Substituted 4-(Thiazol-5-yl)-2-(phenylamino)pyrimidines Are Highly Active CDK9 Inhibitors: Synthesis, X-ray Crystal Structures, Structure—Activity Relationship, and Anticancer Activities

Hao Shao,† Shenhua Shi,† Shiliang Huang,† Alison J. Hole,§ Abdullahi Y. Abbas,‡ Sonja Baumli,§ Xiangrui Liu,† Frankie Lam,†‡ David W. Foley,† Peter M. Fischer,† Martin Noble,§∥ Jane A. Endicott,§∥ Chris Pepper,⊥ and Shudong Wang*†

†School of Pharmacy and Centre for Biomolecular Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, U.K.
‡School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA 5001, Australia
§Department of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K.
∥Northern Institute for Cancer Research, Medical School, Newcastle University, Newcastle upon Tyne NE2 4HH, U.K.
⊥Institute of Cancer and Genetics, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, U.K.

ABSTRACT: Cancer cells often have a high demand for antiapoptotic proteins in order to resist programmed cell death. CDK9 inhibition selectively targets survival proteins and reinstates apoptosis in cancer cells. We designed a series of 4-thiazol-2-anilinopyrimidine derivatives with functional groups attached to the C5-position of the pyrimidine or to the C4-thiazol moiety and investigated their effects on CDK9 potency and selectivity. One of the most selective compounds, 12u inhibits CDK9 with IC50 = 7 nM and shows over 80-fold selectivity for CDK9 versus CDK2. X-ray crystal structures of 12u bound to CDK9 and CDK2 provide insights into the binding modes. This work, together with crystal structures of selected inhibitors in complex with both enzymes described in a companion paper,34 provides a rationale for the observed SAR. 12u demonstrates potent anticancer activity against primary chronic lymphocytic leukemia cells with a therapeutic window 31- and 107-fold over those of normal B- and T-cells.

INTRODUCTION

Cyclin-dependent kinases (CDKs) can generally be classified into two main groups based on whether their primary role is in the control of cell cycle progression or regulation of transcription. Multiple CDKs control the cell cycle and are considered essential for normal proliferation, development, and homeostasis. CDK4/cyclin D, CDK6/cyclin D, and CDK2/cyclin E facilitate the G1-S phase transition by sequentially phosphorylating the retinoblastoma protein (Rb), while CDK1/cyclin A, and CDK1/cyclin B are essential for S-phase progression and G2-M transition, respectively.1 Most CDK inhibitors have been developed as potential cancer therapeutics based on the premise that they might counteract the uncontrolled proliferation of cancer cells by targeting the cell-cycle regulatory functions of CDKs. However, in recent years, this understanding of the cellular functions and regulatory roles of CDKs has been challenged.2,3 The observations that cancer cell lines and some embryonic fibroblasts lacking CDK2 proliferate normally and that CDK2 knockout mice are viable35 suggest that this CDK performs a nonessential role in cell-cycle control. Furthermore, redundancy of CDK4 and CDK6 was also suggested in cells that enter the cell cycle normally.6 It has been demonstrated that mouse embryos deficient in CDKs 2, 3, 4, and 6 develop to mid-gestation, as CDK1 can form complexes with their cognate cyclins and subsequently phosphorylate Rb protein. Inactivation of Rb in turn activates E2F-mediated transcription of proliferation factors.7 In cells depleted of CDK1/cyclin B, CDK2/cyclin B is readily detectable and can facilitate G2/M

Received: October 11, 2012
Published: January 9, 2013
progression. These studies suggest that specifically targeting individual cell-cycle CDKs may not be an optimal therapeutic approach because of a high level of functional redundancy and compensatory mechanisms.

By contrast, the hypothesis that inhibition of transcriptional CDKs might be an effective anticancer strategy has gained considerable support following the observation that many cells rely on the production of short-lived mitotic regulatory kinases and apoptosis regulators such as Mcl-1 for their survival. The transcriptional CDKs, particularly CDK9/cyclin T and CDK7/cyclin H, are involved in the regulation of RNA transcription. CDK7/cyclin H is a component of transcription factor III (TFIIH) that phosphorylates the serine-5 residues within the heptad repeats of RNA polymerase II (RNAPII) C-terminal domain (CTD) to initiate transcription. CDK9/cyclin T, the catalytic subunit of positive transcription elongation factor P-TEFb, phosphorolyzes two elongation repressors, i.e., the DRB-sensitive-inducing factor (DSIF) and the negative elongation factor (NELF), and position serine-2 of the CTD heptad repeat to facilitate productive transcription elongation. While CDK7 is also recognized as a CDK-activating kinase (CAK), CDK9 appears to have a minimal effect on cell-cycle regulation.

During the past decade an intensive search for pharmacological CDK inhibitors has led to the development of several clinical candidates and to the realization that inhibition of the transcriptional CDKs underlies their antitumor activity. Flavopiridol (alvocidib), the first CDK inhibitor to enter clinical trials, is the most potent CDK9 inhibitor identified to date and has demonstrated marked antitumor activity in chronic lymphocytic leukemia (CLL). Flavopiridol has been shown to inhibit multiple CDKs and other kinases, but the primary mechanism responsible for its observed antitumor activity in CLL appears to be the CDK9-mediated down-regulation of transcription of antiapoptotic proteins.

R-Roscovitine (seliciclib) is the first orally bioavailable CDK inhibitor that targets CDK2, CDK7, and CDK9 (IC50 ≈ 0.1, 0.5, and 0.8 μm, respectively). During evaluation in phase I oncology monotherapy and combination chemotherapy clinical trials it was shown to be well tolerated and some evidence of disease stabilization was reported. Phase II clinical trials are underway in non-small-cell lung cancer (NSCLC) patients. R-Roscovitine has demonstrated selective induction of apoptosis in cancer cells by down-regulation of antiapoptotic proteins through transcriptional CDK inhibition. Other CDK inhibitors including AZD5438, R547, and AT51930 have also been evaluated in clinical trials.

While there are several pan-CDK inhibitors in clinical studies, CDK9 inhibitors with good potency and selectivity have only recently emerged. To further exploit the sensitivity of the 4-heteroarylpyrimidine pharmacophore (type I, Figure 1) that specifically targets the CDK9-ATP

![Figure 1. 4-(Thiazol-5-yl)-2-(phenylamino)pyrimidine derivatives.](image-url)
### Table 1. Structure and Biological Activity Summary

| compd | R' | R          | 12a: CDK9/cyclin T1 | 12b: CDK1/cyclin B | 12c: CDK2/cyclin A | 12d: CDK7/cyclin H | cytotoxicity GI<sub>50</sub> (μM)<sup>b</sup> HCT-116 |
|-------|----|------------|---------------------|-------------------|-------------------|-------------------|-------------------------------------------|
| la    | H  | -NO<sub>2</sub> | 2                   | 5                 | 3                 | 417               | 0.09                                      |
| 12a   | CN | -NO<sub>2</sub> | 6                   | 6                 | 1                 | 260               | 0.04                                      |
| 12b   | OH | -NO<sub>2</sub> | 932                | 1424              | >5000             | >5000             | 3.20                                      |
| lb    | H  | -SO<sub>2</sub>NH<sub>2</sub> | 2                  | 6                 | 4                 | 1960              | 0.05                                      |
| 12c   | CN | -SO<sub>2</sub>NH<sub>2</sub> | 6                  | 12                | 4                 | 114               | 0.55                                      |
| 12d   | OH | -SO<sub>2</sub>NH<sub>2</sub> | >5000             | >5000             | >5000             | >5000             | 2.03                                      |
| 12e   | F  | -SO<sub>2</sub>NH<sub>2</sub> | 4                  | 4                 | 3                 | 91                | <0.01                                     |
| 12f   | Cl | -SO<sub>2</sub>NH<sub>2</sub> | 11                | 19                | 10                | 685               | 0.03                                      |
| 12g   | Me | -SO<sub>2</sub>NH<sub>2</sub> | 5                  | 62                | 34                | 1176              | 0.30                                      |
| 12h   | Et | -SO<sub>2</sub>NH<sub>2</sub> | 98                | 788               | 845               | 1285              | 3.81                                      |
| 12i   | Pr | -SO<sub>2</sub>NH<sub>2</sub> | >5000             | ND                | >5000             | ND                | 4.50                                      |
| 12j   | CN | -4-acetylpirazin-1-yl | 7                 | 43                | 43                | 92                | 0.22                                      |
| 12k   | CN | -piperazin-1-yl      | 5                  | 42                | 56                | 68                | 0.23                                      |
| 12l   | CN | -p-4-acetylpirazin-1-yl | 22               | 45                | 26                | 316               | 0.82                                      |
| 12m   | CN | -p-piperazin-1-yl    | 6                  | 79                | 39                | 71                | 0.03                                      |
| 12n   | CN | -piperidin-1-yl      | 9                  | 35                | 42                | 286               | 0.93                                      |
| 12o   | F  | -4-acetylpirazin-1-yl | 7                 | 26                | 42                | 302               | 0.35                                      |
| 12p   | F  | -piperazin-1-yl      | 4                  | 24                | 20                | 193               | 0.31                                      |
| 12q   | F  | -morpholin-4-yl      | 3                  | 18                | 20                | 473               | 0.16                                      |
| 12r   | Cl | -piperazin-1-yl      | 4                  | 88                | 45                | 155               | 0.26                                      |
| 12s   | F  | -1,4-diazepan-1-yl   | 5                  | 47                | 85                | 111               | 0.64                                      |
| 12t   | CN | -4-acetyl-1,4-diazepan-1-yl | 7             | 91                | 131               | 210               | 0.33                                      |
| 12u   | CN | -1,4-diazepan-1-yl   | 7                  | 94                | 568               | 46                | 0.42                                      |
| 12v   | H  | -1,4-diazepane-1-yl  | 19                 | 195               | 320               | 433               | 0.66                                      |

<sup>a</sup>The ATP concentrations used in these assays were within 15 μM of K<sub>m</sub>, i.e., 45, 45, 90, and 45 μM for CDK1/cyclin B, CDK2/cyclin A, CDK7/cyclin H/MAT1, and CDK9/cyclin T1, respectively. The data given are mean values derived from two replicates. Apparent inhibition constants (K<sub>i</sub>) were calculated from IC<sub>50</sub> values and the appropriate K<sub>m</sub> (ATP) values for each kinase.<sup>35</sup><sup>a</sup>Antiproliferative activity by MTT 48 h assay. The data given are mean values derived from at least three replicates.

### Scheme 1. Synthesis of 3-(Dimethylamino)-2-(4-methyl-2-(methylamino)thiazole-5-carbonyl)acrylonitrile and Derivatives<sup>a</sup>

![Scheme 1](image-url)

<sup>a</sup>Reagents and conditions: (a) di-tert-butyl dicarbonate, 4-dimethylaminopyridine (DMAP), DCM, rt, 1 h, 93%; (b) LDA, MeCN, THF, −78 °C, 1.5 h, 72%; (c) N,N-dimethylformamide–dimethylacetal (DMF–DMA), reflux, overnight or microwave, Δ, 20–45 min, 30–80%; (d) Selectfluor, MeOH, 0 °C, 1 h; or NCS, MeOH, rt 0.5 h, 30%; (e) 1-methylthiourea, MeOH, pyridine, rt 4 h, 88%; (f) LDA, R′CH<sub>2</sub>CHO, MeCN, THF, −78 °C, 1–1.5 h, 53–77%; (g) MnO<sub>2</sub>, CHCl<sub>3</sub>, Δ, 3 h, 65–85%.
bulky alkyl group; thus, attempting the synthesis of 4, where \( R' = \) isopropyl, failed even under harsher reaction conditions because of unfavorable electronic and steric effects of the bulky isopropyl group. Pyrimidine ring formation reaction was performed under conditions similar to those we have developed for the synthesis of 2-anilinoamino-4-(heteroaryl)pyrimidines.35 The limiting factor in the preparation of type II analogues was the efficiency of the condensation reactions between the substituted phenylguanidines (11) and 4 (Scheme 2). In general, microwave-aided protocols were more effective in terms of reducing reaction times and improving yields in the pyrimidine condensation reactions compared to conventional methodology. Analogues 12b and 12d, where \( R' = \) OH, were obtained from condensation of 4 (\( R' = \) Cl) with corresponding phenylguanidines 11, followed by in situ hydrolysis.

