3.22 (t, J = 5.80 Hz, 2H), 3.60 (t, J = 5.65 Hz, 2H), 4.19 (t, J = 5.19 Hz, 2H), 4.45−4.70 (m, 2H), 5.04 (br s, 1H), 6.47 (s, 1H), 7.95 (s, 1H), 7.97 (s, 1H), 9.68 (s, 1H), 10.79 (s, 1H).

Macrocyclic Diarylurea 17e. The desired product was prepared by a procedure similar to that described for **17g**. Analytical LC/MS with two different solvent systems indicated >95% purity. MS (APCI) m/z 431.26 (M + H)$^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.19 (d, J = 6.10 Hz, 6H), 1.52−1.62 (m, 2H), 1.73−1.86 (m, 2H), 1.90−2.02 (m, 2H), 3.70−3.81 (m, 1H), 4.19 (t, J = 5.03 Hz, 2H), 4.52−4.62 (m, 2H), 6.45 (s, 1H), 7.94 (s, 1H), 7.97 (s, 1H), 9.68 (s, 1H), 10.79 (s, 1H).

Macrocyclic Diarylurea 17f. The desired product was prepared by a procedure similar to that described for **17g** by replacing 3-pyridinecarboxaldehyde with isobutylaldehyde. MS (APCI) m/z 445.42 (M + H)$^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.92 (d, J = 6.41 Hz, 6H), 1.54−1.61 (m, 2H), 1.78−1.83 (m, 2H), 1.87−1.99 (m, 3H), 2.99 (t, J = 6.56 Hz, 2H), 4.18 (t, J = 5.19 Hz, 2H), 4.57 (t, J = 8.24 Hz, 2H), 5.08 (t, J = 5.95 Hz, 1H), 6.39 (s, 1H), 7.93 (s, 1H), 7.97 (s, 1H), 9.68 (s, 1H), 10.78 (s, 1H).

Macrocyclic Diarylurea 17g. The desired product was prepared by a procedure similar to that described for **17g**. To a solution of **5f** (20 mg, 0.0514 mmol) in THF (3 mL) at 0 °C was added a fresh mixture (mixture A) of 3-pyridinecarboxaldehyde (0.022 mL), 3 M H_2SO_4 (0.203 mL), and methanol (1 mL), followed by the addition of $NaBH_4$ (4 mg, 0.11 mmol). The reaction mixture was stirred at 0 °C for 30 min and monitored by LC/MS. The addition of mixture A and $NaBH_4$ was repeated until the reaction was complete. Saturated $NaHCO_3$ was added to adjust the pH > 7. The precipitates were collected, washed with water thoroughly, and purified by reverse-phase HPLC, eluted with a gradient of 0−70% of acetonitrile in 0.1% TFA aqueous solution. The desired product was obtained in 90% yield. MS (DCI/NH_3) m/z 480.11 (M + H)$^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.45−1.50 (m, 2H), 1.64−1.69 (m, 2H), 1.86−1.92 (m, 2H), 4.01 (t, J = 5.30 Hz, 2H), 4.47 (d, J = 6.24 Hz, 2H), 4.50 (t, J = 8.11 Hz, 2H), 6.01 (t, J = 6.40 Hz, 1H), 6.33 (s, 1H), 7.33 (dd, J = 7.49, 4.37 Hz, 1H), 7.75−7.77 (m, 1H), 7.84 (s, 1H), 7.97 (s, 1H), 8.43 (dd, J = 4.68, 1.56 Hz, 1H), 8.61 (d, J = 1.87 Hz, 1H), 9.60 (s, 1H), 10.75 (s, 1H). Anal. ($C_{23}H_{22}ClN_7O_3$·0.1H_2O·0.9TFA) C, H, N.

Macrocyclic Diarylurea 17h. The desired product was prepared by a procedure similar to that described for **17g** by substituting 3-pyridinecarboxaldehyde with 4-pyridinecarboxaldehyde. MS (ESI) m/z 480.12 (M + H)$^+$, 478.15(M − H)$^-$. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.42−1.53 (m, 2H), 1.58−1.70 (m, 2H), 1.84−1.95 (m, 2H), 3.96 (t, J = 4.75 Hz, 2H), 4.53 (t, J = 8.81 Hz, 2H), 4.60 (d, J = 4.07 Hz, 2H), 6.23 (s, 1H), 6.28 (br s, 1H), 7.60 (s, 1H), 7.61 (s, 1H), 7.96 (s, 2H), 8.62 (s, 1H), 8.64 (s, 1H), 9.63 (s, 1H), 10.79 (s, 1H). Anal. ($C_{23}H_{22}ClN_7O_3$·0.1H_2O·TFA) C, H, N.

