Published in final edited form as:

Biochem Pharmacol. 2013 September 1; 86(5): 597–611. doi:10.1016/j.bcp.2013.06.024.

Intracellular Targets for a Phosphotyrosine Peptidomimetic include the Mitotic Kinesin, MCAK

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Abstract

SH2 domains are attractive targets for chemotherapeutic agents due to their involvement in the formation of protein-protein interactions critical to many signal transduction cascades. Little is known, however, about how synthetic SH2 domain ligands would influence the growth properties of tumor cells or with which intracellular proteins they would interact due to their highly charged nature and enzymatic lability. In this study, a prodrug delivery strategy was used to introduce an enzymatically stable, phosphotyrosine peptidomimetic into tumor cells. When tested in a human tumor cell panel, the prodrug exhibited a preference for inhibiting the growth of leukemia and lymphoma cells. In these cells, it was largely cytostatic and induced endoreduplication and the appearance of midbodies. Proteomic analyses identified multiple targets that included mitotic centromere-associated kinesin (MCAK). Molecular modeling studies suggested the ATP-binding site on MCAK as the likely site of drug interaction. Consistent with this, ATP inhibited the drug-MCAK interaction and the drug inhibited MCAK ATPase activity. Accordingly, the effects of the prodrug on the assembly of the mitotic spindle and alignment of chromosomes were consistent with the identification of MCAK as an important intracellular target.

Keywords

signal transduction; SH2 domain; drug delivery; peptidomimetic; kinesin; MCAK

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1. Introduction

There has been considerable interest in the targeting of signal transduction pathways with small molecule inhibitors for therapeutic purposes. A common structural feature of many components of signaling pathways is the Src-homology 2 (SH2) domain [1]. SH2 domains mediate signaling-induced protein-protein associations based on their binding specificity for primary sequences containing phosphotyrosyl residues. Consequently, numbered among the repertoire of >100 SH2 domain-containing proteins are multiple signaling molecules including, among others, protein-tyrosine kinases and phosphatases, lipid kinases and phosphatases, adaptor proteins, transcription factors, phospholipases and guanine nucleotide exchange factors [2]. Since many of these proteins play important, fundamental roles in the regulation of cell proliferation, SH2 domains are interesting and possibly very important targets for the development of antitumor agents [3].

While SH2 and functionally related domains mediate protein-protein interactions, small phosphotyrosine-containing peptides are able to compete effectively with larger protein ligands and thus serve as lead compounds for the rational design of inhibitors [3]. The concentrated negative charges on the phosphotyrosyl group of peptide ligands, or peptidomimetics, are essential for high binding affinity, but they pose a serious problem for membrane permeability [4]. Furthermore, once inside a cell, drug molecules with phosphate esters are susceptible to hydrolysis by intracellular phosphatases, which would rapidly render them inactive as SH2 domain ligands. Due to such difficulties, there is a deficit of information regarding how cells would respond to treatment with synthetic SH2 domain ligands. Even less is known regarding the repertoire of proteins that might be targeted by such an agent, as there are numerous functional motifs and catalytic domains capable of binding ligands containing phosphate groups. To gather such information, we generated a cell permeable, but metabolically stable ligand designed to target group I SH2 domains of Src-family tyrosine kinases. To circumvent difficulties with delivery, a prodrug strategy that offers cellular permeability and selective intracellular activation [5] was applied to the delivery of this SH2 domain-targeted phosphopeptidomimetic. The synthetic prodrug was largely cytostatic when administered to cancer cells and was a particularly good inhibitor of the proliferation of leukemia and lymphoma cells. Proteomic and functional analyses indicated that, in its active form, the ligand was capable of engaging multiple targets that included the mitotic kinesin, MCAK [6]. Molecular modeling studies coupled with functional assays indicate that the drug, in its active form, binds to the ATP-binding site on MCAK to inhibit its ATPase activity and disrupt microtubule disassembly during mitosis.

2. Materials and methods

2.1. Preparation of 4-((N-methyl-N-4-chlorobutyl)-O-5-nitrofurfuryl-N-(1-(3-carbamoyl-4-cyclohexylmethoxy phenyl)ethyl)phosphinyl)difluoromethyl phenylacetamide **1**

The phosphinylphenylacetic acid prodrug building block **6** and the aminobenzamide **7** were prepared as previously reported. [7, 16] To a stirred solution of **6** (67 mg, 0.14 mmol) in anhydrous CH₂Cl₂ (1.5 ml) at 0 °C, PyBOP (85 mg, 0.16 mmol) was added, and the solution was stirred for 10 min at 0 °C. Aminobenzamide **7** as its hydrochloride (52 mg, 0.16 mmol) was added followed by addition of iPr₂NEt (56 µl, 0.32 mmol). After stirring at 0 °C for 10 min, the ice bath was removed, and stirring was continued for 30 min. Saturated NH₄Cl was added to quench the reaction, and the mixture was extracted with EtOAc (10 ml × 3), dried over Na₂SO₄ and concentrated in vacuo. The residue was purified by flash chromatography to give **1** (78 mg, 74 %) as a yellow foam. R_f = 0.52 (5:95 MeOH:EtOAc). ¹H NMR (CDCl₃): δ 8.03 (s, 1H), 7.84 (s, 1H), 7.51 (d, 2H), 7.28 (m, 3H), 7.14 (d, 2H), 6.75 (d, 1H), 6.40 (m, 3H), 4.92 (m, 3H), 3.68 (s, 2H), 3.58 (m, 5H), 3.02 (m, 2H), 2.68 (d, 3H), 1.60 (m, 4H) 1.30 (d, 3H). ¹³C NMR (CDCl₃): δ 173.2, 170.3, 158.2, 152.0, 137.8, 133.7, 132.8,

131.5, 130.2, 129.5, 122.3, 121.7, 114.3, 76.1, 43.2, 39.1, 31.2, 27.8, 27.1, 22.4. ^{31}P NMR (CDCl_3): δ -11.0 (dd). ^{19}F NMR (CDCl_3): δ -113.5 (dq). ESI MS $[\text{M}+\text{Na}]$: Calcd. for 775.2446 found 775.2455. HPLC (50:50 CH_3CN : ddH_2O): t_{R} = 14.1 min. mp 64–65 °C. Anal. ($\text{C}_{35}\text{H}_{44}\text{ClF}_2\text{N}_4\text{O}_8\text{P}$) C, H, N.

2.2. Preparation of 4-((1-(3-carbamoyl-4-cyclohexylmethoxy phenyl)ethyl)carbamoyl)methyl) phenyl difluoromethyl phosphonic acid 2

To a stirred solution of **1** (64 mg, 0.085 mmol) in CH_2Cl_2 (0.6 ml) was added BSTFA (0.30 ml, 0.37 mmol) at room temperature. After stirring for 30 min, the mixture was cooled to -20 °C and treated with TMSI (55 μl , 0.24 mmol). The reaction was gradually warmed to room temperature over 3 h and then evaporated at reduced pressure to a brown oil. The residue was desilylated with aqueous TFA in CH_3CN (TFA: H_2O : CH_3CN 1:1:2) for 2 h, concentrated, and partitioned between EtOAc/0.1 M aq. HCl (10 ml each). The organic layer was washed with brine (2 $\times$), dried over Na_2SO_4 and concentrated in vacuo to provide **2** (35 mg, 79%) as a yellow foam. ^1H NMR (CD_3OD): 7.94 (s, 1H), 7.51 (d, 1H), 7.38 (m, 3H), 7.04 (d, 1H), 4.92 (m, 1H), 3.95 (d, 2H), 3.68 (s, 2H), 1.80 (m, 6H) 1.43 (d, 3H), 1.20 (m, 5H). ^{13}C NMR (CD_3OD): δ 172.8, 158.2, 137.8, 132.8, 130.1, 127.9, 114.3, 76.1, 43.8, 39.2, 31.3, 27.8, 27.2, 22.4. ^{31}P NMR (CD_3OD): δ -20.1 (t). ^{19}F NMR (CD_3OD): δ -113.5 (dq).