In order to extend the SARs, we prepared another series of compounds with functional group \( R' \) at the C4-position of the thiazol ring system (type III, Figure 1); the chemistry is outlined in Scheme 3. 2-Methylaminothiazol-5-ylethanone derivative 14 was obtained by bromination of 5 in the presence of PTSA to afford 13, which was then treated with di-\( \text{tert} \)-butyl dicarbamate, followed by reaction with methyl 2,2-difluoro-2-(fluorosulfonyl)acetate in the presence of a catalytic amount of CuI.43 Converting 14 to the corresponding enamino 15 (\( R_1 = \) Boc, \( R'' = \) CH\(_2\)CF\(_3\)) was achieved by reacting 14 with DMF(DMA as described above. To prepare analogue 17, 1,1,1-trifluoropentane-2,4-dione was treated with hydroxy(tosyloxy)iodobenzene,42 followed by reaction with 1-methylthiourea and then di-\( \text{tert} \)-butyl dicarbonate. tert-Butyl (5-acetylthiazol-2-yl)(methyl)carbamate (20) was obtained by cyclization reaction between chloroacetone chloride and N’-carbamothioyl-N,N-dimethylformimidamide (18),43 giving 19, followed by treating the latter with di-\( \text{tert} \)-butyl dicarbonate. To prepare tert-butyl(5-acetyl-4-cyclopropylthiazol-2-yl)(methyl)-carbamate (24), bromination of 1-cyclopropylethanone (21) yielded 2-bromo-1-cyclopropylethanone (22), which was subsequently reacted with 1-methylthiourea and then di-\( \text{tert} \)-butyl dicarbamate to afford 4-cyclopropyl-N-methylthiazol-2-amine (23). Acetylation was achieved by LDA-mediated alkylation reaction between 23 and acetaldehyde,39 followed by oxidation of the resulting thiazol-5-ylethanol intermediate with MnO\(_2\). 4-Phenylthiazol derivative (26) was prepared by condensation reaction between 2-chloro-1-phenylethanone and

**Scheme 2. Synthesis of 4-(4-Methylthiazol-5-yl)-2-(phenylamino)pyrimidine-5-carbonitrile and Derivatives**

Reagents and conditions: (a) 2-methoxyethanol, microwave, 200–300 W, \( \Delta \), 20–45 min.

**Scheme 3. Synthesis of N-Methyl-5-(2-(phenylamino)pyrimidin-4-yl)-4-thiazol-2-amine Derivatives**

Reagents and conditions: (a) pentane-2,4-dione, pyridine, EtOH, rt 4 h; (b) NBS, p-toluenesulfonic acid (PTSA), CHCl\(_3\), 0–5 °C, 1 h, 56%; (c) di-\( \text{tert} \)-butyl dicarbonate, DMAP, DCM, rt 2 h; (d) methyl 2,2-difluoro-2-(fluorosulfonyl)acetate, Cul, DMF, \( \Delta \), 12 h; (e) DMF–DMA, \( \Delta \), overnight, or microwave, \( \Delta \), 45 min; (f) (i) 1,1,1-trifluoropentane-2,4-dione, hydroxy(tosyloxy)iodobenzene, MeCN, \( \Delta \), 1 h; (ii) 1-methylthiourea, \( \Delta \), 4 h, 53%; (g) DMF–DMA, CHCl\(_3\), \( \Delta \), overnight, 98%; (h) chloroacetone chloride, MeCN, \( \Delta \), 4 h, 79%; (i) Br\(_2\), 0 °C to rt 4 h, 77%; (j) (i) LDA, acetaldehyde, THF, –78 °C, 2 h, 49%; (ii) MnO\(_2\), CHCl\(_3\), \( \Delta \), 4 h, 55%; (k) acetyl chloride/AlCl\(_3\), rt, 2 h, 67%; (l) 11, 2-methoxyethanol, microwave 200–300 W, \( \Delta \), 20–45 min.
1-methylthiourea to afford N-methyl-4-phenylthiazol-2-amine, followed by the Friedel–Crafts acylation reaction. Finally, the enaminones (1S) were converted to the corresponding pyrimidines (27a−l) upon treatment with the appropriate phenylguanidines\(^{32,35}\) under microwave irradiation conditions.

## RESULTS AND DISCUSSION

**Inhibitor Design and SAR Analysis.** We previously identified a series of 2-herteroaryloxy-4-anilinopyrimidine CDK inhibitors.\(^{32,35,44}\) Many of these compounds showed potent CDK9 inhibitory activity. The lead compounds demonstrated excellent pharmaceutical properties and in vivo antitumor efficacy.\(^{32}\) However, CDK9 specificity was not achieved, as they cross-reacted with other cell cycle CDKs, in particular CDK2.

Previously established SARs of 2-anilino-4-(thiazol-5-yl)-pyrimidines (type I, Figure 1) with respect to CDK2 suggested the importance of substituents at the C2-position in the thiazole ring.\(^{32,35}\) It was found that introduction of amino functions, in the context of either meta- or para-substituted anilines at the C2-pyrimidine ring, resulted in a significant increase of inhibitory activity not only against CDK2 but also against CDK9. Co-crystal structures of some of these inhibitors bound to CDK2 revealed that the thiazole C2-amino group interacted strongly with the Asp145 residue of CDK2, enhancing the hydrophobic interactions of the thiazol C4-methyl group with the Phe80 gatekeeper residue of CDK2 (Phe103 in CDK9). Additional hydrogen bonding interactions between the thiazole C2-amino groups and Gln131 and Asp86 of CDK2 were also observed. Substitution of the thiazole C2-amino group with a C2-methylamino or C2-ethylamino appeared to have a detrimental effect on CDK2 and CDK4 activity while having only a minimal effect on CDK9 potency.\(^{32,35}\) A bulkier group, such as phenyl, pyridyl, or other heterocycles at this position, however, led to significantly reduced activity against all CDKs. We therefore designed a series of 5-substituted-4-thiazolopyrimidines in the context of the C2-methylamino to improve potency and selectivity against CDK9.

The SAR analysis of the pyrimidinyl C2-aniline moiety was previously described.\(^{32,35}\) It was shown that many meta- or para-substituted anilines in the context of the 4-thiazolopyrimidine, were well tolerated and manipulation of these substituents led to a number of inhibitors possessing varying CDK selectivity profiles. In many cases, meta-substituted anilines gave rise to selectivity for CDK9 over CDK2 compared with their para-substituted aniline analogues. However, substituents in the ortho position abolished CDK-inhibitory activity in all cases.

It is recognized that the ATP-binding sites are highly conserved among kinases,\(^{45}\) but the nonconserved hydrophobic region, which is not occupied by ATP, and the so-called "gatekeeper" region can be exploited for inhibitor design.\(^{46,47−50}\) A co-crystal structure of flavopiridol bound to CDK9 showed that the hydrophobic region that accommodates the chlorophenyl ring of flavopiridol is more open in CDK9 than CDK2. CDK9 Gly112 takes the place of CDK2 Lys89 and creates a less crowded and a different electrostatic environment.\(^{51}\) Analysis of the previously published 2-anilino-4-(thiazol-5-yl)pyrimidine CDK2-bound crystal structures and their corresponding models of CDK9 binding\(^ {32,35}\) suggested that an appropriate functional group at either the C5-pyrimidine or the C4-thiazol moiety might enhance interactions with the CDK9 gatekeeper region. We thus investigated the potency and selectivity of a series of 5-substituted 2-anilino-4-(thiazol-5-yl)-pyrimidines against CDKs and characterized their cellular antitumor activity. The results are summarized in Table 1.

Compound 1a (R′ = H, R = m-NO\(_2\)) is a highly potent pan-CDK inhibitor. Substitution of hydrogen at C5-pyrimidine in 1a with a carbonitrile group results in compound 11a (R′ = CN, R = m-NO\(_2\)) that exhibits a similar potency and selectivity profile. Both compounds inhibit CDK9, CDK1, and CDK2 potently with K\(_i\) values ranging from 1 to 6 nM but are significantly less active toward CDK7. Both compounds are highly effective antiproliferative agents with respective GI\(_50\) values of 90 and 40 nM in the HCT-116 human colon cancer cell line. Replacement of the C5-carbonitrile with a C5-hydroxyl group in 12b (R′ = OH, R = m-NO\(_2\)) results in over 155-fold and 230-fold loss in CDK9 and CDK1 inhibition, respectively. This replacement also abolishes CDK2 and CDK7 inhibitory activity and significantly reduces cellular antiproliferative activity. A compound containing the m-sulfonamidoaniline ring, 12c (R′ = CN, R = m-SO\(_2\)NH\(_2\)), shows similar potencies against CDK1, CDK2, and CDK9 but a 17-fold or a 10-fold loss in CDK7 inhibition and cellular toxicity, respectively, compared to 1b (R′ = H, R = m-SO\(_2\)NH\(_2\)). Again, introducing a hydroxyl group at C5-pyrimidine, in the context of m-sulfonamidoaniline, is not tolerated; 12d (R′ = OH, R = m-SO\(_2\)NH\(_2\)) shows little biological activity in the enzymatic and cellular assays. These results demonstrate the importance of C5-substitution of the pyrimidine and that a protic or hydrogen bond donating function at this position has a detrimental effect on biological activity.

Compound 12e (R′ = F, R = m-SO\(_2\)NH\(_2\)), a potent pan-CDK inhibitor (K\(_i\) = 3−7 nM), is the most potent antiproliferative agent of this series, with GI\(_50\) < 10 nM against HCT-116 cells. Analogue 12f, where R′ = Cl, R = m-SO\(_2\)NH\(_2\), however, displays a >3-fold reduced CDK inhibitory activity and cellular potency compared to 12e. A more interesting trend toward CDK9 selectivity is observed with CS-alkylpyrimidines; 12g (R′ = Me, R = m-SO\(_2\)NH\(_2\)) exhibits a CDK9 inhibitory potency similar to that of 12e with K\(_i\) = 5 nM but enhances selectivity for CDK9 with >7-fold lower effectiveness against other CDKs. However, this selectivity results in 12g showing over 30-fold reduced cytotoxicity in HCT-116 cells compared to 12e. With further introduction of a bulkier alkyl group, CDK9 inhibitory activity dramatically decreases; thus, 12h (R′ = Et, R = m-SO\(_2\)NH\(_2\)) is 20-fold less potent against CDK9 than 12g, while 12i (R′ = Pr, R = m-SO\(_2\)NH\(_2\)) is not active against CDKs at concentrations up to 5 μM. As expected, 12h and 12i are also less cytotoxic in cancer cells with respective GI\(_50\) values of 3.81 and 4.50 μM.

Retaining the C5-carbonitrile pyrimidine core but replacing the m-sulfonamide with a bulkier 1-(piperazin-1-yl)ethanone or piperazine leads to the corresponding 12j (R′ = CN, R = m-1-(piperazin-1-yl)ethanone) or 12k (R′ = CN, R = m-piperazine). This not only maintains CDK9 potency but also increases selectivity (~10-fold) over CDK2 compared to 12c, indicating the tolerance of a large ring system in the corresponding CDK9 binding region. Compounds 12m–r, bearing heterocyclic piperidine, 1-(piperazin-1-yl)ethanone, piperazine, or morpholine at the meta- or para-position of the aniline in the context of a C5-carbonitrile or C5-halogen pyrimidine moiety, display favorable CDK9 inhibitory activity with low nanomolar potencies and possess over 4-fold selectivity for CDK9. The exception is compound 12l (R′ = CN, R = p-1-(piperazin-1-yl)ethanone), which shows a >3-fold loss of potency against
CDK9. All these analogues demonstrate excellent antiproliferative activity with GI50 values ranging from 0.03 to 0.93 μM.

Introduction of a bulkier heterocyclic (1,4-diazepan-1-yl)-ethanone or 1,4-diazepane at the meta-position of the aniline affords 12s−u, displaying appreciable selectivity for CDK9 versus CDK2. Compound 12u, in particular, shows a >80-fold enhanced CDK9 selectivity over CDK2. Compounds 12s−u effectively inhibit tumor cell growth with GI50 values of 0.64, 0.33, and 0.42 μM, respectively. Replacement of the C5-carbonitrile or C5-fluorine with a C5-hydrogen affords 1c (R′ = H, R = m-1,4-diazepane). However, this replacement results in a >2-fold loss in CDK9 inhibitory activity but a more significant drop in CDK2 selectivity when compared with 12s and 12u. These further support the role of the carbonitrile or fluorine substitution at the C5-pyrimidine in favoring potency and selectivity against CDK9 over CDK2.