Macrocyclic Diarylurea 17i. The desired product was prepared by a procedure similar to that described for **17g**. MS (APCI) m/z 478.83 (M + H)$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.38−1.57

(m, 2H), 1.58−1.73 (m, 2H), 1.81−1.97 (m, 2H), 3.97 (t, J = 5.06 Hz, 2H), 4.43 (d, J = 5.83 Hz, 2H), 4.48−4.59 (m, 2H), 5.99 (t, J = 5.98 Hz, 1H), 6.30 (s, 1H), 7.22 (t, J = 7.06 Hz, 1H), 7.32 (t, J = 7.67 Hz, 2H), 7.35−7.41 (m, 2H), 7.92 (s, 1H), 7.96 (s, 1H), 9.62 (s, 1H), 10.76 (s, 1H). Anal. ($C_{24}H_{23}ClN_6O_3 \cdot 0.1H_2O \cdot 0.1TFA$) C, H, N.

Macrocyclic Diarylurea 17j. The desired product was prepared by a procedure similar to that described for **17g**. MS (APCI) *m*/*z* 468.87 $(M + H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.48−1.62 (m, 2H), 1.70−1.83 (m, 2H), 1.87−2.02 (m, 2H), 4.13 (t, J = 5.03 Hz, 2H), 4.50 (d, J = 4.58 Hz, 2H), 4.53−4.61 (m, 2H), 5.83 (s, 1H), 6.47 (s, 1H), 7.58 (s, 1H), 7.97 (s, 2H), 8.93 (s, 1H), 9.68 (s, 1H), 10.81 (s, 1H), 14.21 (br s, 2H). Anal. ($C_{21}H_{21}ClN_8O_3 \cdot 0.2H_2O \cdot$ TFA) C, H, N.

Macrocyclic Diarylurea 17k. The desired product was prepared by a procedure similar to that described for **17g**. MS (APCI) *m*/*z* 485.97 $(M + H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.36−1.60 (m, 2H), 1.63−1.81 (m, 2H), 1.84−2.04 (m, 2H), 4.10 (t, J = 5.19 Hz, 2H), 4.50−4.60 (m, 2H), 4.69 (s, 2H), 6.06 (br s, 1H), 6.51 (s, 1H), 7.89 (s, 1H), 7.93 (s, 1H), 7.96 (s, 1H), 8.94 (s, 1H), 9.66 (s, 1H), 10.79 (s, 1H). Anal. ($C_{21}H_{20}ClN_7O_3S \cdot 0.4H_2O \cdot 0.1TFA$) C, H, N.

Macrocyclic Diarylurea 17l. The desired product was prepared by a procedure similar to that described for **17g**. MS (APCI) *m*/*z* 470.1 $(M + H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.49−1.61 (m, 2H), 1.71−1.85 (m, 2H), 1.86−2.04 (m, 2H), 4.11−4.21 (m, 2H), 4.52 (d, J = 6.41 Hz, 2H), 4.54−4.60 (m, 2H), 5.87 (t, J = 6.10 Hz, 1H), 6.54 (s, 1H), 7.09 (s, 1H), 7.94 (s, 1H), 7.97 (s, 1H), 8.27 (s, 1H), 9.67 (s, 1H), 10.79 (s, 1H). Anal. ($C_{21}H_{20}ClN_7O_4 \cdot 0.5H_2O$) C, H, N.

Macrocyclic Diarylurea 17m. The desired product was prepared by a procedure similar to that described for **17g**. Analytical LC/MS with two different solvent systems indicated >95% purity. MS (APCI) *m*/*z* 485.22 $(M + H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.44−1.58 (m, 2H), 1.63−1.77 (m, 2H), 1.85−1.98 (m, 2H), 4.05 (t, J = 5.19 Hz, 2H), 4.40 (d, J = 6.10 Hz, 2H), 4.49−4.63 (m, 2H), 5.86 (t, J = 6.10 Hz, 1H), 6.41 (s, 1H), 7.11 (d, J = 4.88 Hz, 1H), 7.39 (d, J = 2.14 Hz, 1H), 7.45−7.49 (m, 2H), 7.91 (s, 1H), 7.96 (s, 1H), 9.63 (s, 1H), 10.78 (s, 1H). HRMS (ESI-TOF) calcd for $C_{22}H_{22}ClN_6O_3S$ $(M + H)^+$ 485.1163; found 485.1160.