2.3. Preparation of 5-acetyl-2-cyclohexylmethoxy benzoic acid 8

To a stirred solution of methyl-5-acetylsalicylate (0.936 g, 4.82 mmol) in anhydrous CH_3CN (20 ml) was added Cs_2CO_3 (1.726 g, 5.30 mmol), followed by the addition of cyclohexylmethyl bromide (0.74 ml, 5.30 mmol). The reaction mixture was heated under reflux overnight and then cooled to room temperature. The solvent was removed, and the residue was dissolved in EtOAc and filtered. The combined organic layers were washed with brine (2 $\times$), dried, and concentrated in vacuo to provide the methyl ester intermediate (1.36g, quantitative yield) as a pale yellow oil, which was used without purification.

To a stirred solution of the methyl ester (2.74 g, 9.44 mmol) in THF (20 ml), aqueous LiOH (1 M, 10.4 ml) was added dropwise and stirred overnight. The volatiles were removed, and the residue was washed with Et_2O (10 ml). The residue was acidified with aqueous HCl (1 M) to pH 3 and extracted with EtOAc (20 ml $\times$ 3). The combined organic layer was washed with brine, dried and concentrated to afford **8** (2.14 g, 81%) as a white solid. R_{f} = 0.29 (4:1 EtOAc:Hexanes). ^1H NMR (CDCl_3): δ 8.62 (s, 1H), 8.13 (d, 1H), 7.08 (d, 1H), 4.08 (d, 2H), 2.57 (s, 3H), 1.68 (m, 6H), 1.23 (m, 5H). ^{13}C NMR (CDCl_3): δ 196.0, 165.3, 161.2, 134.7, 132.3, 130.8, 117.4, 112.8, 75.8, 37.2, 29.6, 26.4, 26.1, 25.4. HRMS (ESI): calculated for 299.1529 $[\text{M}+\text{Na}]^+$, found 299.1562

2.4. Preparation of 6-(5-acetyl-2-cyclohexylmethoxy benzoylamino) hexanoic acid methyl ester 9

To a stirred solution of **8** (455 mg, 1.65 mmol) in anhydrous CH_2Cl_2 (20 ml) at 0 °C, PyBOP (944 mg, 1.81 mmol) was added and stirred for 10 min. Then methyl 6-aminocaproate hydrochloride (328 mg, 1.81 mmol), prepared from 6-aminocaproic acid according to the method of Lin [8], was added followed by the addition of $i\text{Pr}_2\text{NEt}$ (0.63 ml, 3.62 mmol). The reaction was stirred at 0 °C for 10 min and then warmed to room temperature. After 30 min, saturated NH_4Cl solution was added to quench the reaction. The aqueous layer was extracted with CH_2Cl_2 (15 ml $\times$ 3). The combined organic layers were washed with brine, dried, and concentrated. The crude compound was purified by flash chromatography to afford **9** (0.56 g, 84%) as a white solid. R_{f} = 0.31 (1:1 EtOAc:Hexanes). ^1H NMR (CDCl_3): δ 8.76 (s, 1H), 8.12 (d, 1H), 7.88 (s, 1H), 7.01 (d, 1H), 4.00 (d, 2H), 3.65 (s, 3H), 3.46 (q, 2H), 2.61 (s, 3H), 2.31 (t, 2H), 1.68 (m, 10H), 1.23 (m, 7H). ^{13}C NMR (CDCl_3): δ 196.8, 173.9, 164.4, 160.5, 133.7, 132.5, 130.4, 121.1, 112.3,

74.9, 51.5, 39.7, 37.7, 30.0, 29.3, 26.6, 26.5, 26.2, 25.6, 24.6. HRMS (ESI): calculated for 404.2437 [M+H]⁺, found 404.2439.

2.5. Preparation of 6-[5-(1-aminoethyl)-2-cyclohexylmethoxy benzoylamino] hexanoic acid methyl ester **10**

To a stirred solution of **9** (0.56g, 1.4 mmol) in anhydrous MeOH (5 ml), titanium (IV) isopropoxide (0.82 ml, 2.8 mmol) was added, followed by the addition of ammonia-saturated MeOH (7.0 M, 1.0 ml) [9]. The reaction mixture was stirred in a capped flask for 12 hr. Then NaBH₄ (79 mg, 2.1 mmol) was added and the resulting cloudy mixture was stirred for an additional 3 hr. The reaction was quenched by pouring into aqueous ammonium hydroxide (0.27 ml, 6.9 mmol). The resulting inorganic precipitate was filtered off and washed with EtOAc (10 ml × 2). The organic layer was separated and the remaining aqueous layer was extracted with EtOAc (10 ml × 4). The combined organic solution was then extracted with aqueous HCl (1 M, 6.0 ml) to separate the neutral materials. The acidic aqueous extracts were washed with EtOAc (15 ml), then treated with aqueous NaOH (2 M) to pH 12, and extracted with EtOAc (15 ml × 4). The combined organic extracts were washed with brine, dried and concentrated in vacuo to afford **10** (622.6 mg, 62 %). R_f = 0.31 (1:1 EtOAc:Hexanes). ¹H NMR (CDCl₃): δ 8.12 (d, 1H), 7.98 (s, 1H), 7.47 (d, 1H), 6.87 (d, 1H), 4.10 (q, 1H), 3.89 (d, 2H), 3.62 (s, 3H), 3.46 (q, 2H), 2.26 (t, 2H), 1.68 (m, 10H), 1.23 (m, 10H). ¹³C NMR (CDCl₃): δ 173.8, 165.1, 155.8, 140.2, 129.6, 129.3, 121.0, 112.2, 74.4, 51.3, 50.4, 39.4, 37.7, 33.7, 30.0, 29.2, 26.5, 26.1, 25.5, 25.4, 24.5. HRMS (ESI): calculated for 405.2753 [M+H]⁺, found 405.2750

2.6. Preparation of 4-[(diethoxy phosphoryl)difluoromethyl] phenylacetic acid **11**

To a stirred solution of **5** (446 mg, 1.33 mmol) in THF (20 ml) and H₂O (5 ml), aqueous LiOH (0.3 M, 6.6 ml) was added dropwise at 0 °C and then stirred for 20 min. The reaction was quenched with the addition of 1 % citric acid to adjust the pH to 4. The volatiles were removed and the residue was extracted with EtOAc. (20 ml × 3). The combined organic layer was washed with brine (1×), dried, and concentrated to afford **11** (2.14 g, 81%) which was used without purification. R_f = 0.29 (4:1 EtOAc:Hexanes). ¹H NMR (CDCl₃): δ 7.62 (d, 2H), 7.32 (d, 2H), 4.18 (m, 4H), 3.58 (s, 2H), 1.28 (t, 6H). ³¹P NMR (CDCl₃): δ -19.1 (t). ¹⁹F NMR (CDCl₃): δ -113 (d)

2.7. Preparation of 6-{2-cyclohexylmethoxy-5-[1-(2-{4-[(diethoxyphosphoryl)difluoromethyl]-phenyl}acetyl amino)ethyl]benzoylamino}hexanoic acid **12**

To a stirred solution of **11** (227 mg, 0.70 mmol) in anhydrous CH₂Cl₂ (8 ml) at 0 °C, PyBOP (367 mg, 0.70 mmol) was added and stirred for 10 min. Then the solution of amine **10** (285 mg, 0.70 mmol) in CH₂Cl₂ (2 ml) was added, followed by the addition of iPr₂NEt (0.19 ml, 1.06 mmol). After 10 min, the reaction mixture was warmed to room temperature and stirred for 30 min. The reaction was quenched with the addition of saturated NH₄Cl and the aqueous layer was extracted with CH₂Cl₂ (10 ml × 3). The combined organic extracts were washed with brine (1×), dried, and concentrated in vacuo. The residue was purified by flash chromatography to afford the coupled methyl ester (0.29 g, 58 %). R_f = 0.13 (4:1 EtOAc:Hexanes). ¹H NMR (CDCl₃): δ 8.12 (d, 1H), 8.05 (t, 1H), 7.47 (d, 2H), 7.27 (d, 2H), 6.80 (d, 1H), 6.58 (d, 1H), 5.00 (m, 1H), 4.10 (m, 4H), 3.81 (d, 2H), 3.62 (s, 3H), 3.50 (s, 2H), 3.46 (q, 2H), 2.26 (t, 2H), 1.68 (m, 10H), 1.23 (m, 13H). ¹³C NMR (CDCl₃): δ 173.9, 169.4, 165.2, 156.1, 138.2, 136.1, 131.3, 130.8, 129.2, 128.9, 126.3, 121.0, 112.2, 77.2, 74.4, 64.8, 64.7, 51.4, 48.3, 42.9, 39.4, 37.7, 33.7, 29.9, 29.2, 26.5, 26.2, 25.5, 24.4, 21.6, 16.2, 16.1. ³¹P NMR (CDCl₃): -19.2 (t). ³¹F NMR (CDCl₃): -113.4 (d). HRMS (ESI): calculated for 731.3249 [M+Na]⁺, found 731.3250.