In general, all C5-substituted pyrimidine analogues are also potent CDK1 inhibitors, with activity comparable to that of CDK2 as shown in Table 1. An exception is compound 12u which targets CDK1 and CDK2 with Ki values of 94 and 568 nM, respectively, being 6-fold more potent for CDK1 than for CDK2. It is apparent that this combined inhibition of CDK9, CDK1, and CDK2 results in significant cytotoxicity in cancer cells. 12e, a nanomolar CDK9, CDK1, and CDK2 inhibitor, for example, is the most potent cytotoxic agent of this chemical class, with GI50 < 10 nM against HCT-116 cells. This is consistent with the finding that cancer cells expressing shRNA targeting a combination of CDK2, CDK1, and CDK9 were most effective in induction of apoptosis of cancer cells, and targeting CDK9, CDK1, and CDK2 has been proposed as an anticancer strategy.3

Most of the analogues described here are significantly less effective as CDK7 inhibitors when compared to their activity against other CDKs, suggesting that CDK7 inhibition is not a requirement for the observed cellular cytotoxicity: many compounds demonstrate excellent antiproliferative activity irrespective of modest CDK7 inhibition (Table 1). Compounds 1b and 12g, for example, inhibit CDK7 with Ki > 1 μM, but both exhibit excellent antiproliferative activity with GI50 = 0.05 and 0.30 μM, respectively.

In order to assess whether modification of the C4-methyl of the thiazole is tolerated, we prepared a series of substituted C4-thiazolopyrimidine derivatives; the SAR is summarized in Table 2. Replacement of the C4-methyl with phenyl (27a, R′ = Ph, R = m-NO2) is not tolerated, and no inhibitory activity against CDKs is detected up to 5 μM. However, this compound exhibits potent antiproliferative activity with a GI50 of 60 nM, indicating potential non-CDK kinase targets. Substitution of C4-trifluoroethyl, in the context of m-nitroanilinopyrimidine, yields 27b (R′ = CH3CF3, R = m-NO2). This compound exhibits excellent selectivity for CDK9 (Ki = 0.134 μM), being inactive against other CDKs at concentrations up to 5 μM. Despite its high selectivity, 27b still displays good antiproliferative activity with GI50 < 1 μM in HCT-116 cancer cells. However, analogues 27c (R′ = CH3CF3, R = m-OH), 27d (R′ = CH3CF3, R = p-OH), and 27e (R′ = CH3CF3, R = m-SO2NH2) reduce CDK9 inhibition and selectivity when compared to 27b. Keeping the benzenesulfonamide moiety but replacing the 4C-trifluoroethyl with a C4-trifluoromethyl, C4-hydrogen, or C4-cyclopropyl affords compounds 27f–k. All of these compounds possess significantly enhanced inhibitory activity not only against CDK9 but also against other CDKs. As expected, these compounds are extremely cytotoxic to cancer cells with GI50 values in the range of 0.01–0.41 μM. However, substitution of the benzenesulfonamide with 1,4-diazepan-1-yl aniline yields 27j (R′ = cyclopropyl, R = m-1,4-diazepane). This analogue shows a significant loss of activity which suggests that the benzenesulfonamide moiety is a key contributor to optimal CDK inhibition and cellular potency of this series.

Table 2. Structure and Biological Activity Summary

| compd | R′    | R      | CDK9T1 (μM) | CDK1B (μM) | CDK2A (μM) | CDK7H (μM) | cytotoxicity GI50 (μM) |
|-------|-------|--------|------------|------------|------------|------------|-----------------------|
| 27a   | Ph    | m-NO2  | >5000      | >5000      | >5000      | >5000      | 0.06                  |
| 27b   | CH3CF3| m-NO2  | 134        | >5000      | >5000      | >5000      | 0.90                  |
| 27c   | CH3CF3| m-OH   | 245        | 556        | 282        | 3474       | 2.60                  |
| 27d   | CH3CF3| p-OH   | 278        | 334        | 347        | 1543       | 3.70                  |
| 27e   | CH3CF3| m-SO2NH2| 156       | 154        | 226        | 1984       | 0.91                  |
| 27f   | CF3   | m-SO2NH2| 2         | 1.5        | 2          | 47         | 0.05                  |
| 27g   | CF3   | p-SO2NH2| 3         | 0.5        | 1.5        | 64         | 0.01                  |
| 27h   | H     | p-SO2NH2| 5         | 1          | 1          | 54         | 0.13                  |
| 27i   | H     | m-SO2NH2| 3         | 4          | 5          | 40         | 0.02                  |
| 27j   | cyclopropyl | m-SO2NH2| 16        | 18         | 2          | 273        | 0.41                  |
| 27k   | cyclopropyl | p-SO2NH2| 8         | 2          | 13         | 255        | 0.08                  |
| 27l   | cyclopropyl | m-1,4-diazepan-1-yl| 66        | 176        | 326        | 464        | 0.77                  |

“a”The ATP concentrations used in these assays were within 15 μM of K. The data given are mean values derived from two replicates. Apparent inhibition constants (Ki) were calculated from I(a) values and the appropriate K (ATP) values for each kinase. Antiproliferative activity by MTT 48 h assay. The data given are mean values derived from at least three replicates.
CDK9/cyclin T and CDK2/cyclin A in order to explain the observed SAR. The crystal structure and refinement data are summarized in Table 3. A more thorough rationalization of the SARs provided by the determination of five additional inhibitor cocystal structures bound to CDK9/cyclin T and CDK2/cyclin A is provided in the companion paper. As shown in Figure 2, 12u adopts a similar binding mode within the CDK9 and CDK2 ATP binding sites located between the N- and C-terminal lobe, and the thiazole, pyrimidine, and aniline moieties occupy similar positions. In both CDK9 and CDK2, 12u hydrogen-bonds with the kinase hinge regions. The N1-pyrimidine accepts a hydrogen bond from the peptide nitrogen of Cys106 (Leu83 in CDK2), while the C2-NH of the pyrimidine ring donates a hydrogen bond to the peptide.

Table 3. Crystallographic Parameters of CDK2/Cyclin A/12u and CDK9/Cyclin T/12u

|                          | CDK2/cyclin A/12u | CDK9/cyclin T/12u |
|--------------------------|-------------------|-------------------|
| Data Collection          | Diamond I-03      | Diamond I-03      |
| space group              | P2,12,2,1         | P2,12,2,1         |
| unit cell (Å)            | a = 73.81, b = 134.55, c = 149.17 | a = b = 172.80, c = 98.88 |
| resolution (highest resolution shell) (Å) | 29.83–2.26 (2.38–2.26) | 86.40–3.08 (3.25–3.08) |
| total observations       | 308028 (43211)    | 70331 (10036)     |
| unique observations      | 69656 (9807)      | 20079 (2956)      |
| Rmerge                   | 0.071 (0.496)     | 0.058 (0.511)     |
| multiplicity             | 4.4 (4.4)         | 3.5 (3.4)         |
| mean I/σI                | 13.4 (2.8)        | 15.4 (2.4)        |
| completeness (%)         | 99.2 (96.6)       | 99.0 (99.6)       |

Figure 2. Cocystal structures of 12u bound to CDK9/cyclin T1 (PDB code 4BCG) and CDK2/cyclin A (PDB code 4BCP). The structures of CDK9/T1/12u (A, C) are shown. Compound 12u bound to CDK2/cyclin A (B, D) and showing two binding orientations. Electron density around 12u is shown as a wire mesh (A, B). Selected CDK9 and CDK2 residues are drawn in ball-and-stick representation. Hydrogen bonds in all panels are depicted by dotted lines.
carbonyl of Cys106. At the back of the ATP binding site the C5-carbonitrile group exploits the hydrophobic region close to the gatekeeper residue Phe103 (Phe80 in CDK2) to form a favorable lone pair–π interaction. The CDK2/cyclin A/12u structure was determined at a higher resolution and shows a water molecule trapped in a pocket behind the C5-carbonitrile (Figure 2B and Figure 2D). This water molecule forms a hydrogen-bond network with the backbone of residue Asp145 and with the side chain of Glu51. In the adenine site the pyrimidine ring is sandwiched between the hydrophobic side chains of Ala46 (Ala31 in CDK2) and Leu156 (Leu134 in CDK2), with which it forms extensive van der Waals interactions. The hydrogen of the C2-methylaminothiazole binds to Asp167 in CDK9 and to the corresponding residue Asp145 in CDK2. At the front of the ATP binding pocket, the aniline ring is contacted from above by Ile25 (Ile10 in CDK2) to make favorable van der Waals interactions with both enzymes.

The very weak electron density of the 1,4-diazepan-1-ylaniline moiety of 12u suggests that it is not bound tightly to CDK2. Two conformations of 12u were consistent with the observed electron density and represent possible alternative binding modes. Neither of these modes suggests either favorable or unfavorable interactions, correlating to the relative absence of electron density for the 1,4-diazepane ring. In the CDK9 complex, however, the 1,4-diazepane ring clearly adopts an “inward” conformation oriented toward the thiazole ring. This may be more favorable for 12u because of a reduced solvent exposure of the hydrophobic 1,4-diazepane ring in this orientation.

Although the inhibitor interactions with CDK2 and CDK9 are mostly conserved, there are, however, two significant differences. First, as seen for other CDK9/cyclin T inhibitor structures described in the companion paper,54 the binding of the inhibitor induces a lowering of the glycine-rich loop into the ATP binding site. The loop adopts several conformations, and this inherent flexibility is reflected in the higher β-factors. By contrast, the conformation of the glycine rich loop in CDK2/cyclin A appears to be relatively unaltered on inhibitor binding (PDB 2GZ). Second, in comparison with the apo structure of CDK9/cyclin T (PDB 3BLH)51 the backbone of the hinge region adapts to inhibitor binding by shifting away from 12u. This shift enables a hydrogen bond to form between the C2-NH of the pyrimidine with the peptide carbonyl of Cys106 at an optimal length of 2.8 Å. These two observations support the hypothesis that CDK9 has a more flexible ATP binding pocket than CDK2.

We propose that the greater flexibility of the ATP-binding site of CDK9 enables the large flexible anilino-1,4-diazepine of 12u, in the context of the C5-carbonitrilepyrimidino moiety, to be well accommodated by CDK9. In contrast, the crystal structure of 12u bound to CDK2 shows that this ring adopts an orientation either “inward” or “outward”, suggesting that the CDK2 binding pocket is too crowded for 12u. This variation in the ability of the kinases to adapt and readily accommodate inhibitors offers an explanation for the high potency and selectivity of 12u toward CDK9.

**Compound 12u Is a Potent Antiproliferative Agent.** Compound 12u was screened against a panel of kinases using biochemical assays and showed no inhibitory activity at concentrations up to 5 μM against a panel of kinases, including BCR-Abl, CaMK1, IKK, Lck, MARK2, PKA, PKB, PKC, and cSRC (Table 4). The antiproliferative effects of 12u against a panel of nine tumor cell lines and three nontransformed cell lines were examined using a 48 h MTT assay as summarized in Table 5A. To investigate cell-type sensitivity, we included HCT-116 colon carcinoma (wild-type and mutant p53, respectively), MCF-7 breast carcinoma (wild-type p53, pRb positive, ER positive and containing CDK4/cyclin D and CDK6/cyclin D), and MDA-MB-468 breast carcinoma cells (mutant p53, pRb negative, ER negative and lacking CDK4/cyclin D and CDK6/cyclin D),52 and other cell lines. Similar sensitivity is observed for cells with different p53, Rb, and CDK4/6 status. Compound 12u suppresses tumor cell proliferation with GI50 values ranging from 0.38 to 0.78 μM, irrespective of the tumor cell type. However, all nontransformed cell lines, i.e., microvascular endothelial cell line HMEC-1 and embryonic lung fibroblasts MRC-5 and WI-38, are significantly less sensitive to 12u treatment (GI50 = 3.12–5.96 μM). The time-course assays were performed using A2780 ovarian cancer and MRC-5 and HMEC-1 nontransformed cell lines. As shown in Table 5B, 24 h treatment with 12u, as well as with flavopiridol, is sufficient to achieve maximal growth inhibition of 12u in A2780 cancer cells. Again, 12u is significantly less toxic in the HMEC-1 and MRC-5 nontransformed cells. In contrast, flavopiridol fails to demonstrate any significant differential effects between the cancer cells and noncancerous cell lines.