Macrocyclic Diarylurea 17n. The desired product was prepared by a procedure similar to that described for **17g**. Analytical LC/MS with two different solvent systems indicated >98% purity. MS (APCI) *m*/*z* 483.28 $(M + H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.52−1.61 (m, 2H), 1.73−1.82 (m, 2H), 1.86−2.02 (m, 2H), 3.87 (s, 3H), 4.16 (t, J = 5.06 Hz, 2H), 4.48−4.66 (m, 4H), 5.95 (br s, 1H), 6.51 (s, 1H), 7.61 (s, 1H), 7.98 (s, 1H), 7.98 (s, 1H), 9.00 (s, 1H), 9.69 (s, 1H), 10.79 (s, 1H), 14.12 (br s, 1H).

Macrocyclic Diarylurea 18a. To a mixture of **5f** (10 mg, 0.026 mmol) and pyridine (1 mL) in dichloromethane (10 mL) was added acetyl chloride (0.011 mL, 0.154 mmol) dropwise. The reaction mixture was stirred for 3 h, and methanol (1 mL) was added to quench the reaction. The resulting mixture was concentrated, and the residue was triturated with a mixture of dichloromethane and methanol to provide the desired product (9.8 mg, 89%) as an off-white solid. MS (DCI/NH_3) *m*/*z* 430.96 $(M + H)^+$, 429.01$(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.58−1.64 (m, 2H), 1.78−1.84 (m, 2H), 1.90−1.99 (m, 2H), 2.08 (s, 3H), 4.11−4.14 (m, 2H), 4.60 (t, J = 8.29 Hz, 2H), 7.50 (s, 1H), 7.99 (s, 1H), 8.22 (s, 1H), 9.45 (s, 1H), 9.91 (s, 1H), 10.91 (s, 1H). Anal. ($C_{19}H_{19}ClN_6O_4$) C, H, N.

Macrocyclic Diarylurea 18b. The desired product was prepared by a procedure similar to that described for **18a** by substituting acetyl chloride with 2-dimethylacetyl chloride. Analytical LC/MS with two different solvent systems indicated >95% purity. MS (ESI) *m*/*z* 474.10 $(M + H)^+$, 472.02 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.60−1.66 (m, 2H), 1.80−1.87 (m, 2H), 1.93−2.00 (m, 2H), 2.87 (s, 6H), 4.15−4.17 (m, 4H), 4.62 (t, J = 7.98 Hz, 2H), 7.47 (s, 1H), 8.01 (s, 1H), 8.29 (s, 1H), 9.81 (s, 1H), 9.96 (s, 1H), 10.24 (s, 1H), 10.97 (s, 1H). HRMS (ESI-TOF) calcd for $C_{21}H_{25}ClN_7O_4$ $(M + H)^+$ 474.1657; found 474.1661.

Macrocyclic Diarylurea 18c. To a mixture of **5f** (50 mg, 0.13 mmol) and pyridine (5 mL) in dichloromethane (25 mL) was added 3-chloropropionyl chloride (0.037 mL, 0.39 mmol) dropwise at 0 °C. The reaction mixture was stirred at 0 °C until **5f** was consumed completely. Careful removal of solvent at low temperature (<10 °C) provided a residue. To this residue was added cyclopentylamine (3 mL), and the mixture was heated at 80 °C for 1 h and concentrated. The residue was purified by reverse-phase HPLC, eluted with a gradient of 0−70% acetonitrile in 0.1% TFA aqueous solution to provide the desired product (TFA salt, 66.8 mg, 80% for two steps). Analytical LC/MS with two different solvent systems indicated >95% purity. MS (ESI) *m*/*z* 528.07 $(M + H)^+$, 526.16 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.50−1.66 (m, 8H), 1.66−1.74 (m, 2H), 1.79−1.87 (m, 2H), 1.92−2.03 (m, 4H), 2.82−2.85 (m, 2H), 3.21 (m, 1H), 3.52 (m, 1H), 4.13 (t, J = 4.30 Hz, 2H), 4.61 (t, J = 7.98 Hz, 2H), 7.53 (s, 1H), 8.01 (s, 1H), 8.26 (s, 1H), 8.40 (br s, 1H), 9.76 (s, 1H), 9.93 (s, 1H), 10.94 (s, 1H). HRMS (ESI-TOF) calcd for $C_{25}H_{31}ClN_7O_4$ $(M + H)^+$ 528.2126; found 528.2130.