The methyl ester (0.29 g, 0.41 mmol) was dissolved in THF (9 ml) at 0 °C, and aqueous LiOH solution (0.3 M, 4.1 ml) was added dropwise and stirred for 30 min. The reaction mixture was warmed to room temperature and stirring was continued for 1 h. The reaction was quenched with the addition of citric acid to adjust the pH to 4, and the aqueous layer was extracted with EtOAc (10 ml × 3). The combined organic extracts were washed with brine (1×), dried, and concentrated in vacuo to afford **12** (0.29 g, 58 %). R_f = 0.13 (4:1 EtOAc:Hexanes). ^1H NMR (CDCl_3): δ 8.12 (d, 1H), 8.05 (t, 1H), 7.47 (d, 2H), 7.27 (d, 2H), 6.80 (d, 1H), 6.58 (d, 1H), 5.00 (m, 1H), 4.10 (m, 4H), 3.81 (d, 2H), 3.62 (s, 3H), 3.50 (s, 2H), 3.46 (q, 2H), 2.26 (t, 2H), 1.68 (m, 10H), 1.23 (m, 13H). ^{13}C NMR (CDCl_3): δ 173.9, 169.4, 165.2, 156.1, 138.2, 136.1, 131.3, 130.8, 129.2, 128.9, 126.3, 121.0, 112.2, 77.2, 74.4, 64.8, 64.7, 51.4, 48.3, 42.9, 39.4, 37.7, 33.7, 29.9, 29.2, 26.5, 26.2, 25.5, 24.4, 21.6, 16.2, 16.1. ^{31}P NMR (CDCl_3): -19.2 (t). ^{19}F NMR (CDCl_3): -113.4 (d). HRMS (ESI): calculated for 689.2779 $[\text{M}+\text{Na}]^+$, found 689.2792.

2.8. Preparation of immobilized ligand 3

To a stirred solution of **12** (96 mg, 0.19 mmol) in anhydrous CH_2Cl_2 (4 ml) at 0 °C, PyBOP (148 mg, 0.29 mmol) was added and stirred for 10 min. Then the reaction mixture was added to LCAA-CPG (0.625g, 80 $\mu\text{mol/g}$) followed by the addition of $i\text{Pr}_2\text{NEt}$ (0.13 ml, 0.76 mmol). The resultant mixture was shaken gently at room temperature for 40 min and then filtered. The beads were washed with CH_2Cl_2 , then treated with Ac_2O :NMP:pyridine (1:2:2) for 1 h to cap any free amines. The beads were then washed with CH_2Cl_2 , Et_2O , and dried in vacuo to give the immobilized phosphonate ester. To a suspension of these beads (0.375 g) in CH_2Cl_2 (20 ml) was successively added TBAI (1.84 g, 5 mmol), BSTFA (1.33 ml, 5 mmol), and $\text{BF}_3\cdot\text{Et}_2\text{O}$ (65 μl , 0.5 mmol) at room temperature. The mixture was shaken for 1.5 h and then washed with CH_2Cl_2 , DMF, Et_2O , and dried in vacuo to afford **3** (loading ~10 $\mu\text{mol/g}$).

2.9. Preparation of 6-[2-cyclohexylmethoxy-5-(1-phenylacetyl-aminoethyl)-benzoylamino]-hexanoic acid 13

To a stirred solution of phenylacetic acid (88 mg, 0.65 mmol) in anhydrous CH_2Cl_2 (8 ml) at 0 °C, PyBOP (338 mg, 0.65 mmol) was added and stirred for 10 min. Then a solution of **10** (264 mg, 0.65 mmol) in CH_2Cl_2 (2 ml) was added, followed by the addition of $i\text{Pr}_2\text{NEt}$ (0.17 ml, 0.98 mmol). After 10 min, the reaction mixture was warmed to room temperature and stirred for 30 min. The reaction was quenched with addition of saturated NH_4Cl , and the aqueous layer was extracted with CH_2Cl_2 (10 ml × 3). The combined organic extracts were washed with brine (1×), dried, concentrated in vacuo, and purified via flash chromatography to afford the coupled methyl ester (0.32 g, 94 %). R_f = 0.30 (3:1 EtOAc:Hexanes). ^1H NMR (CDCl_3): δ 8.12 (d, 1H), 8.05 (t, 1H), 7.47 (d, 2H), 7.27 (m, 5H), 6.70 (d, 1H), 6.58 (d, 1H), 5.98 (d, 1H), 5.05 (m, 1H), 3.81 (d, 2H), 3.62 (s, 3H), 3.50 (s, 2H), 3.46 (q, 2H), 2.32 (t, 2H), 1.68 (m, 10H), 1.23 (m, 13H). HRMS (ESI): calculated for 545.2991 $[\text{M}+\text{Na}]^+$, found 545.2997.

To a solution of the methyl ester (36 mg, 0.069 mmol) in THF (2 ml) at 0 °C, aqueous LiOH (0.3 M, 0.70 ml) was added dropwise and stirred for 30 min. The reaction mixture was warmed to ambient temperature and stirred for 1 h. The reaction was quenched with the addition of citric acid to adjust pH to 4, and the aqueous layer was extracted with EtOAc (5 ml × 3). The combined organic extracts were washed with brine, dried and concentrated in vacuo to afford **13** (32 mg, 92 %). R_f = 0.42 (5:95:0.3 MeOH:EtOAc:HOAc). ^1H NMR (CDCl_3): δ 8.12 (d, 1H), 8.05 (t, 1H), 7.34 (m, 6H), 6.82 (d, 1H), 6.38 (d, 1H), 5.00 (m, 1H), 3.89 (d, 2H), 3.52 (s, 3H), 3.45 (q, 2H), 2.32 (t, 2H), 1.68 (m, 10H), 1.23 (m, 13H). HRMS (ESI): calculated for 509.3015 $[\text{M}+\text{H}]^+$, found 509.3008.

2.10. Preparation of immobilized control ligand **4**

To a stirred solution of **13** (64 mg, 0.13 mmol) in anhydrous CH₂Cl₂ (3 ml) at 0 °C, PyBOP (65 mg, 0.13 mmol) was added and stirred for 10 min. Then the reaction mixture was added to LCAA-CPG (0.39g, 80 μmol/g) followed by addition of iPr₂NEt (66 μl, 0.38 mmol). The resulting mixture was shaken at room temperature for 40 min and then passed through a filter. The remaining beads were washed with CH₂Cl₂, then treated with Ac₂O:NMP:pyridine (1:2:2) for 1 h to cap any free amines. The beads were then washed with CH₂Cl₂, Et₂O, and dried in vacuo to afford **4** (loading ~10 μmol/g).

2.11. Instrumentation, apparatus and techniques

All NMR spectra were recorded on a 300 MHz Bruker instrument equipped with a multinuclear (³¹P, ¹³C, ¹H and ¹⁹F) probe. ¹H chemical shifts are reported in parts per million using tetramethylsilane (TMS) as an internal reference. All ³¹P NMR spectra were acquired using broadband gated decoupling. ³¹P chemical shifts are reported in parts per million using 1% triphenylphosphine oxide in benzene-d₆ as the coaxial reference. Mass spectral data were obtained from the Purdue University Mass Spectrometry Service. Elemental analyses were performed by the Purdue University Microanalysis lab.