**Compound 12u Effectively Induces Cancer Cell Apoptosis.** Cell death induced by therapeutic agents can occur through caspase-dependent or -independent apoptosis or by necrosis. To assess whether apoptosis is contributing to the cytotoxic effect of 12u, we used annexin V/PI (propidium iodide) surface staining in A2780 cancer cells following treatment with 12u for 48 h (Figure 3A). Compound 12u induced cell apoptosis at the GI50 (the concentration of 12u required to inhibit 50% of cell proliferation by MTT assay) in a dose-dependent manner. At the GI50 concentration 12u causes 38% annexin V-positive cells and the percentage increases to 50% at 5GI50. With flavopiridol the same treatments results in 39% and 54% apoptotic cells at GI50 and 5GI50 respectively. Concurrent treatment with 5GI50 of either 12u or flavopiridol together with 50 μM of the pan-caspase inhibitor Z-VAD-fmk suppresses apoptosis, suggesting a caspase-dependent mechanism of apoptosis induction.18

![Table 4. Inhibitory Activity of 12u against Protein Kinases](Image)

*The ATP concentrations used in these assays were within 15 μM of K_i. The data given are mean values derived from two replicates.*
Activation of caspase-3 activity by 12u was confirmed in A2780 cancer cell following exposure to drug for 24 h and was used to compare that in HMEC-1 untransformed endothelial cells (Figure 3B). Compound 12u significantly activates caspase-3 activity in the tumor cells starting at GI50 concentration (p < 0.001), and the effect is further enhanced at higher concentrations. In contrast, no such activity is detected in the HMEC-1 cells up to 10GI50 concentrations of 12u. These results confirm that the cytotoxicity induced by 12u is mediated through the preferential induction of apoptosis in cancer cell lines and corroborates the MTT cytotoxic potency.

As 12u showed potent CDK1 inhibition in biochemical kinase assays, we next investigated its effects on cell cycle progression. A2780 cells were treated with 12u (or flavopiridol) for a period of 24 h at GI50 and 5GI50 concentrations, respectively (Figure 3C). The cells showed no alteration in cell cycle distribution at concentrations less than 5GI50 12u, at which concentration accumulation of cells in G2/M phase of the cell cycle was detected. This confirms that 12u has a lower cellular CDK1 inhibitory activity compared to that of CDK9. A similar cell cycle profile is observed with flavopiridol (Figure 3C).

**Compound 12u Inhibits CDK9 Activity and Down-Regulates Mcl-1 in Cancer Cells.** We next investigated the cellular mode of action of 12u by Western blot analysis (Figure 3D). Treatment of A2780 cells with 12u for a period of 24 h showed that phosphorylation at Ser-2 CTD of RNAPII was reduced at the GI50 and abrogated at 5GI50, confirming cellular CDK9 inhibition. The same treatment caused down-regulation of Mcl-1 and HDM2 but had little effect on the levels of Bcl-2 expression. Induction of apoptosis was indicated by PARP cleavage. Analogous results were obtained with flavopiridol, with inhibition of the phosphorylation of Ser-2 of RNAPII CTD, reduction of Mcl-1 and HDM2, and induction of cleaved PARP being observed.

**Ex Vivo Antitumor Activity in Primary Chronic Lymphocytic Leukemia Cells.** The potency and selectivity of 12u were further evaluated in patient-derived CLL cells (Table 6), as well as age-matched normal B-cells and T-cells, using an annexin V-FITC apoptosis assay. As shown in Figure 4A, the compound exhibits excellent activity with a mean LD50 of 2.60 μM ± 1.1 μM against CLL cells (the concentration of 12u required to kill 50% of the CLL cells following exposure for 48 h). Figure 4B shows that 12u induces a dose-dependent increase in apoptosis in CLL cells as denoted by an increased annexin V positivity. In contrast, little toxicity is observed in the normal B- and T-cells with LD50 of >80 and >280 μM, respectively (Figure 4A). To determine whether the cytotoxicity induced by 12u is caspase-dependent, primary CLL cells were incubated with various concentrations of 12u for 24 h, followed by flow cytometric assessment of active caspase-3. As shown in Figure 4C, the caspase-3 activity is significantly induced at 1.0 μM 12u (P < 0.05) and is further enhanced in a dose-dependent manner at 5 μM (P < 0.0001) and 10 μM (P < 0.0001) when compared with untreated controls. These data support the conclusion that 12u-induced cytotoxicity is mediated via the activation of the effector caspase-3.

CLL cells are characterized by resistance to apoptosis mediated by up-regulation of Bcl-2 family proteins. Mcl-1 is the most important antiapoptotic member of the Bcl-2 protein family and is overexpressed in the majority of patients with CLL at baseline. Increased levels of Mcl-1 are associated with both drug resistance and inferior survival. Down-regulation of Mcl-1 is sufficient to induce apoptosis in CLL cells. A correlation between lower Mcl-1 protein and mRNA levels with known biologic prognostic markers and improved outcomes in
patients with CLL has been reported.53,56 In the present study, primary CLL cells derived from 10 patients were cultured with 1 μM 12u for 8 h and examined for the effect on Mcl-1 protein. Figure 4D shows that the levels of Mcl-1 protein expression are consistently inhibited by 12u in all CLL patient samples (P < 0.0001) irrespective of stages of the disease. The change in Mcl-1 protein expression precedes evidence of apoptosis induction suggesting that the inhibition is a trigger for apoptosis rather than a consequence of apoptosis induction.

**CONCLUSION**

In this communication we describe the synthesis and SAR of a series of S-substituted-4-(thiazol-5-yl)-2-(phenylamino)-pyrimidines and 4-(4-substituted-thiazol-5-yl)-N-phenylpyrimidin-2-amines. Many compounds inhibit CDK9 activity at low nanomolar concentrations and exhibit very potent antiproliferative activity in tumor cells. Optimization led to the discovery of 12u, one of the most selective CDK9 inhibitors in the series, being >80-fold more potent for CDK9 versus CDK2. The cocrystal structures of 12u bound in CDK9/cyclin T and CDK2/cyclin A provide a rationale for the observed potency and selectivity. Compound 12u was examined in more detail regarding its cellular mode of action. The study demonstrates that by inhibiting cellular RNAPII transcriptional activity, 12u mediates down-regulation of the antiapoptotic protein Mcl-1, thereby rendering cells sensitive to apoptosis. Significantly, 12u exhibits excellent antitumor activity in primary CLL cells but shows little toxicity in healthy normal B- and T-cells. In keeping with this finding, Mcl-1 is not detectable in normal B- and T-cells (data not shown), indicating that Mcl-1 may not be a critical regulator of survival in normal lymphocytes. In contrast, CLL cells appear to have a requirement for this protein in order to maintain viability.53 This study provides a rationale for further development of CDK9 inhibitors for the treatment of CLL and other human malignancies.

**EXPERIMENTAL SECTION**

**Chemistry.** Chemical reagents and solvents were obtained from commercial sources. When necessary, solvents were dried and/or purified by standard methods.1H NMR and 13C NMR spectra were obtained using a Bruker 400 Ultrashield spectrometer at 400 and 100 MHz, respectively. These were analyzed using the Bruker TOPSPIN 2.1 program. Chemical shifts are reported in parts per million relative to internal tetramethylsilane standard. Coupling constants (J) are quoted to the nearest 0.1 Hz. The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. High resolution mass spectra were obtained using a Waters 2795 single quadrupole mass spectrometer/micromass LCT platform. Purity for final compounds was greater than 95% and was measured using Waters high performance liquid chromatography (Waters 2487 dual λ absorbance detector) with Phenomenex Gemini-NX 5μ C18 110A 250 mm x 4.60 mm column, UV detector at 254 nm, using system A (10% MeOH containing 0.1% TFA for 4 min, followed by linear gradient 10−100% MeOH over 6 min at a flow rate of 1 mL/min), system B (10% MeCN containing 0.1% TFA for 2 min, followed by linear gradient 10−100% over 10 min at a flow rate of 1 mL/min), and system C (10% MeCN containing 0.1% TFA for 4 min, followed by linear gradient 10−100% over 10 min at a flow rate of 1 mL/min).

**Table 6. Clinical Characteristics of the CLL Patients (n = 10) in This Study**

| patient characteristics | number |
|--------------------------|--------|
| mean age (years)         | 68     |
| sex (male/female)        | 7/3    |
| previously untreated/treated | 10/0 |
| Binet stage (A/B/C)      | 8/2/0  |
| IGHV gene mutation (mutated/unmutated) | 8/2 |
| CD38 expression (<20%/≥20%) | 7/3 |
| ZAP-70 expression (<20%/≥20%) | 7/3 |

dx.doi.org/10.1021/jm301475f / J. Med. Chem. 2013, 56, 640−659
Flash chromatography was performed using either a glass column packed with silica gel (200–400 mesh, Aldrich Chemical) or prepacked silica gel cartridges (FlashMaster systems, Biotage). Melting points were determined with an Electrothermal melting point apparatus.

**Ethyl 4-Methyl-2-(methylamino)thiazole-5-carboxylate 1.** To a solution of ethyl 2-chloroacetoacetate (13.8 mL, 100 mmol) in 100 mL of MeOH were added 1-methylthiourea and 3 mL of pyridine, and the mixture was stirred at room temperature for 4 h. The mixture was concentrated and the precipitate was washed with saturated NaHCO₃ solution, filtered, and dried to give the title compound as a white solid (17.46 g, 85% yield), mp 88–90 °C. ¹H NMR (CDCl₃): δ 1.35 (t, 3H, J = 7.2 Hz, CH₃), 2.53 (s, 3H, CH₃), 2.99 (s, 3H, CH₃), 4.28 (q, 2H, J = 7.2 Hz, CH₂). ¹³C NMR (CDCl₃): δ 14.78, 17.67, 31.27, 60.28, 107.47, 159.79, 162.37, 171.08. HR-MS (ESI⁺): m/z [M+H]+ calcd for C₈H₁₃N₂O₂S, 201.0698, found 201.0463.

**Ethyl 2-(tert-Butyloxycarbonyl(methyl)amino)-4-methylthiazole-5-carboxylate 2.** To a solution of ethyl 4-methyl-2-(methylamino)thiazole-5-carboxylate (10.0 g, 50.0 mmol) in DCM were added 4-dimethylaminopyridine (DMAP) (1.0 g) and di-tert-butyl dicarbonate (12.0 g, 55.0 mmol), and the reaction was continued for 8 h at room temperature. After completion of the reaction, the mixture was washed with 5% aqueous HCl, followed by saturated NaHCO₃ solution, brine, dried over MgSO₄, and filtered. The organic solution was concentrated to dryness, and the title compound was obtained via recrystallization from hexane as a white solid (14 g, 93%), mp 148–150 °C. ¹H NMR (CDCl₃): δ 1.34 (t, 3H, J = 7.2 Hz, CH₃), 1.60 (s, 9H, 3 × CH₃), 2.64 (s, 3H, CH₃), 3.55 (s, 3H, CH₃), 4.29 (q, 2H, J = 7.2 Hz, CH₂). ¹³C NMR (CDCl₃): δ 14.35, 17.37, 28.14, 33.94, 60.54, 83.85, 116.19, 153.13, 156.56, 162.68, 163.10. HR-MS (ESI⁺): m/z [M+H]+ C₁₃H₂₁N₂O₄S, 301.1222, found 301.1312.

**tert-Butyl 5-(2-Cyanoacetyl)-4-methylthiazol-2-yl(methyl)carbamate 3.** To a solution of 2 (6.0 g, 20.0 mmol) in 6 mL of anhydrous THF was added 1.50 mL of acetonitrile (1.3 mmol). The mixture was cooled at −78 °C, and LDA was added dropwise over 10 min. The reaction was continued for 2 h. After completion of the reaction, 10 mL of H₂O was added and the mixture was acidified with dilute HCl solution and extracted with CHCl₃ (3 × 50 mL). The extract was washed with saturated NaHCO₃ solution, brine, dried over MgSO₄, and filtered. The organic solution was concentrated to dryness, and the title compound was obtained via recrystallization from MeOH as a white solid (5.0 g, 87% yield), mp 156–158 °C. ¹H NMR (CDCl₃): δ 1.79 (s, 3H, CH₃), 3.96 (s, 3H, CH₃), 4.29 (q, 2H, J = 7.2 Hz, CH₂). ¹³C NMR (CDCl₃): δ 14.35, 17.37, 28.14, 33.94, 60.54, 83.85, 116.19, 153.13, 156.56, 162.68, 163.10. HR-MS (ESI⁺): m/z [M+H]+ C₁₃H₂₁N₂O₄S, 301.1222, found 301.1312.
combined organic phase was washed with brine, dried over MgSO$_4$ and concentrated to dryness. The mixture was purified by using PE/EtOAc as eluant to afford the title compound as a white solid (4.25 g, 72%), mp 119–121 °C. H NMR (CDCl$_3$): δ 1.61 (s, 9H, 3 × CH$_3$), 2.68 (s, 3H, CH$_3$), 3.59 (s, 3H, CH$_3$), 3.86 (s, 2H, CH$_2$). C$_3$ NMR (CDCl$_3$): δ 18.71, 28.09, 32.23, 34.26, 84.76, 113.76, 122.37, 153.11, 159.30, 163.19. HR-MS (ESI$^+$): m/z [M + H]$^+$ calcd for C$_{14}$H$_{21}$N$_2$O$_3$, 301.1586, found 301.1584.