Macrocyclic Diarylurea 18d. To a solution of **5f** (20 mg, 0.051 mmol) and pyridine (3 mL) in dichloromethane (10 mL) was added methanesulfonyl chloride (0.016 mL, 0.24 mmol) at 0 °C. The reaction mixture was stirred for 6 h, and saturated $NaHCO_3$ was added to adjust pH value to 9. The precipitate was collected by filtration, washed with water thoroughly, and recrystallized from methanol to provide the desired product (20.2 mg, 85%). MS (DCI/NH_3) *m*/*z* 467 $(M + H)^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.66−1.73 (m, 2H), 1.87−1.93 (m, 2H), 1.98−2.06 (m, 2H), 3.10 (s, 3H), 4.24 (t, J = 5.22 Hz, 2H), 4.68 (t, J = 7.98 Hz, 2H), 7.19 (s, 1H), 8.07 (s, 1H), 8.35 (s, 1H), 9.48 (s, 1H), 10.01 (s, 1H), 11.02 (s, 1H). HRMS (ESI-TOF) calcd for $C_{18}H_{18}ClN_6O_5S$ $(M - H)^-$ 465.0753; found 465.0753. Anal. ($C_{18}H_{19}ClN_6O_5S$) C, H, N.

Macrocyclic Diarylurea 19a. To a solution of **5f** (36 mg, 0.0925 mmol) in THF (6 mL) at 0 °C was added a fresh mixture of formaldehyde (0.0345 mL, 0.463 mmol), 3 M H_2SO_4 (0.05 mL), and THF (0.5 mL), followed by the addition of $NaBH_4$ (11 mg, 0.28 mmol). The reaction mixture was stirred at 0 °C for 3 h. Saturated $NaHCO_3$ was added to adjust the pH > 7. The precipitates were collected, washed with water thoroughly, and purified by reverse-phase HPLC, eluted with a gradient of 0−70% acetonitrile in 0.1% TFA aqueous solution. The desired product (33 mg, 86%) was obtained as a yellow solid. Analytical LC/MS with two different solvent systems indicated >95% purity. MS (ESI) *m*/*z* 415.35 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.34−1.41 (m, 2H), 1.55−1.61 (m, 2H), 1.66−1.76 (m, 2H), 2.49 (s, 6H), 3.97 (t, J = 5.06 Hz, 2H), 4.37 (t, J = 7.98 Hz, 2H), 6.62 (s, 1H), 7.74 (s, 1H), 7.88 (s, 1H), 9.59 (s, 1H), 10.62 (s, 1H). HRMS (ESI-TOF) calcd for $C_{19}H_{22}ClN_6O_3$ $(M + H)^+$ 417.1442; found 417.1442.

Macrocyclic Diarylurea 19b. To a solution of **5f** (20 mg, 0.051 mmol) in THF (5 mL) at 0 °C was added a fresh mixture of acetylaldehyde (0.014 mL, 0.255 mmol), 3 M H_2SO_4 (0.034 mL), and THF (0.5 mL), followed by the addition of $NaBH_4$ (7.7 mg, 0.20 mmol). The reaction mixture was stirred at 0 °C for 1 h. Saturated $NaHCO_3$ was added to adjust the pH > 7. The mixture was concentrated, and the precipitates were collected, washed with water thoroughly, and dried to provide the desired product (22 mg, 97%) as a yellow solid. Analytical LC/MS with two different solvent systems indicated >95% purity. MS (DCI/NH_3) *m*/*z* 445.16 $(M + H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.96 (t, J = 7.02 Hz, 6H), 1.57−1.64 (m, 2H), 1.79−1.84 (m, 2H), 1.92−1.99 (m, 2H), 3.06 (q, J = 7.02 Hz, 4H), 4.20 (t, J = 4.88 Hz, 2H), 4.61 (t, J = 7.93 Hz, 2H), 6.89 (s, 1H), 7.98 (s, 1H), 8.15 (s, 1H), 9.83 (s, 1H), 10.90 (s, 1H). HRMS (ESI-TOF) calcd for $C_{21}H_{26}ClN_6O_3$ $(M + H)^+$ 445.1755; found 445.1756.