Flash chromatography using silica gel grade 60 (230–400 mesh) was carried out for all chromatographic separations. Thin layer chromatography (TLC) was performed using Analtech glass plates precoated with silica gel (250 microns). Visualization of the plates was accomplished using UV and/or the following stains: 1% 4-(p-nitrobenzyl)pyridine in acetone followed by heating and subsequent treatment with 3% KOH in methanol; or p-anisaldehyde dip (1.85% p-anisaldehyde, 20.5% sulfuric acid, 0.75% acetic acid in 95% EtOH) followed by heating; or Hanessian's dip (5.0 g Ce(SO₄)₂, 25.0 g (NH₄)Mo₇O₂₄·4H₂O, 50 ml H₂SO₄, 450 ml H₂O) followed by heating. All organic solvents were distilled prior to use unless specified.

2.12. Cellular assays

Leukemia or lymphoma cells seeded at a density of 5×10⁴ cells/ml were treated with the indicated concentration of compound **1** or with DMSO carrier alone (0.2% final concentration) for 48 h. The number of live cells as indicated by Trypan blue exclusion was counted. For cell cycle analyses, cells were fixed in ice-cold 75% ethanol in PBS and treated with Vindelov's propidium iodide staining solution (10 mM Tris, 10 mM NaCl, 0.7 U/ml RNase, 50 μg/ml propidium iodide and 0.1% NP-40) on ice for 30 min. The amount of DNA was analyzed in the Purdue Analytical Cytometry facility on a Cytomics FC500 flow cytometer (Beckman Coulter, Inc., Indianapolis, IN). DG75 cells were synchronized by incubation in media containing 0.5 mM mimosine for 24 h. After removal of mimosine, cells were collected at different time points and then fixed and stained. For immunofluorescence studies of lymphoid cells, cells were transferred to poly-lysine coated cover slips, fixed in 10% formaldehyde in PBS, and permeabilized with 0.5% Triton X-100 in PBS. Nuclei were stained with 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) (Sigma-Aldrich, Inc., St. Louis, MO). For immunostaining, cells were blocked with 5% goat serum in PBS at room temperature for 1 h after permeabilization, incubated with primary anti-α-tubulin antibody (Sigma-Aldrich, Inc., St. Louis, MO) for 1–2 h or at 4°C overnight and then with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR) for 1 h and examined by fluorescence microscopy.

For comparison of the effects of compound **1** relative to MCAK RNAi, HeLa cells were plated at 20,000 cells/ml onto poly-lysine coverslips in 6 well plates. Cells were transfected with negative control siRNA or MCAK siRNAs at 20 nM using lipofectamine RNAi max. At 24 h post-transfection, cells were treated with control DMSO or 5 μM compound **1** for an

additional 24 h. Cells were then fixed with 4% formaldehyde, 0.1% glutaraldehyde in PHEM (25 mM Hepes, 2 mM MgCl₂, 10 mM EGTA, pH 6.9) and processed for immunofluorescence using 1/500 dilution DM1- α (Sigma-Aldrich, Inc., St. Louis, MO), followed by 1 μ g/ml Alexa-488 secondary antibodies (Life Technologies Corporation, Carlsbad, CA). DNA was stained with 2 μ g/ml Hoechst, and coverslips were mounted in prolong gold. Images were collected with a Nikon 90i microscope with a 100X 1.4 NA plan-apo objective and a Photometrics CoolSnap HQ CCD camera, all controlled by Metamorph software. Image stacks (0.5 μ m step size) were collected through the cell volume and then processed for deconvolution using AutoQuantX for 30 iterations. Mitotic stage distribution was quantified in 4 independent experiments by counting the percentage of cells in each mitotic stage in which at least 100 mitotic cells were counted per condition in each experiment.

2.13. Analysis of protein phosphorylation in treated cells

CEM6 cells were treated with compound **1** (7 μ M) or DMSO carrier alone for 48 h prior to stimulation with anti-CD3 antibody for 5 min at 37°C. Cells were harvested and lysed in buffer containing 1% NP-40, 25 mM Hepes, pH 7.5, 1 mM EDTA, 150 mM NaCl, 2 mM sodium orthovanadate, 100 μ g/ml each of aprotinin and leupeptin, and 625 μ M phenylmethylsulfonyl fluoride (PMSF). Proteins in supernatants collected following centrifugation at 18,000 $\times g$ for 10 min were separated by SDS-PAGE and analyzed by Western blotting with antibodies against phosphotyrosine (4G10, EMD Millipore Corporation, Billerica, MA).

For the analysis of histone H3 phosphorylation, DG75 B cells (4×10^5 cells/ml) were treated with DMSO alone or with the indicated concentrations of compound **1** for 24 h or with nocodazole (10 μ M) for 18 h. Cell lysates were separated by SDS-PAGE and blotted using an antibody specific for histone H3 phosphorylated on serine-10 (Cell Signaling Technology, Inc. Danvers, MA).

2.14. Mass spectrometric analyses

Human DG75 cells were lysed in buffer containing 1% NP-40, 150 mM NaCl, 25 mM HEPES, pH 7.5, 1 mM EDTA, 2 mM sodium orthovanadate, 2 mM sodium fluoride, 100 μ g/ml aprotinin, 100 μ g/ml leupeptin and 625 μ M PMSF. Lysates were centrifuged 10 min at 18,000 $\times g$. Supernatants were incubated with the drug-conjugated beads **3** or control beads **4** for 2 h at 4°C. Beads were washed three times with lysis buffer containing 2 M NaCl. Beads were incubated in 0.2% (w/v) RapiGestTM SF (Waters Corporation, Milford, MA) in 50 mM ammonium bicarbonate (NH₄HCO₃) and heated at 90°C for 5 min. 10% of the eluted proteins were separated by SDS-polyacrylamide gel and visualized by silver staining using the ProteoSilverTM Silver stain kit (Sigma-Aldrich, Inc., St. Louis, MO) or by Western blotting using a variety of specific antibodies. Trypsin was added to the remaining proteins and incubated at 37°C overnight. The solution was acidified to a pH below 2 and centrifuged to remove insoluble material (products from the degradation of RapiGest). The supernatant was dried using a Speedvac and reconstituted in 0.1% formic acid. Tryptic peptides were analyzed on an Agilent 1100 nanoflow liquid chromatograph (Agilent Technologies, Wilmington, DE) connected to an LTQ linear ion trap mass spectrometer (Thermo Fisher, San Jose, CA). The mass spectrometer was operated in the data-dependent mode, in which a full scan MS was followed by MS/MS scans of the 10 most abundant ions with +2 to +3 charge states. All MS/MS data were searched against the human database using the SEQUEST algorithm [10] on the SorcererTM IDA server (SageN, Inc, San Jose, CA). A PeptideProphet [11] and ProteinProphet [12] threshold of 0.9 probability score was used for all peptide identifications and protein assignment (the estimated false positive rate is 1.1% for a probability score of 0.9).

For the mass spectrometric analysis of prodrug activation, DG75 cells seeded at a density of 6×10^5 cells/ml were treated with 7 μ M of compound **1** for 24 h. Cells were harvested, washed with PBS, resuspended in 10 mM Hepes, pH 7.4, 10 mM KCl, 0.1 mM EDTA, and lysed by sonication. Proteins present in supernatants from a centrifugation at $18,000 \times g$ for 5 min were precipitated using a 1:5 ratio of lysate to acetonitrile. The supernatant of a 5 min, $18,000 \times g$ centrifugation was removed, dried under vacuum and resuspended in 0.1% formic acid. Chromatography was performed using an in-house C18 capillary column packed with 5 μ m C18 Magic beads (Michrom; 75 μ m i.d. and 12 cm of bed length) on an 1100 Agilent HPLC with an eluting buffer of 100% acetonitrile run over a modified gradient of 5–40% acetonitrile for 10 min and 40–80% acetonitrile for 30 min with a flow rate of 0.3 μ l/min. The electrospray ionization emitter tip was generated on the prepacked column with a laser puller (Model P-2000, Sutter Instrument Co.). The HPLC system was coupled online with an LTQ Orbitrap hybrid mass spectrometer (Thermoelectron, San Jose, CA, USA).