**tert-Butyl (5-(2-Cyano-3-(dimethylamino)acryloyl)-4-methylthiazol-2-yl)(methyl)carbamate (4, R = Boc, R$_1$ = CN).**

The compound was prepared by the method described previously. Y6 Yellow solid (80% yield), mp 174–176 °C. H NMR (CDCl$_3$): δ 1.59 (s, 9H, 3 × CH$_3$), 2.56 (s, 3H, CH$_3$), 3.28 (s, 3H, CH$_3$), 3.46 (s, 6H, 2 × CH$_3$), 3.54 (s, 3H, CH$_3$), 7.86 (s, 1H, CH). C$_3$ NMR (CDCl$_3$): δ 18.33, 28.18, 34.09, 38.97, 48.17, 81.56, 83.89, 119.49, 124.63, 152.80, 171.91, 180.77.

**General Procedure for Preparation of 10 (R = Me, Et, or Pr).** A solution of corresponding 9 in CHCl$_3$ (1.5 mmol/mL) was treated with MnO$_2$ (5.0 equiv), and the mixture was refluxed for 3 h. Upon completion of the reaction, the mixture was filtered through Celite, and the filtrate was concentrated to dryness.

**tert-Butyl Methyl(4-methyl-5-propionylthiazol-2-yl)(methyl)carbamate (10, R = Me).** Yellow solid (88%), mp 97–99 °C. H NMR (CDCl$_3$): δ 1.20 (t, 3H, J = 7.2 Hz, CH$_3$), 1.61 (s, 9H, 3 × CH$_3$), 1.72 (m, 2H, CH$_2$), 2.67 (s, 3H, CH$_3$), 2.76 (t, 2H, J = 7.2 Hz, CH$_2$), 3.57 (s, 3H, CH$_3$). C$_3$ NMR (CDCl$_3$): δ 8.36, 18.40, 28.15, 34.08, 60.13, 103.13, 125.02, 153.14, 155.40, 161.17, 194.13. HR-MS (ESI$^+$): m/z [M + H]$^+$ calcd for C$_{16}$H$_{23}$N$_2$O$_3$, 285.1273, found 285.1183.

**tert-Butyl Methyl(4-methyl-4-propionylthiazol-2-yl)(methyl)carbamate (10, R = Et).** Yellow solid (81%), mp 101–103 °C. H NMR (CDCl$_3$): δ 0.99 (t, 3H, J = 7.2 Hz, CH$_3$), 1.61 (s, 9H, 3 × CH$_3$), 1.70–1.82 (m, 2H, CH$_2$), 2.67 (s, 3H, CH$_3$), 2.76 (t, 2H, J = 7.2 Hz, CH$_2$), 3.57 (s, 3H, CH$_3$). C$_3$ NMR (CDCl$_3$): δ 13.79, 17.92, 18.35, 28.15, 34.12, 44.93, 84.06, 125.21, 153.14, 155.37, 161.72, 193.63. HR-MS (ESI$^+$): m/z [M + H]$^+$ calcd for C$_{16}$H$_{25}$N$_2$O$_3$, 299.1492, found 299.1459.

**tert-Butyl Methyl(4-methyl-5-pentanoylthiazol-2-yl)(methyl)carbamate (10, R = Pr).** Yellow solid (65%), mp 82–84 °C. H NMR (CDCl$_3$): δ 0.92 (t, 3H, J = 7.2 Hz, CH$_3$), 1.30–1.43 (m, 2H, CH$_2$), 1.59 (s, 9H, 3 × CH$_3$), 1.62–1.72 (m, 2H, CH$_2$), 2.64 (s, 3H, CH$_3$), 2.75 (t, 2H, J = 7.2 Hz, CH$_2$), 3.55 (s, 3H, CH$_3$). C$_3$ NMR (CDCl$_3$): δ 13.88, 18.38, 22.37, 26.54, 28.14, 34.07, 42.75, 84.01, 125.16, 153.16, 155.50, 161.67, 193.78. HR-MS (ESI$^+$): m/z [M + H]$^+$ calcd for C$_{18}$H$_{29}$N$_2$O$_3$, 313.1586, found 313.1620.

Preparations of 10 (R = Me, Et, or Pr) were done by heating 10 in DMF–DMSO using the method described previously or by heating in a Discovery microwave at 140 °C for 45 min. The mixture was concentrated and used for the pyrimidine formation reaction without further purification.

**General Procedure for Preparation of 12a–u.** A mixture of the appropriate 3-(dimethylamino)-1-(4-methylthiazol-2-yl)acryloyl-4 and 1-phenylnuquamide (11)5,5,5 (2 equiv mmol) in 2-methoxyethanol (0.2 mL/mmol) was heated in a microwave at 100–140 °C for 20–45 min. When the mixture was cooled, the residue was purified by flash chromatography using appropriate mixtures of EtOAc/PE or EtOAc/MEOH as the eluant. The products were further purified by crystallization from EtOAc–MEOH mixtures.

**Preparations of 4 (R1 = Boc, R = Me, Et, or Pr).** Yellow solid (53%). 1H NMR (CDCl$_3$): δ 1.21 (t, 3H, J = 7.2 Hz, CH$_3$), 1.59 (s, 9H, 3 × CH$_3$), 1.67–1.94 (m, 2H, CH$_2$) and 1H, J = 4.4 Hz, NH. 84.83 (s, 1H, Ph–H), 8.88 (s, 1H, Py–H), 10.68 (s, 1H, NH). C$_3$ NMR (CDCl$_3$): δ 20.03, 31.39, 95.05, 114.44, 117.74, 117.99, 126.51, 130.35, 140.84, 148.49, 156.48, 159.11, 161.43, 164.19, 170.99. HR-MS (ESI$^+$): m/z [M + H]$^+$ calcd for C$_{19}$H$_{28}$N$_3$O$_3$, 327.2062, found 327.2049.
(3-(4-Methyl-2-(methylthiazol-5-yl)-thiazol-5-yl)-2-prop-2-en-1-one and 3-guanidinobenzenesulfonamide (12f).

For C_{15}H_{15}N_{6}O_{3}S, 359.0926, found, 359.0688.

(3-(5-Ethyl-4-(4-methyl-2-(methylthiazol-5-yl)-thiazol-5-yl)-pyrimidin-2-yl)amino)benzenesulfonamide (12g).

For C_{16}H_{14}N_{7}O_{2}S, 368.0930, found, 368.0937.

(5-Ethyl-4-(4-methyl-2-(methylthiazol-5-yl)-thiazol-5-yl)-pyrimidin-2-yl)amino)benzenesulfonamide (12h).

For C_{16}H_{19}N_{6}O_{2}S_{2}, 391.1011, found, 391.1020.

(3-(5-Ethyl-4-(4-methyl-2-(methylthiazol-5-yl)-thiazol-5-yl)-pyrimidin-2-yl)amino)benzenesulfonamide (12i). 12i was obtained from tert-butyll (S-(2-((dimethylamino)methylene)pentanoyl)-4-methylthiazol-2-yl)(methyl)carbonate and 3-guanidinebenzenesulfonamide. Light yellow powder (30%); mp 183–185 °C. Anal. RP-HPLC: t_{R} 11.32 min (method A), 9.12 min (method B), purity 100%. 1H NMR (DMSO-d_{6}): δ 1.10 (t, J = 7.6 Hz, CH_{3}), 2.20 (s, 3H, CH_{3}), 2.50 (s, 2H, CH_{2}), 7.78 (br s, 1H, NH), 8.43 (s, 1H, Py-H), 9.82 (s, 1H, NH). 13C NMR (DMSO-d_{6}): δ 15.46, 17.72, 22.93, 31.28, 113.99, 115.61, 114.80, 124.99, 120.51, 125.63, 140.17, 144.95, 149.90, 153.86, 153.46, 159.20, 159.30, 160.98, 170.08. HR-MS (ESI): [m/z + H]^{+} calculated for C_{16}H_{19}N_{6}O_{2}S_{2}, 391.1017, found, 391.1020.
5-Fluoro-2-(3-morpholinophenyl)amino)pyrindin-4-yl-N,4-dimethylthiazol-2-amino 12q. 12q was obtained from 3-(dimethylamino)thiazol-2-yl)pyrimidin-2-yl)prop-2-en-1-one and 1-(3-morpholinophenyl)guanidine. Light pink solid (28%); mp 225–227 °C. Anal. RP-HPLC: tR 11.89 min (method A), 11.32 min (method B), purity 98%. 1H NMR (DMSO-d6): δ 2.44 (3H, CH3), 7.88 (2H, 2 × Ph-H), 7.56 (2H, J = 8.0 Hz, 2 × Ph-H). 13C NMR (DMSO-d6): δ 19.21 (6H), 126.81, 128.38, 129.11, 147.85, 148.74, 151.32, 154.26. 1H/19F NMR (DMSO-d6): δ 6.81 (s, 1H, NH), 7.75 (s, 1H, Ph-H). HR-MS (EST): m/z [M + H]+ calcd for C25H26FN3O2S, 464.1580, found 464.1587.

5-Chloro-2-(3-(piperazin-1-yl)phenyl)amino)pyrimidin-4-yl-N4-dimethylthiazol-2-amine 12r. 12r was obtained from 1-(4-(3-(5-fluoro-4-(4-methyl-2-(methylamino)thiazol-5-yl)pyrimidin-2-yl)phenyl)pyrimidin-1-yl)ethanone. Yellow solid (70%); mp 108–110 °C. Anal. RP-HPLC: tR 11.39 min (method A), 8.75 min (method B), purity 99%. 1H NMR (DMSO-d6): δ 2.33 (3H, CH3), 2.83 (apparent t, 2H, J = 4.8 Hz, 2 × CH2), 3.05 (apparent t, 2H, J = 4.8 Hz, CH2), 3.14 (apparent t, 2H, J = 4.8 Hz, 2 × CH2), 6.55 (dd, 1H, J = 8.0, 1.2 Hz, Ph-H), 7.10 (t, 1H, J = 8.0 Hz, Ph-H), 7.16 (d, 1H, J = 8.4 Hz, Ph-H), 7.36 (s, 1H, Ph-H), 7.97 (q, 1H, J = 4.8 Hz, NH), 8.47 (s, 1H, NH). 13C NMR (DMSO-d6): δ 19.26, 31.30, 46.10, 50.09, 106.99, 106.93, 110.60, 112.65, 127.90, 141.17, 141.73, 146.23 (d, J = 25 Hz), 147.24 (d, J = 11 Hz), 147.69 (d, J = 248 Hz), 151.96, 154.57, 156.49 (d, J = 2 Hz), 170.99 (s, d, J = 4 Hz). HR-MS (EST): m/z [M + H]+ calcd for C27H23FN3O2S, 480.1636, found 480.1647.

5-(Fluoro-2-(3-morpholinophenyl)amino)pyrindin-4-yl-N,4-dimethylthiazol-2-amino 12s. 12s was obtained from 1-(4-(3-(5-fluoro-4-(4-methyl-2-(methylamino)thiazol-5-yl)pyrimidin-2-yl)aminophenyl)pyrimidin-1-yl)ethanone. Yellow solid (37%); mp 180–182 °C. Anal. RP-HPLC: tR 11.19 min (method A), 10.98 min (method C), purity 100%. 1H NMR (MeOD-d4): δ 1.95–2.04 (m, 2H, CH2), 2.48 (3H, d, J = 2.8 Hz, CH3), 2.88 (apparent t, 2H, J = 5.2 Hz, CH2), 3.07 (apparent t, 2H, J = 4.8 Hz, 2 × CH2), 6.57 (dd, 1H, J = 8.0, 1.6 Hz, Ph-H), 7.12 (t, 1H, J = 8.0 Hz, Ph-H), 7.21 (d, 1H, J = 8.0 Hz, Ph-H), 7.38 (s, 1H, Ph-H), 8.10 (q, 1H, J = 4.4 Hz, NH), 8.43 (d, 1H, J = 3.6 Hz, Py-H), 9.36 (s, 1H, NH). 13C NMR (DMSO-d6): δ 19.30, 21.67, 31.29, 41.14, 45.92, 48.98, 49.39, 107.48, 110.32, 112.11, 126.26, 131.34, 132.49, 135.21, 136.51, 151.51, 151.64, 158.11, 158.52, 168.73, 170.20. HR-MS (EST): m/z [M + H]+ calcd for C28H24FN3O2S, 508.1580, found 508.1577.
47.59, 48.70 (5.7 mL, 45.0 mmol) and CuI (2.9 g, 15.0 mmol). The mixture was added to a solution of 2-(3-(4-acetyl-1,4-diazepan-1-yl)phenylamino)-4-(bromomethyl)thiazol-2-yl(methyl)carbamate (8.45 g, 24.0 mmol) in toluene (150 mL) under nitrogen. The mixture was stirred at 120 °C for 15 h. The solvent was removed under reduced pressure, and the residue was purified by chromatography using EtOAc/PE to afford a product (2.66 g, 53%), mp 133-135 °C.