Macrocyclic Diarylurea 19c. MS (APCI) *m*/*z* 477.27 $(M + H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.53−1.65 (m, 2H), 1.72−1.89 (m, 2H), 1.90−2.05 (m, 2H), 3.20 (t, J = 6.56 Hz, 4H), 3.40−3.58 (m, 4H), 4.19 (t, J = 5.19 Hz, 2H), 4.53−4.65 (m, 2H), 7.04 (s, 1H), 7.99 (s, 1H), 8.12 (s, 1H), 9.84 (s, 1H), 10.90 (s, 1H). HRMS (ESI-TOF) calcd for $C_{21}H_{26}ClN_6O_5$ $(M + H)^+$ 477.1653; found 477.1656.

Macrocyclic Diarylurea 19d. The desired product (22 mg, 94%) was prepared by a procedure similar to that described for **19b** by replacing acetylaldehyde with glutaraldehyde. MS (DCI/NH_3) *m*/*z* 457.18 $(M + H)^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.50−1.56 (m, 2H), 1.58−1.63 (m, 2H), 1.63−1.68 (m, 4H), 1.79−1.84 (m, 2H), 1.92−1.98 (m, 2H), 2.92 (t, J = 4.68 Hz, 4H), 4.21 (t, J = 4.99 Hz, 2H), 4.61 (t, J = 8.11 Hz, 2H), 6.82 (s, 1H), 7.98 (s, 1H), 8.13 (s, 1H), 9.82 (s, 1H), 10.86 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 22.8, 23.7, 25.6, 25.8, 26.8, 28.8, 52.3, 68.4, 69.5, 104.8, 106.9, 115.4, 118.1, 121.7, 122.5, 128.2, 146.2, 147.5, 149.3, 150.6, 160.3. HRMS (ESI-TOF) calcd for $C_{22}H_{26}ClN_6O_3$ $(M + H)^+$ 457.1755; found 457.1754.

Macrocyclic Diarylurea 20. To a mixture of **15** (100 mg, 0.26 mmol) and triethylamine (0.039 mL, 0.28 mmol) in DMF (5 mL) at 0 °C was added trifluoromethanesulfonyl chloride (0.03 mL, 0.28 mmol). The reaction mixture was stirred at 0 °C for 30 min, and ice−water was added to quench the reaction. The resulting mixture was concentrated, and the residue was purified by flash chromatography, eluted with 9% ethyl acetate in dichloromethane. The desired product (110 mg, 82%) was obtained as a white solid. MS (ESI) 520.58 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.61−1.67 (m, 2H), 1.81−1.87 (m, 2H), 1.92−1.99 (m, 2H), 4.24 (t, J = 5.22 Hz, 2H), 4.61 (t, J = 7.98 Hz, 2H), 7.38 (s, 1H), 8.01 (s, 1H), 8.46 (s, 1H), 10.03 (s, 1H), 11.06 (s, 1H).

Macrocyclic Diarylurea 21e. To a mixture of **20** (270 mg, 0.52 mmol), 2-prop-2-ynloxytetrahydropyran (0.436 mL, 3.10 mmol), triethylamine (0.217 mL, 1.56 mmol), and $(PPh_3)_4Pd$ (180 mg, 0.156 mmol) in DMF was added CuI (20 mg, 0.10 mmol), followed by the addition of *n*-Bu_4NI (288 mg, 0.78 mmol). The reaction mixture was heated at 70 °C overnight, cooled, and concentrated. The residue was purified by flash chromatography, eluted with 9% ethyl acetate in dichloromethane, to provide the desired product (160 mg, 60%) as an off-white solid. MS (ESI) *m*/*z* 510.02 $(M - H)^-$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.44−1.55 (m, 4H), 1.59−1.65 (m, 2H), 1.66−1.76 (m, 2H), 1.78−1.83 (m, 2H), 1.90−1.96 (m, 2H), 3.48 (m, 1H), 3.76 (m, 1H), 4.21 (t, J = 4.99 Hz, 2H), 4.43−4.54 (m, 2H), 4.60 (d, J = 8.11 Hz, 2H), 4.85 (t, J = 2.96 Hz, 1H), 7.26 (s, 1H), 7.98 (s, 1H), 8.31 (s, 1H), 9.99 (s, 1H), 10.98 (s, 1H).