2.15. Ligand binding assay

The GST-Lck-SH2 fusion protein was expressed in *E. coli* and isolated by affinity chromatography using glutathione linked to Sepharose (Sigma-Aldrich, Inc., St. Louis, MO). GST-Lck-SH2 was eluted with 20 mM glutathione, dialyzed against 20 mM Tris/HCl, pH 7.5, and concentrated using an Amicon centrifugal filter. Fluorescence measurements were taken at room temperature using a Fluoro Max-2 fluorometer (Jobin Yuon-Spex Instruments S. A. Inc., Edison, NJ). The 4-nitrobenzo-2-oxa-1,3-diazole (NBD)-labeled peptide (Ac-Glu-Glu-Glu-Ile-pTyr-Dap(NBD)-Glu-Ile-Glu-Ala-NH₂) was synthesized by Biomer Technology, Pleasanton, CA. Experiments were performed by measuring fluorescence changes upon titrating compound **2** into a solution containing GST-Lck-SH2 (1 μ M) and NBD-labeled peptide (2 μ M) in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 1 mM DTT.

2.16. MCAK ATP binding

MCAK-ligand docking studies used the crystal structure with a PDB identification of 1V8J and Glide software from the Schrödinger package (version 5.6) [13]. The protein was prepared using the Protein Preparation Wizard function, which includes optimization of hydrogen bonds and minimization of the protein to an RMSD of 0.3 Å under the OPLS 2005 force field. The grid where the ligand will be docked was centered at the ATP binding site by selecting the cocrystallized ADP. The ligand was prepared using the LigPrep (version 2.4) software using Epik (version 2.1) to generate possible states in the pH range of 7 (+/−) 2. The maximum number of isomers generated was 32. Once the ligand and grid were prepared, Extra Precision (XP) Glide docking was performed [14].

To monitor the ATP-dependence of the MCAK-drug interaction, detergent lysates from DG75 cells were adsorbed to the immobilized ligand **3** as described above. The beads were then incubated with NP40 lysis buffer containing the indicated concentrations of MgATP for 15 min. The beads were then washed 2 times with lysis buffer. Bound proteins were separated by SDS-PAGE followed by Western blotting with antibodies against MCAK.

To measure MCAK ATPase activity, His-tagged MCAK (0.5 μ M) (Addgene plasmid 25551) expressed and isolated from *E. coli* (cultured at 16°C) was incubated in reaction buffer containing 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT, 5 mM HEPES, pH 7.2, 0.1 mg/ml tubulin (Cytoskeleton), 1 mM [γ -³²P]ATP and the indicated concentration of compound **2** (or DMSO solvent alone) for 90 min at room temperature. Reactions were quenched by the addition of 40 mg of activated charcoal in 50 mM NaH₂PO₄. Charcoal was removed by centrifugation and the supernatant counted by scintillation spectrometry. Values were

corrected for background counts obtained in reactions run in the absence of added MCAK and are expressed as percent of control for reactions containing DMSO only.

3. Results

3.1. Synthesis of prodrug **1**

Building block **6** was synthesized from the phosphonate **5** as previously described [7] (Fig. 1A). PyBOP-catalyzed coupling with aminobenzamide **7**, prepared as described [16], produced the difluoromethylphosphonate prodrug **1**. This prodrug bears both delivery and masking groups to deliver intracellularly a stable SH2 domain inhibitor. Drugs of this class are activated by bioreductive elimination of the delivery group followed by spontaneous liberation of the masking group to release the free phosphonate **2**, the active charged form of the therapeutic agent, within the cell (Fig. 1B). Enhanced stability of the target compound was achieved through the use of an aryl difluoromethylphosphonate as a phosphotyrosine mimic, since it is resistant to both enzymatic and hydrolytic cleavage and generates the appropriately negatively charged species at physiological pH [17].

3.2. Inhibition of tumor cell growth

To evaluate the effects of compound **1** on tumor cells, we submitted the prodrug to the National Cancer Institute Developmental Therapeutics Program for evaluation in the 60 cancer cell line panel (NCI-60) [18]. Compound **1** exhibited broad growth inhibitory activity in the low micromolar range, but was particularly effective against the leukemia/lymphoma subpanel. While **1** was growth inhibitory, it was largely cytostatic and thus did not induce significant cell death ($LC_{50} > 100 \mu M$) (data not shown).

The growth-inhibitory effects of compound **1** were confirmed in our laboratory in several cell lines including CEM6, a human T cell leukemia; DT40, a chicken immature B cell lymphoma; WEHI-231, a mouse immature B cell lymphoma; DG75, a human mature B cell lymphoma, Nalm-6, a human pre-B acute lymphoblastic leukemia, and JCam1, an Lck-deficient derivative of Jurkat human T cell lymphoblast-like cells. In these assays, cells were treated with different concentrations of compound **1** for 48 h in the presence of serum, and the number of viable cells—those excluding trypan blue—was counted. The growth of all the cell lines was inhibited in a dose-dependent manner. For most cell lines, 90% growth inhibition occurred at a drug concentration of 4–7 μM (Fig. 2A, B). No obvious cell death was observed in treated cells, which is consistent with the results from the NCI tumor panel.

Since compound **1** was originally designed to target the SH2 domains of Src-family kinases, we tested its effects on cell lines lacking specific members of this family of enzymes. Compound **1** retained inhibitory activity against the growth of JCam1 cells, which lack Lck (Fig. 2B). The growth-inhibitory effect of the compound was also retained in DT40 cells that had been engineered to lack the expression of the Lyn protein-tyrosine kinase [19], which is the only Src-family kinase readily detectable in DT40 cells [19]. Thus, the inhibitory effects of compound **1** on cell growth were not restricted to cells whose proliferation was dependent on the activities of Src-family kinases. To ensure that the activated form of the inhibitor **2** could bind with high affinity to a group I SH2 domain, we monitored its ability to compete with a fluorescently tagged peptide ligand for binding to an expressed fusion protein consisting of GST linked to the SH2 domain of the Src-family tyrosine kinase, Lck (Fig. 2C). Compound **2** bound with an estimated K_d of 0.1 μM in this assay. To confirm that the prodrug **1** was actually converted into the active form **2** in cultured cells, we prepared extracts from drug-treated DG75 B cells, separated these by HPLC and searched for the active compound by mass spectrometry (Fig. 2D). Active drug **2** was readily detected in the treated cells. It also was possible that the activated drug would

stimulate the activity of Src-family kinases by displacing the phosphorylated, inhibitory C-terminal tail from the SH2 domain or increase overall tyrosine phosphorylation through the inhibition of tyrosine phosphatases. However, the treatment of CEM6 T cells with compound **1** failed to enhance the level of protein-tyrosine phosphorylation in either unstimulated or TCR-stimulated cells (Fig. 3A).

3.3. Cell cycle progression and endoreduplication

Since growth inhibition without prominent cell death was observed in cells treated with compound **1**, we monitored progression through the cell cycle to determine if there was an arrest at a specific stage. Cells were treated with compound **1** or DMSO for 48 or 72 h, and the distribution of cells in each stage of the cell cycle was analyzed by measuring DNA content using propidium iodide staining followed by flow cytometry. None of the cells examined showed an obvious arrest at any single stage of the cell cycle (Fig. 4). However, several cell lines showed an increased DNA content for the entire population of cells following treatment. Drug-treated WEHI-231 and DT40 cells showed twice the normal DNA content of untreated cells (Fig. 4A and C) while DG75 cells exhibited a 8C/16C DNA content after 72 h of treatment (Fig. 4B), consistent with cells undergoing endoreduplication [20]. None of the treated cells exhibited a sub-G1 peak characteristic of apoptotic cells. Drug treatment failed to decrease histone H3 Ser-10 phosphorylation, indicating a lack of effect on the Aurora B kinase, the inhibition of which can induce endoreduplication (Fig. 3B).