1H NMR (400 MHz, CD3OD): δ 7.72 (d, 1 H, J = 4.8 Hz, CH), 7.61 (d, 1 H, J = 7.6 Hz, CH), 7.59 (d, 1 H, J = 6.4 Hz, CH), 7.46 (s, 1 H, CH), 4.97 (s, 2 H, CH2), 4.36 (s, 3 H, CH3), 3.64 (s, 3 H, CH3), 3.49 (s, 3 H, CH3), 3.41 (s, 3 H, CH3), 3.26 (s, 3 H, CH3).

13C NMR (100 MHz, CD3OD): δ 172.26, 160.15, 128.48, 128.34, 127.89, 127.83, 126.08, 125.80, 125.52, 124.48, 122.59, 119.58, 119.21, 54.69, 43.05, 38.90, 32.55, 17.96.

HR-MS (ESI-): m/z [M − H]− calcd for C16H13BrN3O3S, 352.0959, found 352.0960.

tert-Butyl (3-(Dimethylamino)acryloyl)-2-(4-(bromomethyl)thiazol-2-yl)aryl (15, R = Boc, R′ = CF3). 15 (R = Boc, R′ = CF3) was obtained from tert-butyl (5-acetyl-4-(bromomethyl)thiazol-2-yl)(methyl)carbamate and DMF–DMSO. Orange solid (77%); mp 116–118 °C. 1H NMR (400 MHz, CDCl3): δ 1.61 (s, 9H, 3 × CH3), 2.91 (3 H, CH3), 3.16 (s, 3 H, CH3), 3.57 (s, 3 H, CH3), 5.44 (d, 2 H, J = 12.4 Hz, CH2), 7.70 (d, 1 H, J = 12.4 Hz, CH). HR-MS (ESI+): m/z [M + H]+ calcd for C19H19F3N3O3S, 394.1412, found 394.1395.
White solid (94%). 1H NMR (CDCl3): δ 1.62 (s, 9H, 3 × CH3), 2.60 (d, J = 0.8 Hz, CH3), 3.59 (s, 3H, CH3). HR-MS (ESI+): m/z [M – (tert-butyl) + H]+ calculated for C7H12N2O3S, 269.0208, found 269.0265. N,N-Dimethyl-N′-(methylcarbamothioyl)formimidamide (18). A mixture of N-methylthiourea (9.0 g, 100 mmol) and DME-DMA (12 mL, 120 mmol) in 50 mL of CHCl3 was refluxed overnight. The mixture was concentrated and the resulting precipitate was collected by filtration to afford 18 as white solid (14.3 g, 98%), mp 109–110 °C. 1H NMR (CDCl3): δ 3.02 (d, 0.9H, J = 5.2 Hz, CH3), 3.04 (s, 2.1H, CH3), 3.13 (s, 0.9H, CH3), 3.15 (s, 2.1H, CH3), 3.19 (s, 0.9H, CH3), 3.21 (d, J = 5.2 Hz, CH3), 6.88 (brs, 1H, NH), 8.85 (s, 0.3H, CH3), 8.88 (s, 0.7H, CH3). HR-MS (ESI+): m/z [M + H]+ calculated for C9H14N3O2S, 216.0722, found 216.0638.

1-(2-Methylamino)thiazol-5-yl)ethanethione (19). A mixture of N,N-dimethyl-N′-(methylcarbamothioyl)formimidamide (3.62 g, 25.0 mmol) and chloroacetone chloride (2 mL, 25.0 mmol) in 50 mL of acetonitrile was refluxed for 4 h. After completion of the reaction, the mixture was concentrated, neutralized by saturated NaHCO3 solution, and washed with brine, dried over MgSO4, and concentrated to yield 19 as a brown liquid (55%).

tert-Butyl (5-(2-(trimethylamino)acryloyl)-4-thiazolyl)(methyl)carbamate. A solution of 1-(2-(methylamino)thiazol-5-yl)ethanethione and di-tert-butyl dicarbonate. White solid (95%). 1H NMR (CDCl3): δ 1.61 (s, 9H, 3 × CH3), 2.53 (s, 3H, CH3), 3.59 (s, 3H, CH3), 8.00 (s, 1H, thiazol-H). HR-MS (ESI+): m/z [M + H]+ calculated for C16H22N4O3S, 297.1909, found 297.1400.

tert-Butyl (5-(Acetyl-4-cyclopropylthiazol-2-yl)(methyl)carbamate) (20). A solution of 1-(2-(methylamino)thiazol-5-yl)ethanethione and di-tert-butyl dicarbonate was a white solid after recrystallization from PE/EtOAc (5.70 g, 49%). 1H NMR (CDCl3): δ 0.76–0.81 (m, 2H, CH2), 0.81–0.87 (m, 2H, CH2), 1.81–1.90 (m, 1H, CH), 2.95 (s, 3H, CH3), 5.46 (brs, 1H, NH), 7.07 (s, 1H, thiazol-H). HR-MS (ESI+): m/z [M + H]+ calculated for C14H16N2O3S, 257.0960, found 257.0994.

tert-Butyl (4-Cyclopropylthiazol-2-yl)(methyl)carbamate (23). A solution of 1-((3-nitrophenyl)guanidine and tert-butyl (5-(3-nitrophenyl)guanidino)acryloyl)-4-thiazolyl-2-yl)(methyl)carbamate. Yellow solid (18%); mp 215–216 °C. Anal. RP-HPLC: tR 13.84 min (method A), 13.45 min (method B), purity 97%. 1H NMR (CDCl3): δ 2.92 (d, 3H, J = 4.8 Hz, CH3), 6.33 (d, 1H, J = 5.2 Hz, Ph-H), 7.40–7.60 (m, 6H, 6 × Ph-H), 7.76–7.84 (m, 1H, Ph-H), 7.97–8.06 (m, 1H, Ph-H), 8.16 (d, 1H, J = 5.6 Hz, Py-H), 8.27 (q, 1H, J = 4.8 Hz, NH), 8.98 (t, 1H, J = 3.0 Hz, Ph-H), 10.05 (s, 1H, NH). 13C NMR (CDCl3): δ 31.49, 107.90, 112.77, 115.97, 119.41, 125.04, 129.12, 132.37, 130.09, 136.47, 142.47, 165.84, 158.57, 159.21, 159.57, 170.52. HR-MS: m/z [M + H]+ calculated for C19H15N3O3S, 304.1134, found 304.1118.

N-Methyl-(2-(3-nitrophenyl)guanidin-4-yl)-4-phenylthiazol-2-amine (27a). 27a was obtained from tert-butyl (5-(3-(dimethylamino)acryloyl)-4-phenylthiazol-2-yl)(methyl)carbamate. Yellow solid (47%); mp 265–267 °C. Anal. RP-HPLC: tR 13.94 min (method A), 11.85 min (method B), purity 99%. 1H NMR (CDCl3): δ 2.89 (d, 3H, J = 4.4 Hz, CH3), 4.11 (q, 2H, J = 11.2 Hz, CH2), 7.01 (d, 2H, J = 5.2 Hz, Ph-H), 7.58 (t, 1H, J = 8.0 Hz, Ph-H), 7.78–7.85 (m, 1H, Ph-H), 8.05 (dd, 1H, J = 5.2, 1.6 Hz, Ph-H), 8.33 (q, 1H, J = 4.8 Hz, NH), 8.48 (d, 1H, J = 5.6 Hz, Py-H), 8.50 (t, 1H, J = 2.0 Hz, Ph-H), 10.07 (s, 1H, NH). 13C NMR (CDCl3): δ 31.52, 35.33, 35.33 (q, J = 29 Hz), 109.05, 113.11, 112.62, 142.21, 126.21 (q, J = 276 Hz), 125.34, 130.18, 142.18, 144.49, 148.60, 152.26, 159.03, 159.45, 170.07. HR-MS: m/z [M + H]+ calculated for C13H11N3O3S, 211.0643, found 211.0612.