Macrocyclic Diarylurea 21a. A mixture of **21e** (6 mg, 0.014 mmol), HOAc (2 mL), THF (1 mL), and water (0.5 mL) was heated at 45 °C for 3 h and concentrated. The residue was suspended in methanol, and the precipitates were collected by filtration. The desired product was obtained as a white solid in quantitative yield. MS (ESI) *m*/*z* 425.95 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.54−1.67 (m, 2H), 1.74−1.83 (m, 2H), 1.85−1.98 (m, 2H), 4.19 (br s, 2H), 4.35 (d, J = 5.83 Hz, 2H), 4.58 (br s, 2H), 5.37 (t, J = 5.98 Hz, 1H), 7.26 (s, 1H), 7.94 (s, 1H), 8.31 (s, 1H), 10.01 (s, 1H), 10.99 (s, 1H). Anal. ($C_{20}H_{18}ClN_5O_4$) C, H, N.

Macrocyclic Diarylurea 21b. The desired product (25 mg, 60%) was prepared by a procedure similar to that described for **21e** by substituting 2-prop-2-ynloxytetrahydropyran with but-3-yn-2-ol. Analytical LC/MS with two different solvent systems indicated >95% purity. MS (ESI) *m*/*z* 439.97 $(M - H)^-$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.41 (d, J = 6.71 Hz, 3H), 1.60−1.66 (m, 2H), 1.79−1.84 (m, 2H), 1.91−1.97 (m, 2H), 4.21 (t, J = 5.19 Hz, 2H), 4.59−4.66 (m, 3H), 5.51 (d, J = 5.19 Hz, 1H), 7.21 (s, 1H), 8.00 (s, 1H), 8.31 (s, 1H), 10.00 (s, 1H), 11.01 (s, 1H). HRMS (ESI-TOF) calcd for $C_{21}H_{21}ClN_5O_4$ $(M + H)^+$ 442.1282; found 442.1283.

Macrocyclic Diarylurea 21c. The desired product (22 mg, 50%) was prepared by a procedure similar to that described for **21e** by substituting 2-prop-2-ynloxytetrahydropyran with 2-methyl-but-3-yn-2-ol. Analytical LC/MS with two different solvent systems indicated >95% purity. MS (ESI) *m*/*z* 454.03 $(M - H)^-$. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.49 (s, 6H), 1.60−1.66 (m, 2H), 1.79−1.84 (m, 2H), 1.91−1.97 (m, 2H), 4.21 (t, J = 4.88 Hz, 2H), 4.61 (t, J = 7.93 Hz, 2H), 5.50 (s, 1H), 7.17 (s, 1H), 8.00 (s, 1H), 8.31 (s, 1H), 9.99 (s, 1H), 11.01 (s, 1H). HRMS (ESI-TOF) calcd for $C_{22}H_{23}ClN_5O_4$ $(M + H)^+$ 456.1439; found 350.1442.

Macrocyclic Diarylurea 21d. The desired product (66% yield) was prepared by a procedure similar to that described for **21e** by substituting 2-prop-2-ynloxytetrahydropyran with diethylamino-2-propyne. Analytical LC/MS with two different solvent systems indicated >95% purity. MS (ESI) *m*/*z* 482.50 $(M + H)^+$, 481.1 $(M - H)^-$. ^{1}H NMR (400 MHz, DMF-d_7) δ 1.40 (t, J = 7.21 Hz, 5H), 1.72−1.79 (m, 2H), 1.90−1.96 (m, 2H), 2.00−2.09 (m, 2H), 3.48 (q, J = 7.21 Hz, 4H), 4.31 (t, J = 4.91 Hz, 2H), 4.53 (s, 2H), 4.75 (t, J = 7.98 Hz, 2H), 7.44 (s, 1H), 8.18 (s, 1H), 8.50 (s, 1H), 10.16 (s, 1H), 11.00 (s, 1H). HRMS (ESI-TOF) calcd for $C_{24}H_{28}ClN_6O_3$ $(M + H)^+$ 483.1911; found 483.1925.