To examine the effects of compound **1** on cell cycle progression in more detail, we arrested DG75 cells by blocking DNA replication with mimosine. When released from the S phase block, both treated and untreated DG75 cells began to transit through the cell cycle. However, as the drug became activated, the treated cells progressed more slowly, began to accumulate increased ploidy and failed to return to the normal 2C/4C DNA distribution observed in the untreated cells. However, no obvious arrest at any particular stage of the cell cycle was observed (Fig. 4D).

A further examination of DG75 cells showed that, after treatment with compound **1** for 72 h, most cells had two nuclei or a bilobed nucleus as detected by DAPI-staining and fluorescence microscopy (Fig. 5A). However, only single nuclei were present in drug-treated DT40 or WEHI-231 cells (data not shown) even though these cells exhibited a higher than normal DNA content. Interestingly, when examined by phase contrast microscopy, many of the drug-treated cells were attached to one another by thin connections or midbodies (Fig. 5B). These observations suggested defects in both mitosis and cytokinesis, which could contribute to the observed increase in DNA content reported above. We examined, therefore, possible effects of compound **1** on the microtubule network. WEHI 231 cells were treated with compound **1** or with DMSO carrier alone and then fixed and stained with antibodies against α -tubulin. Interestingly, staining of the microtubule network emanating from the microtubule organizing center was markedly enhanced in drug-treated cells as compared to control cells, suggesting that microtubule polymerization was enhanced or depolymerization was inhibited (Fig. 5C).

3.4. Synthesis of immobilized ligands

The inhibitory effect of compound **1** on the growth of cells lacking Src-family kinases indicated the existence of alternative targets. A molecule such as compound **1**, once metabolically activated to the phosphonate **2**, could have multiple intracellular targets, as many proteins contain modules capable of binding phosphotyrosines or other phosphorylated ligands. To determine possible molecular targets, we carried out a screen designed to identify proteins capable of interacting with the active,

difluoromethylphosphonate form of the drug. For this purpose, we synthesized resin **3**, a form of compound **2** that is tethered to a solid support for the isolation by affinity chromatography of potential protein binding partners (Fig. 6). To control for nonspecific interactions or for interactions that might occur independently of the difluoromethylphosphonate moiety, we generated a second affinity resin **4** containing a structurally similar molecule lacking the charged group for use as a control (Fig. 7).

To generate the drug-conjugated resin, compound **8** was prepared from methyl 5-acetylsalicylate (Fig. 6). A PyBOP coupling reaction was carried out between compound **8** and methyl 6-aminocaproate to give compound **9** which was reductively aminated to give the desired linker-modified amine **10**. Phosphonate **5** was hydrolyzed and reacted with compound **7** and the resulting methyl ester hydrolyzed to give the linker-modified phosphonate **12**. Compound **12** was directly coupled to long-chain alkylamine-modified controlled pore glass (LCAA-CPG) to provide the immobilized phosphonate ester, which was hydrolyzed to the immobilized free phosphonate **3**.

To generate the control resin, compound **7** was reacted with phenylacetic acid and the resulting coupling product hydrolyzed to give the free acid **13** (Fig. 7). Acid **13** was coupled to LCAA-CPG to afford the immobilized phosphonate-free ligand **4**. The residual free amines on the beads of resins **3** and **4** were capped with Ac₂O:NMP:pyridine (1:2:2) [22].

3.5. Analysis of drug targets

Lysates from DG75 B cells were incubated with the drug-conjugated beads or with the control beads. Bound proteins were separated by SDS-PAGE and detected by silver staining. Several proteins were observed to bind to the drug-conjugated resin, but few proteins bound to the control resin at levels that could be detected easily by this method (Fig. 8A). To determine if proteins with SH2 domains bound to the drug-conjugated beads, we used antibodies against several known B cell proteins to analyze for their presence by Western blotting. Vav1, PLC- γ 2, Lyn and Syk all were found to bind selectively to the drug-conjugated resin (Fig. 8B). These results confirmed the ability of the activated drug to interact with proteins containing SH2 domains.

Bound proteins were eluted, digested with trypsin and analyzed by mass spectrometry to identify other candidate target proteins. Several proteins (Table 1) bound selectively to the drug-conjugated resin. These included metabolic enzymes, many of which bind phosphorylated metabolites or co-factors; nucleic acid-binding proteins including multiple members of the mini-chromosome maintenance (MCM) complex (MCM3, MCM4, MCM5, MCM6, MCM7 and Cdc45); three protein kinases (Cdc2 (Cdk1), Akt2 and protein kinase N1 (PKN1)); several nucleotide-binding proteins including Rab-8, Ran and Septin 9 (SEPT9); and mitotic centromere-associated kinesin (MCAK), a regulator of microtubule depolymerization. Several proteins, including tubulin, some mitochondrial proteins and heat shock proteins were observed in samples that were eluted from both resins (Table 2), implying that they might be either nonspecific contaminants or were bound to the ligand independently of the difluoromethylphosphonate moiety. A subset of these proteins was examined by Western blotting to confirm their identification and the specificity of their associations. As shown in Fig. 8C, Akt2, MCM3, PKN1, MCAK and SEPT9 all were proteins that bound selectively to the drug-conjugated resin while tubulin was found, as expected, to be bound to both the control and drug-conjugated supports.

3.6. Analysis of MCAK as a drug target

The increase in polymerized microtubules resulting from the treatment of cells with compound **1** suggested that the prodrug's effects on cellular mitosis was mediated, at least in

part, through the modulation of microtubule polymerization, suggesting MCAK as a possible drug target. Examination of the MCAK crystal structure with bound ADP (PDB 1V8J) [23] suggested that the adenine ring of ADP partially filled a deeper pocket in the MCAK surface that might accommodate the cyclohexane ring of the active form of the drug **2**. To explore this possibility, we docked **2** *in silico* at the ATP binding site using the Glide program in the Schrödinger software suite. A calculated G score for the inhibitor of -15.3 compared favorably to those of -14.3 for ATP and -13.4 for ADP, indicating favorable binding energetics. The phosphonate moiety of **2** binds to the same backbone N-H groups (amino acids 165–169) as the terminal phosphate group of ADP, and the side chain N-H of Gln-83 hydrogen bonds to the primary carboxamide carbonyl group in **2** and the 3'-hydroxyl oxygen in ADP. The internal carboxamide N-H of **2** also hydrogen bonds to Asp-175 (Fig. 9A). Consistent with an interaction of compound **2** with the ATP-binding site, MCAK binding to the immobilized drug could be inhibited by increasing concentrations of ATP (Fig. 9B). To determine if compound **2** functioned as a direct inhibitor of MCAK, we expressed a His-tagged globular motor domain of MCAK in bacteria and assessed the effect of the drug on the ATPase activity of the purified protein. As shown in Fig. 9C, the MCAK catalyzed hydrolysis of ATP was inhibited by increasing concentrations of compound **2**.

To determine if the inhibition of MCAK might mediate some of the actions of compound **1** on cultured cells, we compared the effects of the prodrug on mitotic spindle formation in HeLa cells to the effects of MCAK knockdown by RNA interference. Cells treated with prodrug **1** exhibited excess astral microtubules, reduced microtubules at the spindle equator and misaligned chromosomes, phenotypes similar to those observed in MCAK knockdown cells or in cells treated with both compound **1** and MCAK siRNA (Fig. 10A). Treatment with both the drug and siRNA resulted in an increase of mitotic cells in prometaphase (Fig. 10B). These results are consistent with the identification of MCAK as a major intracellular target for the phosphopeptidomimetic **2**.

4. Discussion

As common structural and functional motifs found on multiple participants of signal transduction pathways, SH2 domains are attractive drug targets. However, relatively little is known how small molecules directed against them affect cellular processes. In the current study, we utilized a prodrug delivery strategy to introduce a ligand into cells that was designed to target group I SH2 domains of Src-family kinases [24]. In fact, the drug is able to bind to the isolated SH2 domain of Lck and to interact with Lyn, which is the predominant Src-family kinase in B cells. In the NCI tumor cell panel, this inhibitor is cytostatic and exhibits selectivity for the leukemia/lymphoma cohort of cell lines, which are cell types in which Src-family kinases are particularly important regulators of cell signaling. However, the growth inhibitory effects of compound **1** on these cells appears to be independent of its ability to target Src-family kinases. Thus, it is likely that other proteins account for the drug's ability to inhibit cell growth. Among the proteins that bind to the drug-conjugated resin are additional SH2 domain-containing molecules including Syk, Vav1 and PLC- γ 2. However, it is clear from proteomic analyses that potential candidates for drug targets are not limited to proteins containing SH2 domains, as multiple proteins lacking such a domain are capable of binding to the immobilized ligand.