3-(4-(2-Methylamino)-4-(trifluoromethyl)thiazol-5-yl)pyrimidin-2-yl)benzenesulfonamide (27f). 27f was obtained from tert-butyl (5-(3-(dimethylamino)acryloyl)-4-(trifluoromethyl)thiazol-2-yl)(methyl)carbamate and 3-guanidinobenzensulfonamide. Yellow solid (10%); mp 279–281 °C. Anal. RP-HPLC: tR 13.00 min (method A), 11.32 (method B), purity 100%. 1H NMR (CDCl3): δ 2.91 (d, 3H, J = 4.8 Hz, CH3), 7.03 (dt, 1H, J = 5.2, 1.2 Hz, Py-H), 7.31 (s, 2H, NH2), 7.44 (dt, 1H, J = 8.0, 1.2 Hz, Ph-H), 7.49 (t, 1H, J = 8.0 Hz, Ph-H), 7.88 (d, 1H, J = 8.0 Hz, Ph-H), 8.39 (s, 1H, Ph-H), 8.50 (q, 1H, J = 4.8 Hz, NH), 8.56 (d, 1H, J = 5.2 Hz, Py-H), 10.05 (s, 1H, NH). 13C NMR (CDCl3): δ 31.63, 109.34, 116.19, 119.18, 121.28 (q, J = 276 Hz), 122.27, 125.72, 129.54, 137.80 (q, J = 35 Hz), 141.04, 145.03, 156.11, 159.63, 159.74, 170.54. HR-MS (ESI+): m/z [M + H]+ calculated for C18H12F2N3O3S, 431.0572, found 431.0766.
3-(4-(2-(Methylamino)thiazol-5-yl)pyrimidin-2-yl)amino)-benzenesulfonamide (27i). 27i was obtained from tert-butyl (5-(3-<br>dimethylamino)acryloyl)thiazol-2-yl)(methyl)carbamate and 3-guanidinobenzenesulfonamide. Off-white solid (13%); mp 295 °C. Anal. RP-HPLC: tR 11.09 min (method A), 8.72 (method B), purity 99%.<br>1H NMR (DMSO-d6): δ 2.90 (3H, J = 4.8 Hz, CH3), 7.19 (d, 1H, J = 5.2 Hz, Ph-H), 7.29 (s, 2H, NH2), 7.44 (dt, 1H, J = 8.0, 1.6 Hz, Ph-H), 7.46 (t, 1H, J = 8.0 Hz, Ph-H), 7.78–7.95 (m, 5H, 1H, Ph-H), 8.08 (s, 1H, thiazol-H), 8.20 (q, 1H, J = 4.8 Hz, NH), 8.34 (d, 1H, J = 5.2 Hz, Ph-H), 8.43 (t, 1H, J = 1.6 Hz, Ph-H), 9.81 (s, 1H, NH). 13C NMR (DMSO-d6): δ 31.50, 106.60, 110.08, 116.86, 121.92, 124.27, 129.44, 141.55, 143.64, 144.96, 157.64, 159.07, 159.97, 173.00. HR-MS (ESI+): m/z [M + H]+ calculated for C31H19N2O4S, 630.0698, found 630.0666.<br>3-(4-(4-Cyclopropyl-2-(methylamino)thiazol-5-yl)pyrimidin-2-yl)amino)benzenesulfonamide (27j) was obtained from tert-butyl (4-cyclopropyl-5-(3-(dimethylamino)acryloyl)thiazol-2-yl)(methyl)carbamate and 3-guanidinobenzenesulfonamide. Off-white solid (15%); mp 258–260 °C. Anal. RP-HPLC: tR 11.55 min (method A), 9.92 min (method B), purity 100%. 1H NMR (DMSO-d6): δ 0.93–1.04 (m, 4H, 2CH2), 2.52–2.62 (m, 1H, CH), 2.83 (d, 1H, J = 4.8 Hz, CH3), 7.09 (d, 1H, J = 5.6 Hz, Py-H), 7.28 (s, 2H, NH2), 7.40 (d, 1H, J = 8.0 Hz, Ph-H), 7.46 (t, 1H, J = 8.0 Hz, Ph-H), 7.96 (d, 1H, J = 8.0 Hz, Ph-H), 8.05 (q, 1H, J = 4.8 Hz, NH), 8.33 (s, 1H, Ph-H), 8.36 (d, 1H, J = 5.2 Hz, Py-H), 9.75 (s, 1H, NH). 13C NMR (DMSO-d6): δ 9.27, 13.07, 31.46, 108.32, 116.08, 117.37, 118.67, 122.04, 129.41, 141.56, 144.97, 158.08, 158.50, 159.41, 159.73, 170.37. HR-MS (ESI+): m/z [M + H]+ calculated for C31H21N2O4S5, 403.1011, found 403.0900.<br>Crystallography. CDK9g (residues 1–330)/cyclin T1 (residues 1–259, Q77R, E96G, F241L) compounds were expressed, purified, and crystallized as described previously. Crystals were grown by vapor diffusion against a reservoir containing 14% PEG1000, 100 mM sodium potassium phosphate, pH 6.2, 500 mM NaCl, 4 mM TCEP. A crystal was soaked in mother liquor containing also 1 mM 12u and 15% glycerol for 45 min before cryocooling in liquid nitrogen.<br>CDK2/cyclin A was crystallized and purified as described previously. Purified protein was incubated with 12u, filtered, and crystallized in 1.25 M ammonium sulfate, 0.5 M potassium chloride, 100 mM Heps, pH 7.0, 5 mM DTT at 4 °C. Crystals were cryoprotected and frozen in 7 mM sodium formate in the presence of 1 mM 12u. Diffraction data for the CDK9/cyclin T1/12u and CDK2/cyclin A/12u complexes were collected from single crystals at Diamond Light Source beamline I03. Diffraction data for CDK9/cyclin T1/12u were processed with XDS56 and SCALA (CCP4). PHENIX.refine60 was used for rigid body refinement with a model derived from 3BLH as the initial model. REFMAC51 was used for subsequent TLS and restrained refinement. Jelly body restraints to an external model (3BLH) were applied and restrained using PHASER62 using a search model derived from PDB entry 3DDQ. Ligand restraints were defined using PHENIX, and structures were refined and rebuilt using PHENIX.refine and COOT.63 Kinase Assay. Inhibition of CDKs and other kinases was measured by radiometric assay Millipore’s KinaseProfiler according to the protocols detailed at http://www.millipore.com/DrugDiscovery/dd3/, where ATP concentration for each specific kinase assay was set within 15 μM of the apparent Km for ATP where determined. Half-maximal inhibition (IC50) values were calculated from 10-point dose–response curves, and apparent inhibition constants (Ki) were calculated from the IC50 values and Km (ATP) values for the kinases as described. The assay details can also be found in the Supporting Information. Cell Culture. All cancer cell lines were obtained from the cell bank at the Centre for Biomolecular Sciences, University of Nottingham, U.K. The HMEC-1 cell line was purchased (ECACC), and cells were cultured in essential medium with 10% fetal bovine serum (FBS), 7.5% sodium bicarbonate, 1% 0.1 mM nonessential amino acids, 1% 1 M HEPES, 1% 200 mM 1-glutamine, and 1% penicillin. Other cell lines were maintained in RPMI-1640 with 10% FBS.<br>Proliferation Assays. MTI (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldiazotetrazolium bromide, Sigma) assays were performed as reported previously.35 Compound concentrations required to inhibit 50% of cell growth (GI50) were calculated using nonlinear regression analysis.<br>Caspase-3/7 Assay. Activity of caspase 3/7 was measured using the Apo-ONE homogeneous caspase-3/7 kit (Promega G7790).18 Cell Cycle Analysis and Detection of Apoptosis. Cells (4× 106) were cultured for 48 h in medium alone or with varying concentrations of inhibitor. Cell cycle status was analyzed using a Beckman Coulter EPICS XL MCL flow cytometer, and data were analyzed using EXPO32 software. Apoptosis was also confirmed using FITC annexin V/PI (propidium iodide) staining after cells were cultured in medium only or with varying concentrations of inhibitors according to the protocols (BD Bioscience). The annexin V/PI-positive apoptotic cells were enumerated using flow cytometry. The percentage of cells undergoing apoptosis was defined as the sum of early apoptosis (annexin V-positive cells) and late apoptosis (annexin V-positive and PI-positive cells). The pan-caspase inhibitor Z-Val-Ala-Asp-(OMe)-CH2F (Z-VAD-fmk, Sigma) was dissolved in DMSO and used at 25 μM.<br>Detection of Apoptosis in Primary CLL Cells. Freshly isolated primary CLL cells and normal B- and T-cells were cultured in RPMI with 10% fetal calf serum and 1-glutamine, penicillin, and streptomycin. Cells were maintained at 37 °C in an atmosphere containing 5% air and 5% CO2 (v/v). CLL cells (106/mL) were treated with inhibitor for 48 h. Subsequently, cells were labeled with CD19-APC (Caltag) and then resuspended in 200 μL of binding buffer containing 4 μL of annexin V-FITC (Bender Medsystems, Vienna, Austria). Apoptosis was quantified in the CD19+ CLL cells, CD19- normal B-cells, and CD3- normal T-cells using an Accuri C6 flow cytometer and FlowJo software (TreeStar). LD50 values were calculated from line-of-best-fit analysis of the sigmoidal dose–response curves.<br>Western Blots. Western blotting was performed as described. Antibodies used were as follows: total RNAP-II (8WG16), phosphorylated RNAP II Ser-2 (Covance), Bcl-2 (Dako, Denmark A/S), MDM2 and β-actin (Sigma-Aldrich), McI-P, PARP (Cell Signaling Technology). Both anti-mouse and anti-rabbit immunoglobulin G (IgG) horseradish peroxidase-conjugated antibodies were obtained from Dako.<br>Statistical Analysis. All experiments were performed in triplicate and repeated at least twice, representative experiments being selected for figures. Statistical significance of differences for experiments was determined using one-way analysis of variance (ANOVA), with a minimal level of significance at p < 0.01.
and Bauml. Contributions to or assistance in preparation of the manuscript: Shao, Pepper, Hole, Noble, Endicott, and Fischer.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
This study was supported by Cancer Research UK Grants C21568/A8988 and C21568/A12474.

ABBREVIATIONS USED
ATP, adenosine triphosphate; Bcl-2, B-cell lymphoma 2; CaMK1, calcium/calmodulin-dependent protein kinase type 1; DCM, dichloromethane; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; DTT, dithiothreitol; HRMS, high resolution mass spectrometry; IKKβ, inhibitor of nuclear factor κB kinase subunit β; Lck, lymphocyte-specific protein tyrosine kinase; LDA, lithium diisopropylamide; MeCN, acetonitrile; MAPK2, mitogen-activated protein kinase 2; Mcl-1, myeloid cell leukemia sequence 1; NBS, N-bromosuccinimide; NCS, N-chlorosuccinimide; PDB, Protein Data Bank; PARP, poly ADP-ribose polymerase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; RNAPII, RNA polymerase II; SRC, sarcoma kinase; SelectFluor, 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate)

REFERENCES
(1) Lapenna, S.; Giordano, A. Cell cycle kinases as therapeutic targets for cancer. Nat. Rev. Drug Discovery 2009, 8, 547–566.
(2) Wang, S.; Fischer, P. M. Cyclin-dependent kinase 9: a key transcriptional regulator and potential drug target in oncology, virology and cardiology. Trends Pharmacol. Sci. 2008, 6, 302–313.
(3) Cai, D.; Latham, V. M., Jr.; Zhang, X.; Shapiro, G. I. Combined depletion of cell cycle and transcriptional cyclin-dependent kinase activities induces apoptosis in cancer cells. Cancer Res. 2006, 66, 9270–9280.
(4) Berthet, C.; Allee, E.; Coppola, V.; Tessarollo, L.; Kaldis, P. Cdk2 knockout mice are viable. Curr. Biol. 2003, 13, 1775–1785.
(5) Barriere, C.; Santamaria, D.; Cerqueira, A.; Galan, J.; Martin, A.; Ortega, S.; Malumbres, M.; Dubus, P.; Barbacid, M. Mice thrive without Cdk4 and Cdk2. Mol. Oncol. 2007, 1, 72–83.
(6) Malumbres, M.; Sotillo, R.; Santamaria, D.; Galan, J.; Cerezo, A.; Ortega, S.; Dubus, P.; Barbacid, M. Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. Cell 2004, 118, 493–504.
(7) Santamaria, D.; Barriere, C.; Cerqueira, A.; Hunt, S.; Tardy, C.; Newton, K.; Caceres, J. F.; Dubus, P.; Malumbres, M.; Barbacid, M. Cdk1 is sufficient to drive the mammalian cell cycle. Nature 2007, 448, 811–815.
(8) Shapiro, G. I. Cyclin-dependent kinase pathways as targets for cancer treatment. J. Clin. Oncol. 2006, 24, 1770–1783.
(9) Shiekhattar, R.; Mermelstein, F.; Fisher, R. P.; Drapkin, R.; Dynlacht, B.; Weissling, H. C.; Morgan, D. O.; Reinberg, D. Cdk-activating kinase complex is a component of human transcription factor TFIHII. Nature 1995, 374, 283–287.
(10) Fisher, R. P. Secrets of a double agent: CDK7 in cell-cycle control and transcription. J. Cell Sci. 2005, 118, 5171–5180.
(11) Price, D. H. P-TEFB, a cyclin-dependent kinase controlling elongation by RNA polymerase II. Mol. Cell. Biol. 2000, 20, 2629–1634.
(12) Garriga, J.; Grana, X. Cellular control of gene expression by T-type cyclin/CDK9 complexes. Gene 2004, 337, 15–23.
(13) Marshall, R. M.; Grana, X. Mechanisms controlling CDK9 activity. Front. Biosci. 2006, 11, 2598–2613.
(14) Garriga, J.; Bhattacharya, S.; Calbo, J.; Marshall, R. M.; Truongcao, M.; Haines, D. S.; Grana, X. CDK9 is constitutively expressed throughout the cell cycle, and its steady-state expression is independent of SKP2. Mol. Cell. Biol. 2003, 23, S165–S173.
(15) Fischer, P. M.; Gianella-Borradori, A. CDK9 inhibitors in clinical development for the treatment of cancer. Expert Opin. Investig. Drugs 2003, 12, 955–970.
(16) Byrd, J. C.; Lin, T. S.; Dalton, J. T.; Wu, D.; Phelps, M. A.; Fischer, B.; Moran, M.; Blum, K. A.; Rovin, B.; Brooker-McEldowney, M.; Broening, S.; Schaad, L. J.; Johnson, A. J.; Lucas, D. M.; Heerema, N. A.; Lozanski, G.; Young, D. C.; Suarez, J. R.; Colevas, A. D.; Grever, M. R. Flavopiridol administered using a pharmacologically derived schedule is associated with marked clinical efficacy in refractory, genetically high-risk chronic lymphocytic leukemia. Blood 2007, 109, 399–404.
(17) Christian, B. A.; Grever, M. R.; Byrd, J. C.; Lin, T. S. Flavopiridol in chronic lymphocytic leukemia: a concise review. Clin. Lymphoma Myeloma 2009, 9 (Suppl. 3), S179–S185.
(18) Liu, X.; Shi, S.; Lam, F.; Pepper, C.; Fischer, P. M.; Wang, S. CDK9-71, a novel CDK9 inhibitor, is preferentially cytotoxic to cancer cells compared to flavopiridol. Int. J. Cancer 2012, 130, 1216–1226.
(19) Caracciolo, V.; Laurenti, G.; Romano, G.; Carnevale, V.; Cimini, A. M.; Crozier-Fitzgerald, C.; Gentile, E.; Russo, G.; Giordano, A. Flavopiridol induces phosphorylation of AKT in a human glioblastoma cell line, in contrast to siRNA-mediated silencing of Cdk9: implications for drug design and development. Cell Cycle 2012, 1102–1216.
(20) Chen, R.; Keating, M. J.; Gandhi, V.; Plunkett, W. Transcription inhibition by flavopiridol: mechanism of chronic lymphocytic leukemia cell death. Blood 2005, 106, 2513–2519.
(21) Krystof, V.; Bauml, S.; Furst, R. Perspective of cyclin-dependent kinase 9 (CDK9) as a drug target. Curr. Pharm. Des. 2012, 18, 2883–2890.
(22) Meijer, L.; Raymond, E. Roscovitine and other purines as kinase inhibitors. From starfish oocytes to clinical trials. Acc. Chem. Res. 2003, 36, 417–425.
(23) Wang, S.; McClue, S. J.; Ferguson, J. R.; Hull, J. D.; Stokes, S.; Parsons, S.; Westwood, R.; Fischer, P. M. Synthesis and configuration of the cyclin-dependent kinase inhibitor roscovitine and its enantiomer. Tetrahedron: Asymmetry 2001, 12, 2891–2894.
(24) McClue, S. J.; Blake, D.; Clarke, R.; Cowan, A.; Cummings, L.; Fischer, P. M.; MacKenzie, M.; Melville, J.; Stewart, K.; Wang, S.; Zhelev, N.; Zheleva, D.; Lane, D. P. In vitro and in vivo antitumor properties of the cyclin-dependent kinase inhibitor CYC202 (R-roscovitine). Int. J. Cancer 2002, 102, 463–468.
(25) Lacrima, K.; Valenti, A.; Lambertini, C.; Taborelli, M.; Rinaldi, A.; Zucca, E.; Catapano, C.; Cavalli, F.; Gianella-Borradori, A.; Maccallum, D. E.; Bertoni, F. In vitro activity of cyclin-dependent kinase inhibitor CYC202 (selicilib, R-roscovitine) in mantle cell lymphomas. Ann. Oncol. 2005, 16, 1169–1176.
(26) MacCallum, D. E.; Melville, J.; Frame, S.; Watt, K.; Anderson, S.; Gianella-Borradori, A.; Lane, D. P.; Green, S. R. Selicilib (CYC202, R-roscovitine) induces cell death in multiple myeloma cells by inhibition of RNA polymerase II-dependent transcription and down-regulation of Mcl-1. Cancer Res. 2005, 65, 5399–5407.
(27) Anderson, M.; Andrews, D. M.; Barker, A. J.; Brassington, C. A.; Breed, J.; Byth, K. F.; Culshaw, J. D.; Finlay, M. R.; Fisher, E.; McKiemen, H. H.; Green, C. P.; Heaton, D. W.; Nash, I. A.; Newcombe, N. J.; Oakes, S. E.; Paupiti, R. A.; Roberts, A.; Stanaway, J. J.; Thomas, A. P.; Tucker, J. A.; Walker, M.; Weir, H. M. Imidazoles: SAR and development of a potent class of cyclin-dependent kinase inhibitors. Bioorg. Med. Chem. Lett. 2008, 18, 5487–5492.
(28) Chu, X. J.; DePinto, W.; Bartkovitz, D.; So, S. S.; Vu, B. T.; Packman, K.; Lukacs, C.; Ding, Q.; Jiang, X.; Wang, K.; Goelzer, P.; Yin, X.; Smith, M. A.; Higgins, B. X.; Chen, Y.; Xiang, Q.; Molter, J.; Kaplan, G.; Graves, B.; Lovey, A.; Foutouhi, N. Discovery of [4-amino-2-(1-methanesulfonyl)piperidin-4-ylamino]-pyrimidin-5-yl][2,3-difluoro-6-methylphenyl]-methanone (R47), a potent and selective cyclin-dependent kinase inhibitor with significant in vivo antitumor activity. J. Med. Chem. 2006, 49, 6549–6560.
(29) DePinto, W.; Chu, X. J.; Yin, X.; Smith, M.; Packman, K.; Goelzer, P.; Lovey, A.; Chen, Y.; Qian, H.; Hamid, R.; Xiang, Q.; Tovar, C.; Blain, R.; Nevin, T.; Higgins, B.; Luistro, L.; Kolinsky, K.; Felix, B.; Hussain, S.; Heimbrook, D. In vitro and in vivo activity of RS47: a potent and selective cyclin-dependent kinase inhibitor currently in phase 1 clinical trials. Mol. Cancer Ther. 2006, 5, 2644–2658.