Macrocyclic Diarylurea 22. A mixture of **21e** (45 mg, 0.088 mmol) and Pt/C (5%, 20 mg) in THF (5 mL) was stirred under hydrogen atmosphere (40 psi) for 16 h. The insoluble materials were filtered off, and the filtrate was concentrated. The residue was purified by flash chromatography, eluted with 9% ethyl acetate in dichloromethane, to provide the THP-protected **22** (36 mg, 80%). MS (ESI) *m*/*z* 513.93 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.38−1.53 (m, 4H), 1.56−1.65 (m, 3H), 1.71 (m, 1H), 1.77−1.86 (m, 4H), 1.88−1.98 (m, 2H), 2.65−2.77 (m, 2H), 3.31−3.45 (m, 2H), 3.62−3.78 (m, 2H), 4.17−4.21 (m, 2H), 4.54 (m, 1H), 4.60 (t, J = 8.29 Hz, 2H), 7.08 (s, 1H), 7.97 (s, 1H), 8.17 (s, 1H), 9.88 (s, 1H), 10.87 (s, 1H). A mixture of this intermediate (10 mg, 0.019 mmol), HOAc (4 mL), THF (2 mL), and water (1 mL) was heated at 45 °C overnight and concentrated. The residue was purified by reverse-phase HPLC to provide **22** (7 mg, 85%). MS (ESI) *m*/*z* 430.07 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.59−1.65 (m, 2H), 1.67−1.75 (m, 2H), 1.79−1.86 (m, 2H), 1.91−1.99 (m, 2H), 2.68 (t, J = 7.98 Hz, 2H), 3.42−3.47 (m, 2H), 4.19 (t, J = 4.91 Hz, 2H), 4.50 (t, J = 5.22 Hz, 1H), 4.61 (t, J = 7.98 Hz, 2H), 7.07 (s, 1H), 7.99 (s, 1H), 8.16 (s, 1H), 9.89 (s, 1H), 10.90 (s, 1H). Anal. ($C_{20}H_{22}ClN_5O_4$) C, H, N.

Macrocyclic Diarylurea 23. To a mixture of **20** (50 mg, 0.096 mmol), $PdCl_2(PPh_3)_2$ (8.1 mg, 0.0115 mmol), Ph_3P (15.1 mg, 0.058 mmol), and LiCl (32.6 mg, 0.77 mmol) in DMF (2 mL) was added tributylallyltin (0.059 mL, 0.192 mmol). The reaction mixture was heated at 110 °C for 1 h and cooled. Saturated potassium fluoride aqueous solution (1 mL) was added. The resulting mixture was stirred for 30 min and concentrated. The residue was purified by flash chromatography, eluted with 9% ethyl acetate in dichloromethane, to provide the desired product (26 mg, 65%) as an off-white solid. MS (ESI) *m*/*z* 411.98 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.56−1.65 (m, 2H), 1.75−1.84 (m, 2H), 1.90−1.98 (m, 2H), 3.42 (d, J = 6.14 Hz, 2H), 4.16 (t, J = 4.30 Hz, 2H), 4.60 (t, J = 7.98 Hz, 2H), 5.05−5.09 (m, 2H), 5.83−6.01 (m, 1H), 7.07 (s, 1H), 7.98 (s, 1H), 8.19 (s, 1H), 9.90 (s, 1H), 10.90 (s, 1H).

Macrocyclic Diarylurea 24. A mixture of **23** (10 mg, 0.024 mmol), THF (5 mL), and water (0.55 mL) was treated with *N*-methylmorpholine *N*-oxide (8.4 mg, 0.072 mmol), followed by the addition of 2.5% (wt %) OsO_4 in 2-methyl-2-propanol (0.04 mL) at 0 °C. The reaction mixture was stirred at room temperature overnight and concentrated. The residue was purified by HPLC, eluted with a gradient of 0−70% acetonitrile in 0.1% TFA aqueous solution. The desired product (9.3 mg, 85%) was obtained as a white solid. Analytical LC/MS with two different solvent systems indicated >95% purity. MS (ESI) *m*/*z* 445.99 $(M - H)^-$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.56−1.66 (m, 2H), 1.78−1.86 (m, 2H), 1.90−2.00 (m, 2H), 2.54−2.62 (m, 2H), 2.89 (dd, J = 13.96, 4.76 Hz, 2H), 3.70 (m, 1H), 4.17 (t, J = 4.91 Hz, 2H), 4.61 (t, J = 8.29 Hz, 2H), 7.10 (s, 1H), 8.00 (s, 1H), 8.16 (s, 1H), 9.90 (s, 1H), 10.90 (s, 1H). HRMS (ESI-TOF) calcd for $C_{20}H_{23}ClN_5O_5$ $(M + H)^+$ 448.1388; found 448.1389.