The effect of compound **1** on the growth of a variety of leukemia or lymphoma cell lines is very interesting. The majority of cells treated with compound **1** undergo endoreduplication, which is the replication of DNA in the absence of mitosis. The induction of endoreduplication in the absence of cell cycle arrest is reminiscent of inhibitors of Aurora B, a kinase that regulates multiple aspects of chromosome segregation during mitosis [25, 26]. However, we are unable to detect a drug-induced decrease in the phosphorylation of histone

H3 on serine-10, which is a characteristic signature of an Aurora B kinase inhibitor. Interestingly, one of the important substrates known to be regulated by Aurora B during mitosis is MCAK [27–29]. MCAK is a kinesin-13 family member that plays an important role in microtubule dynamics and spindle assembly by promoting the dissociation of tubulin from the ends of microtubules in an ATP-dependent fashion [30, 31]. Molecular docking, drug-binding and ATPase inhibition assays are consistent with an interaction of the active drug **2** with the ATP-binding site on MCAK. The knockdown of MCAK with siRNA, treatment with compound **1** or treatment with both compound **1** and MCAK siRNA produce similar phenotypes with respect to the increased microtubule content of spindles and misalignment of chromosomes. These data support the identification of MCAK as one of the intracellular targets of the activated prodrug that mediates its effects on cellular mitosis.

The effects of compound **1** on cells are not completely phenocopied by the knockdown of MCAK suggesting the existence of additional targets, which is consistent with the results of our mass spectrometric analyses of drug-binding proteins. Which of these is responsible for the additional phenotypes induced by compound **1** are as yet unclear as several candidates have described functions in cell proliferation, cell cycle regulation, mitosis and cytokinesis. For example, the heterohexameric MCM complex is a critical component of the pre-replication complex that functions to limit DNA replication to one time per cell cycle [32, 33]. Five of the six components of the MCM complex (MCM3–7) as well as Cdc45, a protein that is recruited to the complex in late G1, are proteins that were identified as binding selectively to the immobilized SH2 domain ligand **3**. The knockdown of MCM3 by RNA interference causes a variety of effects on cells, including an incomplete separation of dividing cells leaving them attached through “thin cytoplasmic bridges” [34]. Other candidate targets with roles in mitosis include septin-9, a GTP-binding protein whose inhibition results in an increased percentage of binucleate cells and an increase in newly divided cells remaining attached by midbody bridges [35]; Cdk1, a cyclin-dependent kinase whose inhibition is sufficient in some cellular model systems to promote endoreduplication [36]; Ran, a GTPase critical for both nucleocytoplasmic transport and mitotic spindle assembly [37]; and Akt2 and PKN1, which play important roles in the regulation of cell division [38–41].

Our data indicate that molecules designed as SH2 domain inhibitors may well encounter unexpected protein targets when introduced into cells. Thus, it will be important to investigate the nature of these targets when evaluating the effects of such agents on the growth properties of cells. The critical role for MCAK in regulating proper chromosome attachments and kinetochore microtubule dynamics [27, 42, 43] makes it an attractive candidate for further investigation. Indeed, several recent studies have shown that elevated MCAK levels correlate with poor prognosis in breast, colorectal and gastric cancers [44–46]. Furthermore, knockdown of MCAK in cells derived from multiple cancers is beneficial in reducing cell proliferation [44–46]. Thus, inhibitors of MCAK have considerable potential as novel therapeutics for the treatment of human tumors. Consequently, efforts are underway to design and synthesize more potent and selective inhibitors of MCAK based on the structure of compound **2** as a lead compound.

In summary, we have developed a methodology that allows the delivery into cells of a stable, phosphotyrosine peptidomimetic, providing a unique avenue for investigating the effects of ligands targeted to SH2 domains on the growth properties of tumor cells. This ligand is cytostatic for leukemia cells, slowing progression through the cell cycle and allowing DNA replication in the absence of mitosis without inducing cell death. A proteomics analysis identified multiple potential targets that include the mitotic kinesin, MCAK. The peptidomimetic binds to the MCAK ATP-binding site, inhibits its ATPase activity and disrupts mitotic spindles. This distinctive activity profile and target set suggests

new opportunities for the further development and optimization of a novel class of antitumor agents.

Acknowledgments

This work was supported by National Institutes of Health grants AI098132 and CA115465 (R.L.G.), CA034619 (R.F.B.), GM059618 (C.E.W) and GM088317 (W.A.T.); and an NSF career development award CHE-0645020 (W.A.T.). Support from Purdue Cancer Center Support Grant P30 CA23168 is gratefully acknowledged for services provided by the NMR, Mass Spectrometry and Analytical Cytology shared resources.

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Fig. 1.

Preparation of metabolically activated prodrug **1**. (A) Synthetic scheme for the generation of compound **1**. a) see reference 7 for details. b) **6**, PyBOP, $i\text{Pr}_2\text{NEt}$, CH_2Cl_2 , yield 83%. (B) Mechanism for the activation of prodrug **1**. Prodrug activation is initiated by bioreductive elimination of the delivery group followed by spontaneous liberation of the masking group to yield the active form of the drug **2**.

Fig. 2.

Compound **1** is activated in cells, inhibits cell growth, and binds SH2 domains in vitro. (A, B) Each indicated cell line was treated with increasing concentrations of compound **1** for 48 h. The number of live cells was counted and compared to the number of cells present in a control culture treated with the DMSO solvent carrier alone and the time zero control. (C) GST-Lck-SH2 was incubated with an NBD-labeled ligand peptide and then titrated by the addition of increasing concentrations of compound **2**. The change in fluorescence intensity resulting from the dissociation of the peptide from the SH2 domain was measured. (D) Pure compound **2** (1 nM) (upper panel) or compound **2** present in extracts of DG75 cells treated

with compound **1** (7 μ M) for 24 h (lower panel) was detected ($m/z = 525.200$) using LC-MS. The multiple peaks observed arise due to the propensity for the phosphonates to bind metal ions.

Fig. 3.

Lack of effect of compound **1** on tyrosine phosphorylation in cultured cells. (A) CEM6 T cells were activated through the T cell antigen receptor by cross-linking the receptor with anti-CD3 antibodies in the presence of compound **1** (1) or DMSO carrier alone (Ctrl). Cellular lysates were isolated, separated by SDS-PAGE and analyzed by immunoblotting (IB) with antibodies against phosphotyrosine (pTyr). No significant differences were observed in three replicates. (B) Lysates from DG75 B cells that had been treated with the indicated concentrations of compound **1** or, as a control, nocadazole, were analyzed by

immunoblotting with antibodies against histone H3 phosphorylated on serine-10 or for GAPDH as a loading control.

Fig. 4.

Compound **1** induces polyploidy in cultured cells. (A) WEHI-231 cells were treated with compound **1** (4 μ M) (red line) or DMSO carrier alone (blue line) for 48 h (left panel) or 72 h (right panel). DNA was stained with propidium iodide. Cells were analyzed by flow cytometry. (B) DG75 B cells were treated with compound **1** (7 μ M) (red line) or DMSO carrier alone (blue line) for 72 h, stained and analyzed by flow cytometry. (C) DT40 B cells were treated with compound **1** (7 μ M) (red line) or DMSO carrier alone (blue line) for 48 h, stained and analyzed by flow cytometry. (D) DG75 B cells were released from a mimosine block into media containing DMSO carrier (left panel) or compound **1** (7 μ M) (right panel).

Cells were collected at various time points following release, fixed, stained with propidium iodide and analyzed by flow cytometry.

Fig. 5.