(30) Wyatt, P. G.; Woodhead, A. J.; Berdini, V.; Boulstridge, J. A.; Carson, M. G.; Cross, D. M.; Davis, D. J.; Devine, L. A.; Early, T. R.; Feltell, R. E.; Lewis, E. J.; McNenamin, R. L.; Navarro, E. F.; O’Brien, M. A.; O’Reilly, M.; Reule, M.; Saxy, G.; Seavers, L. C.; Smith, D. M.; Squires, M. S.; Trewartha, G.; Walker, M. T.; Woolford, A. J. Identification of N-(4-piperidinyl)-4-(2,6-dichlorobenzoylamo)lino)pyrazole-3-carboxamide (AT7519), a novel cyclin dependent kinase inhibitor using fragment-based X-ray crystallography and structure based drug design. J. Med. Chem. 2008, 51, 4986–4999.

(31) Malumbres, M.; Barbacid, M. Cell cycle, CDKs and cancer: a changing paradigm. Nat. Rev. Cancer 2009, 9, 153–166.

(32) Wang, S.; Griffiths, G.; Midgley, C. A.; Barnett, A. L.; Cooper, M.; Grabarek, J.; Ingram, L.; Jackson, W.; Kontopidis, G.; McClure, S. J.; McNees, C.; McLachlan, J.; Meades, C.; Mezna, M.; Stuart, I.; Thomas, M. P.; Zheleva, D. I.; Lane, D. P.; Jackson, R. C.; Glover, D. M.; Hobe, D. G.; Fischer, P. M. Discovery and characterization of 2-anilino-4-(thiazol-5-yl)pyrimidine transcriptional CDK inhibitors as anticancer agents. Chem. Biol. 2010, 17, 1111–1121.

(33) Bauml, S.; Hole, A. J.; Noble, M. E.; Endicott, J. A. The CDK9 C-helix exhibits conformational plasticity that may explain the selectivity of CAN508. ACS Chem. Biol. 2012, 7, 811–816.

(34) Hole, A. J.; Bauml, S.; Shao, H.; Shi, S.; Huang, S.; Pepper, C.; Fischer, P. M.; Wang, S.; Endicott, J. A.; Noble, M. E. Comparative structural and functional studies of 4-(thiazol-5-yl)-2-(phenylamino)-1H-pyrazole-3-carboxamide (AT7519), a novel cyclin dependent kinase inhibitor using fragment-based X-ray crystallography and structure based drug design. J. Med. Chem. 2008, 51, 4986–4999.

(35) Wang, S.; Meades, C.; Wood, G.; Owsnoksi, A.; Anderson, S.; Yuill, R.; Thomas, M.; Mezna, M.; Jackson, W.; Midgley, C.; Griffiths, G.; Fleming, I.; Green, S.; McNae, I.; Wu, S. Y.; McNees, C.; Zheleva, D.; Walkinshaw, M. D.; Fischer, P. M. 2-Anilino-4-(thiazol-5-yl)pyrimidine CDK inhibitors: synthesis, SAR analysis, X-ray crystallography, and biological activity. J. Med. Chem. 2004, 47, 1662–1675.

(36) Nyffeler, P. T.; Duron, S. G.; Burkart, M. D.; Vincent, S. P.; Wong, C. H. SelectFluor: mechanistic insight and applications. Angew. Chem., Int. Ed. 2004, 44, 192–212.

(37) Finlay, M. R.; Acton, D. G.; Andrews, D. M.; Barker, A. J.; Dennis, M.; Fisher, E.; Graham, M. A.; Green, C. P.; Heaton, D. W.; Karouchi, G.; Lodick, S. A.; Morgentin, R.; Roberts, A.; Tucker, J. A.; Wein, H. M. Imidazole piperazines: SAR and development of a potent class of cyclin-dependent kinase inhibitors with a novel binding mode. Bioorg. Med. Chem. Lett. 2008, 18, 4442–4446.

(38) Roshchupkina, G. A.; Pervukhina, N. V.; Rybalova, T. V.; Gativol, Y. V.; Burdukov, A. B.; Reznikov, V. A. Heterocyclization reaction of alpha-imino carbonyl compounds. Derivatives of 2,5-dihydro-1H-imidazol-2-Imidazoles. J. Heterocycl. Chem. 1997, 34, 549–550.

(39) Shafiei, A.; Hadizadeh, P. Syntheses of substituted pyrrole[2,3-d]imidazoles. J. Heterocycl. Chem. 1997, 34, 549–550.

(40) Zhang, X. G.; Qing, F. L.; Yu, Y. H. Synthesis of 2,3′-dideoxy-2′-trifluoromethylenecyclohexoside from alpha-trifluoromethyl-alpha-beta-unsaturated ester. J. Org. Chem. 2000, 65, 7075–7082.

(41) Nabana, T.; Togo, H. Reactivities of novel [hydroxy/tosloyloxy]iodo]arenes and [hydroxy/phosphoryloxy]iodo]arenes for alpha-tosloylation and alpha-phosphoryloration of ketones. J. Org. Chem. 2002, 67, 4362–4365.

(42) Kaila, J. C.; Baraiya, A. B.; Pandya, A. S.; Jalani, H. B.; Vasu, K. K.; Sudarsanam, V. A convenient synthesis of di- and trisubstituted 2-aminimidazoles from 1-amidino-3-trityl-thioura. Tetrahedron Lett. 2009, 50, 3955–3958.

(43) Wang, S.; Wood, G.; Meades, C.; Griffiths, G.; Midgley, C.; McNae, I.; McNees, C.; Anderson, S.; Jackson, W.; Mezna, M.; Yuill, R.; Walkinshaw, M.; Fischer, P. M. Synthesis and biological activity of 2-anilino-4-(1H-pyrolyl-3-y] pyrimidine CDK inhibitors. Bioorg. Med. Chem. Lett. 2004, 14, 4237–4240.

(44) Endicott, J. A.; Noble, M. E.; Johnson, L. N. The structural basis for control of eukaryotic protein kinases. Annu. Rev. Biochem. 2012, 81, 587–613.

(45) Cohen, M. S.; Zhang, C.; Shokat, K. M.; Taunton, J. Structural bioinformatics-based design of selective, irreversible kinase inhibitors. Science 2005, 308, 1318–1321.

(46) Noble, M. E.; Endicott, J. A.; Johnson, L. N. Protein kinase inhibitors: insights into drug design from structure. Science 2004, 303, 1800–1805.

(47) Davies, T. G.; Bentley, J.; Arris, C. E.; Boyle, F. T.; Curtin, N. J.; Endicott, J. A.; Gibson, A. E.; Golding, B. T.; Griffin, R. J.; Hardcastle, I. R.; Jewsbury, P.; Johnson, L. N.; Mesguiche, V.; Newell, D. R.; Noble, M. E.; Tucker, J. A.; Wang, L.; Whittle, H. J. Structure-based design of a potent purine-based cyclin-dependent kinase inhibitor. Nat. Struct. Biol. 2002, 9, 745–749.

(48) Xiao, S.; Raleigh, D. P. A critical assessment of putative gatekeeper interactions in the villin headpiece helical subdomain. J. Mol. Biol. 2010, 401, 274–286.

(49) Moholo, D.; Walker, G.; Broojmans, N.; Nilakantan, R.; Denny, R. A.; Dejoannis, J.; Feyant, J.; Kowticwar, R. K.; Mankala, J.; Palli, S.; Punnamantula, S.; Tapitally, M.; John, R. K.; Humblet, C. A protein relational database and protein family knowledge bases to facilitate structure-based design analyses. Chem. Biol. Drug Des. 2010, 76, 142–153.

(50) Tuominen, A.; Lozanski, G.; Byrd, J. C.; Lucas, D. M. Mcl-1 expression predicts prolonged survival in B-cell chronic lymphocytic leukemia. Blood 2005, 102, 2262–2265.

(51) Tuominen, A.; Hua, F.; Dillon, C. P.; Yokoo, R.; Scheiermann, C.; Cardone, M. H.; Barbieri, E.; Recco, L.; Garuti, A.; Wesselsborg, S.; Belka, C.; Brossart, P.; Patrone, F.; Ballestrero, A. Evidence for a protective role of Mcl-1 in proteasome inhibitor-induced apoptosis. Blood 2005, 105, 3255–3262.

(52) Kabsch, W. XDS. Acta Crystallogr., Sect. D: Biol. Crystallogr. 2010, 66, 125–132.
(59) Collaborative computational project, number 4. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr.* 1994, *D50*, 760–763.

(60) Adams, P. D.; Afonine, P. V.; Bunkoczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.; Zwart, P. H. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 2010, *66*, 213–221.

(61) Murshudov, G. N.; Vagin, A. A.; Dodson, E. J. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 1997, *53*, 240–255.

(62) McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. Phaser crystallographic software. *J. Appl. Crystallogr.* 2007, *40*, 658–674.

(63) Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K. Features and development of Coot. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 2010, *66*, 486–501.