Chk1 Enzymatic Inhibition Assay. The Chk1 enzymatic assay was carried out with recombinant Chk1 kinase domain protein covering amino acid residues 1−289 and a polyhistidine tag at the C-terminal end. Human Cdc25c peptide substrate contained a sequence of amino acid residues 204−225. The reaction mixture contained 25 mM Hepes at pH 7.4, 10 mM $MgCl_2$, 0.08 mM Triton X-100, 0.5 mM DTT, 5 μM ATP, 4 nM [^{33}P]-ATP, 5 μM Cdc25c peptide substrate, and 6.3 nM recombinant Chk1 protein. Compound vehicle DMSO was maintained at 2% in the final reaction. After 30 min at room temperature, the reaction was stopped by addition of an equal volume of 4 M NaCl and 0.1 M EDTA, pH 8. A 40 μL

aliquot of the reaction was added to a well in a Flash Plate (NEN Life Science Products, Boston, MA) containing 160 μL of phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride, and the plate was incubated at room temperature for 10 min. The plate was then washed three times in PBS containing 0.05% Tween-20 and counted in a Packard TopCount counter (Packard BioScience Company, Meriden, CT).

Kinase Selectivity Assays. Kinase assays were conducted in 24 μL volumes on 384-well microplates via Tecan liquid-handling automation. Ser/Thr-kinase assays were performed with a radioactive FlashPlate-based assay platform. In this format, biotinylated substrate peptide (2 μM), [γ-^{33}P]-ATP (5 μM, 2 mCi/μmol), Chk1 inhibitors (3−10 000 nM in 2% DMSO), and enzyme were incubated for 1 h in buffer containing 25 mM Hepes, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 100 μM Na_3VO_4, and 0.075 mg/mL Triton X-100. The reaction was stopped with 80 μL of stop buffer containing 100 mM EDTA and 4 M NaCl, and samples were transferred to streptavidin-coated 384-well FlashPlates (Perkin-Elmer, Boston, MA), which were then washed 3 times and read with a TopCount microplate reader (Perkin-Elmer). Tyrosine kinase reactions were performed with a time-resolved fluorescence (HTRF) platform. In this format, biotinylated substrate peptide (0.5 μM), ATP (10 μM−1 mM), inhibitors (3−10 000 nM in 2% DMSO), and enzyme were incubated for 1 h in buffer containing 50 mM Hepes, pH 7.4, 1 mM DTT, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 100μM Na_3VO_4, and 0.01% BSA. Reactions were stopped with 50 μL of revelation buffer containing (final concentrations) Eu-conjugated anti-pY-PT66 antibody (0.05 μg/mL) (CisBio), PycolLink streptavidin−allophycocyanin conjugate (0.001 μg/mL) (Prozyme), and 60 mM EDTA in a buffer containing 25 mM Hepes, pH 7.4, 250 mM KF, 0.005% Tween-20, and 0.05% BSA. Following a 60 min incubation with revelation buffer, the reactions were read with an Envision fluorescence microplate reader (Perkin-Elmer) with 615 nm excitation and 665 nm emission.

Cell Proliferation Assay (MTS Assay). p53-deficient cancer cells (HeLa or SW620 cells) were seeded in 96-well plates and treated with the indicated doses of CPT or Dox with or without the indicated doses of Chk1 inhibitor for 48 h. MTS reagents that measure the amount of live cells (Promega, Madison, WI) were then added to the cells, and the reactions were allowed to develop for 20 min−2 h. Colorimetric measurements were taken at 490 nm on a Spectra MAX 190 instrument from Molecular Devices (Sunnyvale, CA).

Cell Cycle Analysis. Cell cycle analysis was performed as described.[47] Briefly, after the indicated treatments, cells were washed one time in PBS and fixed in 70% ethanol. The fixed cells were washed twice with PBS and treated with RNase A at 37 °C for 30 min. The cells were then stained with propidium iodide and incubated in the dark for 60 min or overnight before analysis. The samples were analyzed through flow cytometry on a fluorescence-activated cell sorter manufactured by BD Bioscience (San Jose, CA) by use of the Cell-Quest program.

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