Compound **1** causes a multi-nucleated phenotype, the appearance of midbodies and altered microtubules in cultured cells. (A) DG75 B cells were treated with or without compound **1** (7 μ M) for 72 h. Cells were examined by phase microscopy (right panels) or by staining with DAPI (left panels) to visualize the nuclei. Examples of cells with more than one nucleus are indicated by arrows. (B) Intercellular connections between drug-treated WEHI-231 cells. WEHI-231 cells were treated with or without compound **1** (7 μ M) for 72 h and visualized by phase microscopy. (C) WEHI-231 cells were treated with or without compound **1** (4 μ M) for

48 h, fixed and stained with antibodies against α -tubulin, and examined by fluorescence microscopy. (C) Chemical structure of the drug-conjugated resins **3** and **4**.

Fig. 6.

Preparation of an immobilized ligand for the analysis of binding proteins. (A) Synthetic scheme for the preparation of linker-modified amine **10**. a) cyclohexylmethyl bromide, Cs_2CO_3 , CH_3CN , reflux, yield 96%; b) LiOH , THF, quantitative yield; c) PyBOP, iPr_2NEt , yield 84%; d) $\text{Ti}(\text{OPr})_4$, NH_3 , MeOH, then NaBH_4 , yield 64%. (B) Synthetic scheme for the preparation of the drug-conjugated resin **3**. a) LiOH , THF, 0°C , yield 91%; b) PyBOP, THF, iPr_2NEt , **10**, 0°C to rt, yield 52%; c) LiOH , THF, quantitative yield; d) PyBOP, LCAA-CPG, iPr_2NEt ; e) TBAI, BSTFA, $\text{BF}_3\text{ET}_2\text{O}$, CH_2Cl_2 .

Fig. 7. Synthetic scheme for the preparation of the control resin **4**. a) PyBOP, $i\text{Pr}_2\text{NEt}$, **10**, yield 94%; b) LiOH, THF, quantitative yield; c) PyBOP, LCAA-CPG, $i\text{Pr}_2\text{NEt}$.

Fig. 8. Multiple proteins bind to the immobilized, active drug **3**. (A) Lysates from DG75 B cells were passed over control beads **4** or beads containing immobilized drug **3**. After extensive washing, proteins remaining attached to the beads were eluted, separated by SDS-PAGE and analyzed by silver-staining. (B, C) Lysates from DG75 B cells were passed over control beads **4** (left lanes) or beads containing immobilized drug **3** (right lanes). After extensive washing, proteins remaining attached to the beads were eluted, separated by SDS-PAGE and analyzed by Western blotting with antibodies against the indicated proteins.

A **B** **C** **Fig. 9.**

Compound **2** interacts with and inhibits MCAK. (A) Compound **2** was docked onto structure of MCAK at the position occupied by ADP in the crystal structure (PDB 1V8J) using Glide from the Schrödinger package (Extra Precision mode). (B) The immobilized ligand **3** was incubated with DG75 B cell lysates, washed extensively and then incubated with a buffer containing the indicated concentrations of ATP for 15 min. Bound MCAK was visualized by SDS-PAGE followed by Western blot analysis. (C) The hydrolysis of [γ - ^{32}P]ATP (1 mM) by the isolated globular domain of MCAK in the presence of tubulin was measured in the

presence of increasing concentrations of compound **2**. Data represent the means and standard errors of 3 replicate assays.

Fig. 10.

Compound **1** affects mitotic spindle morphology similar to MCAK RNAi. (A) HeLa cells were treated with control or MCAK siRNAs +/- compound **1** and then processed for immunofluorescence to visualize microtubules (green) or DNA (blue). Representative spindle structures are shown for each condition. Scale bar, 10 μ m. (B) The percentage of cells in each mitotic stage was scored for 100 cells per condition in 4 independent experiments. Values represent mean $\pm$ SEM, and asterisks indicate $p < 0.05$.

Table 1

Proteins identified by mass spectrometry to bind selectively to the drug-conjugated resin

Number	Protein IP	Percent Coverage	Number unique peptides	Description
MCM complex proteins				
1	IPI00013214	25.4	14	DNA replication licensing factor MCM3
2	IPI00018349	5.6	3	DNA replication licensing factor MCM4
3	IPI00018350	7.6	4	DNA replication licensing factor MCM5
4	IPI00031517	3.8	2	DNA replication licensing factor MCM6
5	IPI00376143	11.6	6	DNA replication licensing factor MCM7
6	IPI00025695	5.8	3	Cdc45
Other nucleic acid-binding proteins				
7	IPI00377114	22.6	7	RNA-binding motif protein 39
8	IPI00016610	36.5	5	Poly(rC)-binding protein
9	IPI00334175	26.7	7	Polypyrimidine tract-binding protein 1
10	IPI00293655	10.9	6	ATP-dependent helicase DDX1
11	IPI00644712	21.4	12	ATP-dependent DNA helicase II, 70 kDa subunit
12	IPI00220834	21.2	11	ATP-dependent DNA helicase II, 80 kDa subunit
13	IPI00306369	11.1	7	tRNA (cytosine-5-)-methyltransferase
Protein kinases				
14	IPI00002803	10.2	6	Protein kinase N1
15	IPI00026689	17.9	4	Cdc2
16	IPI00012870	6.2	4	AKT2
Nucleotide-binding proteins				
17	IPI00552801	17.9	6	Kinesin-like protein KIF2C/MCAK
18	IPI00455033	14.3	6	Septin 9
19	IPI00028481	12.6	6	Ras-related protein Rab-8A
20	IPI00643041	27.5	8	GTP-binding nuclear protein RAN
21	IPI00644674	22.9	4	Nucleotide binding protein 2
22	IPI00025447	59	13	Elongation factor 1-alpha
23	IPI00025491	22.2	7	Eukaryotic initiation factor 4A-I
24	IPI00465160	15.4	8	ATP-binding cassette, sub-family F
Metabolic enzymes				
25	IPI00008982	20.3	14	Delta 1-pyrroline-5-carboxylate synthetase
26	IPI00219018	25.4	7	Glyceraldehyde-3-phosphate dehydrogenase
27	IPI00303568	35.8	10	Prostaglandin E synthase
28	IPI00291646	6.9	5	Methylenetetrahydrofolate dehydrogenase
29	IPI00219452	12.9	5	Acyl coenzyme A thioester hydrolase
Others				
30	IPI00017184	29.2	10	EH-domain containing protein 1

Number	Protein IP	Percent Coverage	Number unique peptides	Description
31	IPI00152998	22.8	9	Leucine-rich repeat containing protein

Table 2

Proteins identified by mass spectrometry to bind to both control and drug-conjugated resins

Number	Protein IP	Percent Coverage	Number unique peptides	Description
1	IPI00645452	70.7	10	Tubulin, beta
2	IPI00180675	40.8	10	Tubulin alpha
3	IPI00382470	12.3	6	Heat shock protein 90-alpha
4	IPI00334775	21.6	14	Heat shock protein 90-beta
5	IPI00025512	36.1	5	Heat-shock protein beta-1
6	IPI00643188	37.9	17	Heat-shock protein 70kDa
7	IPI00472102	31.3	12	Heat-shock protein 60 kDa
8	IPI00017334	55.1	12	Prohibitin
9	IPI00440493	19.5	8	ATP synthase alpha chain
10	IPI00303476	56.9	25	ATP synthase beta chain
11	IPI00024976	55.3	5	Mitochondrial import receptor subunit TOM22
12	IPI00016676	22.8	3	Mitochondrial import receptor subunit TOM20
13	IPI00025086	30.7	7	Cytochrome c oxidase polypeptide Va
14	IPI00218463	39.2	4	Mitochondria-associated GM-CSF signaling molecule
15	IPI00025796	47	9	NADH-ubiquinone oxidoreductase
16	IPI00029264	16	4	Cytochrome c1
17	IPI00554722	16.4	6	Solute carrier family 3
18	IPI00384051	31.9	5	Proteasome activator complex subunit 2
19	IPI00219445	32.3	8	Proteasome activator complex subunit 3
20	IPI00022744	8.7	6	Importin-alpha
21	IPI00329200	6.6	6	Importin-beta
22	IPI00003362	19.4	9	78 kDa glucose-regulated protein
23	IPI00003881	21.4	6	Heterogeneous nuclear ribonucleoprotein F